BLACKHEAD DISEASE: A NEW UNDERSTANDING OF AN OLD DISEASE

by

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(Under the Direction of Jeanna Wilson)

ABSTRACT

Histomonas meleagridis, is a unicellular protozoan parasite and the causative agent of blackhead disease. After the parasite enters turkey flocks by means of the paratenic host, *Heterekis gallinarum*, it can be transmitted from infected birds to uninfected birds by means of cloacal uptake of contaminated feces. Blackhead outbreaks usually result in less then 10% mortality though in some instances whole flocks succumb to the disease. Prior research has not directly addressed this variation of disease transmission. To better understand this variation in disease transmission *in vivo* turkey trials were performed with treatments designed to induce a loosening of the stool. The results of this research suggest that disease transmission may be correlated with alterations in reverse peristalsis, loosening of the stool and intestinal health. Additionally, two products, Natustat a natural plant derivative previously reported as an anti-histomonal and a yeast fermentation by-product were independently investigated as alternative means of preventing blackhead disease. Under the conditions tested neither of these products were able to ameliorate the effects of blackhead disease. This work sheds new light on the mode of blackhead disease transmission, suggest that intestinal health may be directly correlated with turkeys' susceptibility to the disease and provides a model for future research to address the connection between these two.

INDEX WORDS: *Histomonas meleagridis,* transmission, disease, *in vivo*

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

In 1893 Cushman described the first case of Blackhead disease in a turkey flock in Rhode Island (Cushman, 1893). It was not until 1920 that Tyzzer identified the protozoan parasite *Histomonas meleagridis* as the causative agent (Tyzzer, 1920). Blackhead disease has been reported in other gallinaceous species including chickens (Tyzzer, 1934), quail (McDougald, Abraham and Beckstead, 2012; Zeakes, Hansen and Robel, 1981), ostriches (Borst and Lambers, 1985), rheas (Dhillon, 1983), pheasants (Lund and Chute, 1972) and other game birds. Many gallinaceous species are asymptomatic when infected with *H. meleagridis*, however, turkeys are highly susceptible to the disease (Powell, Rothwell, Clarkson and Kaiser, 2009) and chickens are reported to suffer performance losses(Liebhart, Windisch and Hess, 2010). Most research on blackhead disease has been performed in turkeys and chickens.

Clinical symptoms of blackhead disease include loss of appetite, weight loss, a hunched posture, ruffled feathers, sulfur-colored droppings and listlessness (Tyzzer and Fabyan, 1920). Physiological sign of the disease can be seen in both the ceca and liver (Tyzzer, 1934). Infections of the ceca can cause moderate thickening of the cecal wall, followed by a continued swelling and enlargement of the ceca, and eventually results in hemorrhagic lesions. As the infection progresses, the lumen of the ceca begins to fill with blood and caseous abnormal contents which eventually form a dense cecal core (McDougald and Hu, 2001). In severe cases the inflammation and formation of the cecal core cause the cecal wall to become brittle and rupture, and in some

cases lead to the death of the bird. In the liver, signs of blackhead disease are observed as pin-point lesions that increases in number and size as the disease progresses (McDougald and Hu, 2001). An infection of the liver can eventually lead to loss of liver function and the ultimate demise of the bird.

In turkeys, an outbreak of Blackhead disease is initiated through the ingestion of embryonated *Heterakis gallinarum* eggs contaminated with *H. meleagridis* (Graybill and Smith, 1920; Wehr, 1954). Once infected, turkeys can spread the parasite from one bird to another without an intermediate host by means of cloacal uptake or drinking (Hu and McDougald, 2003; McDougald and Fuller, 2005). Blackhead outbreaks in commercial turkey facilities in France have been reported to generally result in less then 10% mortality, but in some cases mortality reaches 100%(Callait-Cardinal, Leroux, Venereau, Chauve, Le Pottier and Zenner, 2007). It is still not understood why there is a variation in mortality associated with outbreaks. However, wet litter, improper sanitation and diarrhea have been correlated with blackhead disease(Callait-Cardinal, Gilot-Fromont, Chossat, Gonthier, Chauve and Zenner, 2010).

Blackhead disease was entirely controlled by arsenical drugs until 1950 at which point nitromidazole drugs became available for use (Joyner, 1963; Joyner, Davies and Kendall, 1963). Their effectiveness in treating blackhead outbreaks in the 1960's led to a sharp decline in research related to the blackhead disease (Avian Diseases, Vols. 10-31). However, in 1995 dimetridazole and in 1997 nitromidazoles were banned for use in food animals due to their being suspect carcinogens, leaving only Histostat 50 as a preventative treatment. In 2015, Histostat50 was removed from the market due to carcinogenic concerns relating to the arsenic contained within the drug(Nachman, Love, Baron, Nigra, Murko, Raber, Francesconi and Navas-Acien, 2017). As a consequence, entire flocks of turkeys, including meat-type and breeders, had to be

destroyed in 2016 and 2017 resulting in financial losses to the poultry industry and growers and an animal welfare problem for the veterinarians. Currently, plant derivatives and essential oils are been utilized as treatment strategies for blackhead disease, but there is limited research to support their effectiveness (Duffy, Sims and Power, 2004; Duffy, Sims and Power, 2005; Grabensteiner, Liebhart, Arshad and Hess, 2008).

Research presented in this dissertation is focused on expanding our knowledge of the *H. meleagridis* life cycle and exploring methods to controlling the disease in turkeys and chickens. The objectives of this dissertation are:

1. To identify factors that play a role in the lateral transmission of *H. meleagridis*
2. Understand the mode of action of Natustat in controlling blackhead disease
3. Determine whether the immune response of turkeys and broilers can be stimulated using a fermentation by-product so that turkeys can survive an infection of a virulent strain of *H. meleagridis* and broilers can overcome performance losses.

Literature Review

H. meleagridis

Histomonas meleagridis, the causative agent of blackhead disease, is a unicellular anaerobic protozoan parasite typically ranging from 8-17 μm in diameter (Tyzzer, 1920). The protozoan parasite has been described to exist in four different forms: invasive, vegetative, resistant and cyst-like. The invasive form is an amoeboid like form that is found in the lumen of the ceca and liver tissues (Olsen, 1986; Tyzzer, 1920). The vegetative form is found lodged within the inflamed tissues and the resistant form is associated with old lesions (Tyzzer, 1920). The cyst-like form, which has a double membrane and is smaller then the amoeboid form, is believed to survive better in the environment (Zaragatzki, Hess, Grabensteiner, Abdel-Ghaffar, Al-Rasheid and Mehlhorn, 2010), though there has been no research to show this to be the case.

Early work mistakenly identified various organisms as the causative agent of blackhead disease including coccidia (Cole and Hadley, 1908), *Trichomonas* (Hadley, 1920), and *Candida albicans* (Ceretto, 1954). This is not surprising considering that blackhead disease has been reported to be associated with various parasitic and bacterial infections (Rad, 2004). However, the actual causative agent *H. meleagridis* was determined by Tyzzer (Tyzzer, 1920). Subsequent research, based on antigen analysis determined that *H. meleagridis* was closely related to *Dientamoeba fragillis* (Dwyer, 1974). Further phylogenetic characterization using small subunit rRNA sequence confirmed the relation of *H. meleagridis* to *D. fragillis* as well as another common ancestor, *Trichomonas foetus* (Gerbod, Edgcomb, Noel, Zenner, Wintjens, Delgado-Viscogliosi, Holder, Sogin and Viscogliosi, 2001). This data allowed for *H. meleagridis* to be placed in the Phylum - Parabasala, Class - Trichomonadae and Family - Monocercomonadidae according to the taxonomic system of Cavalier-Smith (Cavalier-Smith, 1998).

As an anaerobe, *H. meleagridis* is not well suited to living outside of birds or secondary vectors. When excreted from birds the exposed protozoan is only capable of surviving for a few hours (Graybill and Smith, 1920; Lotfi, Abdelwhab and Hafez, 2012; Tyzzer and Collier, 1925). This is due to the toxicity of oxygen and the need of *H. meleagridis* to be maintained above a certain temperature (Gerhold, Lollis, Beckstead and McDougald, 2010). Inside the gastrointestinal tract of the bird *H. meleagridis* is only found within the ceca. In the ceca, the invasive form of the parasite will penetrate the mucosal lining which results in lesions and inflammation (McDougald and Hu, 2001). From there *H. meleagridis* is able to enter the portal veins connecting the ceca to the liver (Clarkson, 1961). Blood drawn from the veins proximal to the ceca of infected birds can result in blackhead infections if injected intravenously into healthy hosts (McGuire and Cavett, 1952; McGuire Wc Fau - Morehouse and Morehouse). This suggests that *H. meleagridis* has a mechanism of surviving in the oxygen rich blood. Although *H. meleagridis* has been detected in multiple tissues (Huber, Reynaud, Callait and Zenner, 2006), it is primarily in the liver that the parasite begins to replicate and cause extensive damage (McDougald and Hu, 2001).

H. meleagridis can be grown outside of the host in culture. Several different medias have been used for the culturing of the parasite, though a modified Dwyer's media is most frequently used by researchers (Dwyer, 1970; van der Heijdena and Landman, 2007). This media consists of M199 with Hank's balanced salts, horse serum, sodium bicarbonate and rice (van der Heijdena and Landman, 2007). Research has shown that various starch sources including cornstarch, oat flour, rye flower or buckwheat flour could be substituted for rice flour (Hauck, Armstrong and McDougald, 2010). The abundance of starch in the diet, and consequently in the ceca, may be one reason why *H. meleagridis* is able to thrive in chickens and turkeys.

H. meleagridis cannot be grown as an axenic culture. Bacteria is required for *H. meleagridis* to be grown in culture (van der Heijdena and Landman, 2007). Ganas et al (2012) looked at the importance of different bacteria in sustaining the growth and virulence of the parasite (Ganas, Liebhart, Glosmann, Hess and Hess, 2012). Their work describes bacteria as a food source for *H.* meleagridis as it can be visualized within the food vacuoles of the parasite. They also found that different bacterial including *E. coli* and *P. aeruginosa* were sufficient for sustained *in-vitro* growth while other bacterial sources like *Salmonella enterica*serovar and Typhimurium were less capable of sustaining growth. Prior work described the virulence of the parasite to be related to the bacteria within the ceca, as gnotobiotic turkeys were not able to contract the disease (Franker and Doll, 1964). In turkeys, bacterial species such as *E. coli*, *Clostridium perfringens*, or *Bacillus subtilis* are important for *H. meleagridis* virulence (Bradley, Johnson and Reid, 1964; Bradley and Reid, 1966). Research has also shown that *H. meleagridis* cultures may prefer the bacterial profile of turkey's ceca compared to that of the chicken, though both are able to sustain growth (Lesser, 1964).

There is limited genomic and proteomic information available about *H. meleagridis*. Up until 2008, much of what was known about *H. meleagridis* was inferred from other phylogenetically related species with no genomic data to support those inferences (Hess, Liebhart, Bilic and Ganas, 2015). In 2008, the confirmation of the hydrogenosome occurred when three genes involved in the anaerobic energy metabolism pathway were successfully cloned (Mazet, Diogon, Alderete, Vivares and Delbac, 2008). The following year several partial protein sequences were identified that showed considerable homology to the related species *Trichomonas vaginalis* (Bilic, Leberl and Hess, 2009). In 2012, the first regulatory DNA sequences were identified by means of Splinkerette PCR (Lynn and Beckstead, 2012) and in

2013 Klodnicki et al identified 3425 *H. meleagridis* genes from the sequencing of a cDNA library (Klodnicki, McDougald and Beckstead, 2013). More recently our laboratory attempted to sequence the entire genome of *H. meleagridis*. However, the excessive amount of bacterial DNA that was co-isolated with the parasite resulted in only two sequences of gDNA greater then 20kb (data not shown). With limited genomic information available there is limited understanding of the pathways that play a role in the virulence of the parasite.

Heterakis gallinarum

H. gallinarum, a common cecal worm, in turkeys, chickens and various game fowls is a paratenic host for *H. meleagridis*. In the 1920s, Graybill and Tyzzer, showed that turkeys fed *H. gallinarum* eggs contracted blackhead disease (Graybill and Smith, 1920; Tyzzer, 1926). Both mature worms and embryonated larvae have been found to house the parasite in their intestinal walls (Lee, 1969b). In mature worms, *H. meleagridis* can also found proximal to and within oocytes, though reduced in size to 3-4 μm in diameter (Lee, 1969a). Its proposed that *H. gallinarum* becomes contaminated upon consuming *H. meleagridis* growing in the ceca, after which the *H. meleagridis* is able to enter females' germinal ridges and penetrate oocytes (Lee, 1969a). Others have suggested that *H. meleagridis* found in the male testis is transmitted to the female through the act of copulation (Lee, 1971; Springer, Johnson and Reid, 1969). Thus, like *Trichomonas vaginalis,* a sexually transmitted disease in humans, *H. meleagridis* may also follow the same transmission pattern.

After birds ingest a *H. gallinarum* egg, the larva will hatch and descend into the lower portions of the intestines where it ultimately takes residence in the ceca (Lund and Chute, 1974). Thus, infected *H. gallinarum* transport *H. meleagridis* directly to the ceca, where the parasite it is

released upon molting of the worm (Fine, 2009; Lund and Chute, 1974). Thus the life cycle of the *H. gallinarum* allows for the fragile *H. meleagridis* to survive in the environment and be transmitted from one bird to another.

As mature *H. gallinarum* worms infected with the parasite procreate within the ceca, eggs contaminated with the *H. meleagridis* parasite can be shed into the environment where they can remain infective for several years (Farr, 1961). Eggs in the environment can be carried on or in paratenic host such as the sow-bug, house flies or grasshoppers (Ackert, 1917; Frank, 1953; Spindler, 1967) or mechanical vectors such as farm equipment or boots and subsequently gain entrance to facilities where susceptible birds are housed. Earthworms can be of particular concern since they can host the contaminated cecal worm for long periods of time (Kemp and Franson, 1975) and can be driven indoors by heavy rains (McDougald, 2005). Life expectancy for earthworms can vary greatly but one of the most common earthworms, *Lumbricus terrestris* or night crawler can live up to 10 years . Proper biosecurity and sanitation may aide in preventing blackhead outbreaks, however, with so many reservoirs of *H. meleagridis* in the environment, prevention of blackhead outbreaks is difficult, especially since chickens serve as a reservoir for both *H. gallinarum* and *H. meleagridis*. Research has shown that free range poultry are positive, though asymptomatic, for *H. meleagridis* (Grafl, Liebhart, Windisch, Ibesich and Hess, 2011) and *H. gallinarum* cecal worms (Lund and Chute, 1970; Lund and Chute, 1973). The combination of these two factors make free range chickens a problem for turkey production (Lund and Chute, 1973). Similarly, broiler breeder facilities, where chickens are raised on the ground, can also serve as reservoirs for infection.

Lateral transmission of blackhead disease

Digestion, relating to nutrient absorption, in mammals is generally a function of intestinal length. However, avians, who have a much shorter digestive tract, have developed a means of being able to over come what would appear to be an evolutionary disadvantage through reverse peristalsis. Peristalsis is the movement of food in the direction of the esophagus toward the cloaca. Anti-peristalsis, more commonly referred to as reverse peristalsis, is a phenomenon observed in avian species where food particles, nutrients and/or water move in a retrograde direction within the digestive tract (Duke, 1994). Reverse peristalsis occurs in four different locations of the gastrointestinal tract; the gizzard to the proventriculous, the small intestine to the gizzard, the rectum into the small intestines, and the rectum into the cloaca(Duke, 1994). In turkeys, Duke (1994) described reverse peristalsis into the ceca as a continuous low amplitude of waves that results in materials being brought into the ceca (Duke, 1994). Reverse peristalsis into the ceca from the large intestine plays a crucial role in the transport of proteins, salt and water contained within the urine that is secreted into the cloaca (Duke, 1989). Reverse peristalsis also allows the bird to take up substances from the environment through the cloaca and transport them to the ceca.

Prior to 2003, it was believed that the only route of *H. meleagridis* infection was due to the ingestion contaminated *H. gallinarum*. This belief was due to the unsuccessful and low rates of blackhead disease contracted after naked *H. meleagridis* was orally ingested (Hortonsmith and Long, 1956; Lund, 1956). However, Hu and McDougal in 2003 showed that in the absence of a secondary vector *H. meleagridis* could be transmitted from infected to uninfected birds (Hu and McDougald, 2003). The following year they reported that turkeys could have a drop of media containing *H. meleagridis* placed on the vent and through reverse peristalsis the birds would

uptake the parasite and contract blackhead disease (Hu, Fuller and McDougald, 2004). The researchers concluded that blackhead disease was transmitted when uninfected turkeys took up contaminated feces through reverse peristalsis. The implications for this model suggest that the separation of infected birds from uninfected birds through culling or dividing turkey houses into smaller sections may aide in controlling the spread of blackhead disease.

Immune response

A limited amount of research has been performed looking at the immune response of turkeys and chickens to *H. meleagridis* (Powell, Rothwell, Clarkson and Kaiser, 2009). Immune responses are classified into two categories the innate and adaptive immune responses. The innate immune response is the initial response that is responsible for recognizing and clearing infections, removing debris, presenting antigens and the activation of the adaptive immune response. The adaptive immune response is a secondary response that provides a long-term protection by creating an immunological memory that can lead to an enhanced immune response should subsequent infections occur.

The only report investigating the immune response of turkeys and chickens to blackhead disease was performed by Powell et al. in 2009 (Powell, Rothwell, Clarkson and Kaiser, 2009). In their report the ceca of chickens infected with blackhead disease had an increase expression of IL-1β, CXCLi2 and IL-6, which are genes that play a role in controlling the innate immune response. In turkeys there was evidence of an increase in IL-1β, CXCLi2 and IL-6, however, this expression did not occur until 5 days post infection, 3 days later then the chickens immune response. The authors hypothesized that the turkey's lack of a functional innate immune response

allowed the parasite to replicate in the cecal tissue, escape immune detection and migrate to the liver.

Turkeys and chickens also showed an increased transcription of the adaptive immune response genes IFN-γ, IL-13, IL-4 and IL-10 suggesting that both may be able to mount an adaptive immune response to *H. meleagridis*. However, as with the innate immune response, the onset of the transcription of these adaptive immune genes in the turkey was delayed compared to the chicken (Powell, Rothwell, Clarkson and Kaiser, 2009). It is possible that by the time the turkeys is able to mount an immune response to *H. meleagridis* the damage caused by the infection has already caused the death of the bird. It is important to note that these conclusions are only based on one paper based on gene expression.

Much of what we hypothesize about virulence of *H. meleagridis* is extrapolated from related organisms. One such organism that manifests similar physiological effects in humans is the parasite *Entomoeba histolytica. E. histolytica* infections cause intestinal hemorrhage which allows for the parasite to enter the circulatory system, disseminate throughout the body, and form granulomatous in soft tissues, more especially in the liver (Campbell and Chadee, 1997). If left untreated *E. histolytica* infections can be fetal due to its ability to alter the immune function of the body (Walsh). *E. histolytica'*s extra cellular secretions are reported to inhibit T cells' ability to secrete IFN-γ, IL-2, and TNF-B which prevents macrophage activation (Denis and Chadee, 1988). If *H. meleagridis* is able to act in a similar manner to *E. histolytica* then it may be possible that the insufficient immune response seen in turkeys could be attributed to *H. meleagridis* modifying the hosts immune response.

Early attempts by Tyzzer and many years later Lund, showed that turkeys could be immunized for blackhead disease if infected with an attenuated avirulent strain of *H.*

meleagridis. However, they also observed that the long-term passage of the parasite in culture, which continues to attenuate the parasite, resulted in loss of protection (Lund, Augustine and Chute, 1967; Lund, Augustine and Ellis, 1966; Tyzzer, 1933; Tyzzer, 1936). In the last decade using more defined media conditions researchers have been able to develop an attenuated strain of *H. meleagridis,* which four weeks after an oral or cloacal inoculation, provides protection against a challenge of a virulent strain of *H. meleargridis* (Hess, Liebhart, Grabensteiner and Singh, 2008; Liebhart, Windisch and Hess, 2010; Sulejmanovic, Bilic, Hess and Liebhart, 2016). No success has been reported using an intramuscular injection of *H. meleagridis* (Hess, Liebhart, Grabensteiner and Singh, 2008). In each of these reports *H. meleagridis* was detected to be present in the ceca of the immunized birds throughout the trial. Tyzzer proposed the theory that protection from infections occurs when birds remain positive for the protozoan parasite (Tyzzer, 1936). This could lead to the assumption that long-term immunity of turkeys to blackhead may be a factor of both an adaptive immune response and a competitive exclusion of virulent *H. meleagridis*.

In-vitro compound screening

H. meleagridis in-vitro compound screens were developed by Calliat (2002) in order to test the effect of drugs on the growth of *H. meleagridis* in culture (Callait, Granier, Chauve and Zenner, 2002). His group showed that nitromidazole drugs killed the parasite in culture. Thus drugs known to treat the disease in the bird also had activity under culture conditions. This methodology established a means of screening compounds and drugs that might have activity against *H. meleagridis* without having to test each compound in live bird trials. Several reports have investigated the *in-vitro* activity of plant extracts and essential oils against *H. meleagridis* (Grabensteiner, Liebhart, Arshad and Hess, 2008; Hauck and Hafez, 2007; Thofner, Liebhart,

Hess, Schou, Hess, Ivarsen, Frette, Christensen, Grevsen, Engberg and Christensen, 2012; van der Heijden and Landman, 2008a; Zenner, Callait, Granier and Chauve, 2003). Despite the reported *in-vitro* success of several of these products their in vivo effects could not be replicated in the bird. Although *in-vitro* compound screening tells us whether compounds have activity against *H. meleagridis* few products have been shown to prevent or treat blackhead disease.

Preventatives and therapeutics

With the removal of arsenicals and nitromidazole drugs a multitude of alternative products have been introduced into the market including antibiotics, essential oils and plant extracts. Antibiotics have been shown to be efficacious in regulating the microbiome and boosting bird health. The antibiotic Tiamulin has been proposed to have preventative properties in relation to blackhead disease (Burch, Young and Watson, 2007). *In vitro* compound screens have reported both success and failure when using relatively the same concentration of antibiotics in the first study, though a higher concentration of *H. meleagridis* was used in the study where the antibiotic was reported to have had no effect (Hauck, Lotfi and Hafez, 2010; van der Heijden, De Gussem and Landman, 2011). Case reports involving Tiamulin also report both successes and failures of the antibiotic (Burch, Young and Watson, 2007; Hauck, Lotfi and Hafez, 2010). This indicates that Tiamulin may act in a dose dependent manner in order to control blackhead disease.

The antibiotic Paromomycin was reported by Lindquist in 1962 (Lindquist, 1962) and more recently confirmed by other researchers to act as an anti-histomonal (Bleyen, De Gussem, Pham, Ons, Van Gerven and Goddeeris, 2009; Hafez, Hauck, Gad, De Gussem and Lotfi, 2010; van der Heijden, De Gussem and Landman, 2011). Paromomycin has shown the ability to reduce

mortality, the severity of liver and cecal lesions, and reduce the lateral transmission of the disease from infected birds to uninfected birds (Bleyen, De Gussem, Pham, Ons, Van Gerven and Goddeeris, 2009; Hafez, Hauck, Gad, De Gussem and Lotfi, 2010; van der Heijden, De Gussem and Landman, 2011). Paromomycin is not an effective treatment for birds that have already contracted blackhead disease but rather it is acts as a preventative for contracting and spreading the disease (Hu and McDougald, 2004). Despite its reported effectiveness as a preventative, the need for Paromomycin to be added continually to feed at therapeutic levels raises a concern about potential health risks associated with bacterial resistance, which makes its use in an industrial setting unrealistic.

Two plant extracts, Natustat and Protophyt, have been reported to provide some in vivo protection against blackhead infections (Duffy, Sims and Power, 2004; Duffy, Sims and Power, 2005; Hafez and Hauck, 2006). Natustat, a proprietary plant blend, was reported to provide chickens with a reduction in ceca lesions associated with blackhead infections and mitigated performance losses associated with body weight gain and feed conversion(Duffy, Sims and Power, 2004). In a trial using turkeys, Natustat reduced cecal lesions as well as liver lesion scores at a similar rate to the anti-histomonal drug nitarsone (Duffy, Sims and Power, 2005). However, this report had a low rate of disease transmission in the control group and failed to determine whether turkeys had been exposed to *H. meleagridis.* Protophyt, a blend of essential oils from cinnamon, garlic, lemon and rosemary, was reported by Hafez (2006) to reduce mortality in turkeys from 50% to 20% (Hafez and Hauck, 2006), however, another report showed it to provide no protection against infections even if a low challenge dose of *H. meleagridis* was used (van der Heijden and Landman, 2008b). In addition to the experimental report, field reports also indicate that protophyt may not be effective in treating blackhead

outbreaks (Aka, Hauck, Blankenstein, Balczulat and Hafez, 2011; Popp, Hauck, Balczulat and Hafez, 2011). This may indicate that protophyt could only be used as a preventative of blackhead disease rather than a treatment for infection. Though both plant derivative products have been reported to aid in the control of blackhead disease, the limited amount of data concerning their mode of action and the variability in their ability to prevent transmission of blackhead disease necessitates additional research.

Intestinal health

With the removal of antibiotics and several drugs there has been an increased focus on products that improve the gastrointestinal tract's natural ability to fight off infection. This area of research has often been referred to as gut health. In poultry, goblet cells, from within the intestinal epithelium, secrete the glycoprotein mucin that acts as a protective layer for the intestines (Forder, Nattrass, Geier, Hughes and Hynd, 2012). This mucosal lining has an outer layer where microorganisms can embed and colonize, and an inner lining that repels bacteria and other organisms (Johansson, Phillipson, Petersson, Velcich, Holm and Hansson, 2008). This lining is part of the gut's innate immune response and performs the function of preventing organisms from invading through the intestinal wall (Brisbin, Gong and Sharif, 2008). Additionally, this lining helps maintain a unique microbiome that interacts with the host to regulate many aspects of the bird's health (Pan and Yu, 2014). Under normal conditions birds are highly efficient at retaining water with 10-12% being retained through the ceca, 3-5% in the rectum and the remainder being reabsorbed through the kidneys (Bjornhag, 1989). However, damage to the mucosal lining or alterations of the microbiome can result in poor nutrient uptake, osmotic or homeostatic imbalances, or impairment of gastrointestinal tract functionality, which

can lead to a reduction in water retention and result in flushing events (Azahan and Sykes, 1980; Barnes and Guy, 2003; Ferket and Veldkamp, 2015). Flushing is a term used to describe excessively wet litter, and is caused by diuresis or diarrhea. Diuresis is excessive urination and is the host's way of clearing excess nutrients, electrolytes or toxins (Ferket and Veldkamp, 2015). Diarrhea is an excessive loss of fluids from the gastrointestinal tract as evidenced by dark fecal excretions and is brought on by a broad range of factors including infection, stress and osmotic imbalances (Ferket and Veldkamp, 2015). Additionally, a survey on blackhead disease in France reported turkeys to be at greater risk for an outbreak if water pH was too low, there was improper sanitation, wet litter or diarrhea present(Callait-Cardinal, Gilot-Fromont, Chossat, Gonthier, Chauve and Zenner, 2010). These factors suggest that severe blackhead outbreaks may be related to a compromised gastrointestinal tract.

Flushing can be caused by a variety of infectious microorganisms including protozoans (ie. Coccidia, Trichomonas, Histomonas), parasites (ie. Round Worms, Tape Worms, Heterekis) or proteolytic bacteria (ie. Salmonella, Clostridium, *E. coli).* These microorganisms have been shown to cause damage to the mucosal lining and alter the microbiome (Burgess, Gilchrist, Lynn and Petri, 2017; Ferket and Veldkamp, 2015). Blackhead disease outbreaks with high levels of mortality are often associated with or follow infections of the above microorganisms (Rad, 2004; Stokholm, Permin, Bisgaard and Christensen, 2010). Thus the severity of a blackhead outbreak may be associated with a co-infection of other pathogenic microorganisms.

Conclusions

There is still a lack of understanding regarding the variation seen in mortality associated with blackhead disease. In addition, many products seem to work under one condition but not

under another. This variation suggests that we don't have a complete understanding of the life cycle of *H. meleagridis.* Based on the published research there appears to be a likely connection between gut health and the transmission of blackhead disease. However, there is still a lack of data directly correlating gut health with blackhead transmission. Research in this dissertation is aimed at looking at this connection and how products that boost gut health may play a role in the transmission of blackhead disease.

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CHAPTER 2

THE ROLE OF FLUSHING IN THE TRANSMISSION OF BLACKHEAD DISEASE[A]

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Abstract

Blackhead disease, histomoniasis, is cause by the protozoan parasite *Histomonas meleagridis*. Transmission of the disease between infected and uninfected turkeys is generally below 10% but in some cases entire flocks can be infected. Although several factors such as sanitation and diarrhea have been associated with the severity of the outbreak, the exact mechanism is not understood. To better understand the role of lateral transmission of blackhead disease outbreaks in turkeys both floor pen and battery trials were conducted. 2 floor pen trials using $1ft^2$ and $0.6ft^2$ of space per bird had no lateral transmission from infected to uninfected birds. We hypothesized that lateral transmission was not seen because the diet used in these experiments promoted firm fecal droppings. To test the dietary effect or the role of flushing in the lateral transmission of blackhead disease a battery trial was conducted. Treatment diets were designed to increase fecal moisture, induce flushing, or promote reverse peristalsis. These treatment diets increased the percent of birds that contracted blackhead disease after a direct inoculation of *H. meleagridis* and lateral transmission occurred. This data suggest that the susceptibility of turkeys and the rate of blackhead disease transmission may be closely related to turkeys' gut health and integrity. Additionally, this model established a means of being able studying the lateral transmission of blackhead disease.

Introduction

Blackhead disease is caused by the protozoan parasite, *H. meleagridis.* Upon infection, turkeys can spread the parasite to uninfected birds by uptake of contaminated feces through the cloaca by means of reverse peristalsis (Hu, Fuller and McDougald, 2004; Hu and McDougald, 2003; McDougald and Fuller, 2005). Blackhead outbreaks in commercial turkey facilities generally cause less then 10% mortality, but in some cases mortality reaches 100% (Callait-Cardinal, Leroux, Venereau, Chauve, Le Pottier and Zenner, 2007). It is still not understood why there is a variation in mortality associated with outbreaks. However, the correlation of outbreaks with wet litter, improper sanitation and diarrhea suggests that a compromised gastrointestinal tract may be a factor (Callait-Cardinal, Gilot-Fromont, Chossat, Gonthier, Chauve and Zenner, 2010).

Intestinal health and integrity play a crucial role in nutrient uptake, water retention, homeostasis and the prevention of infection. However, damage to the mucosal lining or alterations of the microbiome can impair gastrointestinal tract functionality and lead to flushing (diarrhea) events (Azahan and Sykes, 1980; Barnes and Guy, 2003; Ferket and Veldkamp, 2015). Flushing occurs for a variety of reasons including feed withdraw, environmental stress, nutritional imbalances, excess of ions or infections with microorganisms. Blackhead disease outbreaks with high levels of mortality have been reported to accompany or follow infections of coccidia, *H. gallinarum* or *E. coli* (Rad, 2004; Stokholm, Permin, Bisgaard and Christensen, 2010). Thus the severity of blackhead disease outbreaks may be associated with a co-infection of other pathogenic microorganisms. Based on the published research there appears to be a likely connection between intestinal health and the transmission of blackhead disease, however, there is still a lack of data directly correlating the two. The following study was performed to determine

whether intestinal health and integrity play a role in the transmission of blackhead disease in turkeys.

Materials and Methods

All animal trials were approved by the North Carolina State Animal Care and Use Committee.

Parasites

H. meleagridis strains used in this study were obtained from field outbreaks in Buford Georgia and Zeeland Michigan. Field isolations were frozen in liquid nitrogen for long-term storage. 7 days prior to infection, *H. meleagridis* was thawed and propagated in modified Dwyers media (van der Heijdena and Landman, 2007).

Experimental designs

For all trials, birds were given ad libitum access to feed and water from the time of placement until termination of the trial. The diet formulations can be found in 2.1.

Floor pen trial 1. 200 day-old turkeys were placed on fresh litter in 8 pens containing 25 birds per pen. Pens were 4ftx8ftx2.5ft with wire netting enclosing the top. Treatment groups consisted of non-challenged and challenged Basal diet 1. On day 18, 5 birds from each of the challenged group pens were tagged and intercloacally inoculated with 1mL of media containing $1x10^5$ *H. meleagridis* from the Buford field isolation. Birds were monitored daily for mortality and morbidity. Any birds manifesting severe morbidity or found dead were necropsied and scored for

signs of blackhead disease. The trial was terminated 30 days post infection and all remaining birds were euthanized, necropsied and scored for signs of blackhead disease.

Floor pen trial 2. This trial was performed the same as above except pens were reduced in size to 4ftx4.5ftx2.5ft and an inoculation of $1x10^5$ *H. meleagridis* from the Zeeland field isolation was administered one day after the Buford inoculation.

Battery trial. 396 birds were placed in clean battery cages 2ftx2ftx1.5ft with 9 birds per pen with 7 pens per infected treatment groups and 2 pens per uninfected control group. Infected treatments consisted of Diet 1, Diet 1 supplemented with $MgSO_4$ or $MgCl_2$, Diet 1 orally administered 0.45g Dextran sulphate suspended in 1mL of water 3 and 4 days post challenge, Diet 2 or Diet 3. The Control Uninfected group was fed diet 3. On day 7, 2 inches of fresh litter shavings were placed on the bottom of the batteries over a sheet of paper. On day 9, 5 birds from each challenge pen were wing banded and intercloacally inoculated with 1ml of media containing $1x10^5$ *Histomonas meleagridis* from the Buford field isolation. Birds were monitored daily stated as above. 19 days post infection, the study was terminated and all birds were necropsied and scored for signs of infection.

Lesion Scoring

Lesions were scored on a scale of 0-4 as previously described by McDougal (McDougald and Hu, 2001). Ceca lesions are characterized by 0:Normal ceca with normal contents, 1:Thickening of the cecal wall with normal contents, 2:moderate thickening of cecal wall with small cores forming, 3:severe thickening of the cecal wall with caseous cores partially filling the lumen of

the ceca, and 4: severe thickening of the cecal wall with necrosis and caseous core completely filling the lumen of the ceca. Liver lesions are characterized by 0: normal liver, 1: 1-5 small foci, 2: numerous small foci, 3: numerous small and large foci and 4: numerous large foci and necrosis of the liver.

Statistics

Data were subject to GLM procedures for completely randomized designs by using the general linear models procedure for SAS software. The least significant difference multiple comparisons procedure was used to determine differences among treatments.

Results

For floor pen trials 1 and 2 birds were fed treatment diet 1 and cloacally inoculated with *H. meleagridis* on day 18 and in the case of trial 2 a second inoculation was performed on day 19. Turkeys that were directly inoculated with *H. meleagridis* contracted blackhead disease at a rate of 22% and 47% respectively (figure 2.1) with no signs of infection in the uninfected control group. In both floor pen trials no uninfected birds that were comingled with the direct infected birds contracted blackhead disease (0 of 68 birds for each trial). Comparison of diet 1 with diet 3, which was used in previous blackhead disease research, demonstrated that diet 3 had an amino acid imbalance and 1.5 time higher levels of sodium(Hu and McDougald, 2003).

To determine if diet can have an effect on rates of direct infection and lateral transmission treatment diets were formulated that encouraged loosening of the stool (Diet 1 supplemented with $MgSO_4$ or $MgCl_2$ or Diet 3), caused a flushing event (Diet 1 plus administration of dextran sulphate), or increased reverse peristalsis (Diet 2) (Ferket and

Veldkamp, 2015; Kuttappan, Vicuna, Latorre, Wolfenden, Tellez, Hargis and Bielke, 2015; Sacraine, 2006; Smith, Rose, Wells and Pirgozliev, 2000; van der Hoeven-Hangoor, van de Linde, Paton, Verstegen and Hendriks, 2013). 5 of 9 birds in each cage were intercloacally inoculated with *H.* meleagridis on day 9. Direct infection rates were 74% for Diet 1, 83% for Diet 1 $MgCl_2$, 76% for Diet 1 $MgSO_4$, 94% for Diet 1 Dextran, 83% for Diet 2 and 94% for Diet 3 and 0% for the uninfected control group (Figure 2). Significant differences in the rate of direct infection were observed between Diet 1-Diet 1 Dextran, Diet 1-Diet 3, Diet 1 $MgSO_4$-Diet 1 Dextran and Diet 1 $MgSO_4$-Diet 3 at p=0.05. There was no significant difference seen in the average direct ceca and liver lesion scores for birds that contracted blackhead disease in any of the treatment groups (Figure 3). As seen in figure 4 lateral transmission was only observed in Diet 1 $MgCl_2$ (4%), Diet 1 $MgSO_4$ (4%), Diet 1 Dextran (8%), Diet 2 (8%) and Diet 3 (21%). With a significant difference observed between Diet 1-Diet 3, Diet 1 $MgCl_2$-Diet 3 and Diet 1$MgSO_4$-Diet3 at p=0.05.

Discussion

Previous research in turkeys has demonstrated that lateral transmission of *H. meleagridis* occurs through the cloacal uptake of contaminated fecal dropping from infected birds (Hu, Fuller and McDougald, 2004; Hu and McDougald, 2003). The variation in the number of birds infected within a flock suggests that other factors may effect transmission of *H. meleagridis* from infected to uninfected birds. The work presented here demonstrates that diets that induce diarrhea or increase peristalsis lead to higher direct infection rates and lateral transmission. In addition, damage to the gut that leads to a flushing event has a similar outcome. Thus we propose that the transmission of blackhead disease is related to the gut health.

We were surprised that even under crowded conditions no lateral transmission occurred in either of the floor pen trials or in the battery trial when birds were fed Diet 1. In addition, direct infection rates were also lower than previously published (Abraham, McDougald and Beckstead, 2014; Armstrong and McDougald, 2011). Diet 1 is a standard turkey starter ration that contains no antiprotozoal drugs. Thus the effect that we observed is not due to anti-protozoal activity but was due to an ability of the bird to be infected with the parasite. Lateral transmission of *H. meleagridis* occurs through the process of cloacal uptake of contaminated feces (Hu, Fuller and McDougald, 2004; McDougald and Fuller, 2005). Turkeys, especially when ill, will huddle together, which increases their chance of taking up contaminated feces. Our data suggests that birds with firmer feces have a lower rate of *H. meleagridis* transmission into the ceca. Presumably, a tighter fecal dropping would be more difficult for birds to uptake through the cloaca, thus protecting uninfected birds. However, stimulation of reverse peristalsis by adding coarse corn (Diet 2) increased the percentage of birds that were directly infected and allowed for lateral transmission to occur (Sacranie, 2006). This suggests that stimulation of retrograde passage of intestinal contents plays a role in the transmission of blackhead disease.

In order to ascertain whether diets that promote a looser fecal dropping can increase blackhead disease infection rates turkeys were fed one of two different magnesium conjugates, $MgSO_4$ and $MgCl_2$, in Diet 1 or fed Diet 3. Magnesium, a common laxative, administered in excess causes an osmotic imbalance that results in reduced water retention in the ceca, with the conjugate $MgCl_2$ inducing a greater effect compared to $MgSO_4$ (van der Hoeven-Hangoor, van de Linde, Paton, Verstegen and Hendriks, 2013). Addition of magnesium to diet 1 increased the direct infection rate of *H. meleagridis* from 74% in diet 1 to 76% in diet 1 $MgSO_4$ and 84% in diet 1 $MgCl_2$ (figure 2.2). Diet 3 has an amino acid imbalance and contains 1.5 times more

sodium, which leads to increased water consumption, which results in the litter becoming more wet (Smith, Rose, Wells and Pirgozliev, 2000). Diet 3 had the highest rates of direct infection (94%) and lateral transmission (21%). These high rates of infection associated with diet 3 along with the magnesium data suggest that diet can be a contributing factor to the transmission of blackhead disease. Additionally, poor dietary formulations can lead to metabolic imbalances that reduce water retention through the ceca or stimulate excessive drinking (Ferket and Veldkamp, 2015; Sacranie, 2006; Smith, Rose, Wells and Pirgozliev, 2000).

To model insults caused by secondary pathogens, dextran sulphate was given to birds three and four days after direct cloacal infection. Dextran sulphate induces intestinal damage that results in leakage similar to that of feed restricted birds (Kuttappan, Vicuna, Latorre, Wolfenden, Tellez, Hargis and Bielke, 2015) and may resemble intestinal damage similar to that of infectious organisms. Even though the birds were infected three days prior to dextran sulfate administration there was a significant increase in the percentage of directly infected birds, suggesting that intestinal integrity may play a role in the ability of *H. meleagridis* to colonize and cause disease in the turkey. Thus organisms such as coccidia, chlostridia or *E. coli*, lack of feed and environmental stressors may be contributing factors to blackhead disease outcomes. Future research aimed at understanding the relation between gut health and *H. meleagridis* infections is merited in order to determine key factors that may predispose turkeys to severe blackhead outbreaks.

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Tables and Figures

Table 2.1. Dietary formulations

Ingredient/Supplement %	Diet 1	Basal 1-MgCl2	Basal 1-MgSO4	Diet 2-Coarse Corn	Diet 3
Corn	46.5	46.5	46.5	23.25	54.3
Coarse Corn	-	-	-	23.25	-
Soy Bean Meal	35.3	35.3	35.3	35.3	39.5
Soy Bean oil	-	-	-	-	2.424
Poultry meal	10	10	10	10	-
Limestone	1.808	1.808	1.808	1.808	0.444
Mono-Dicalcium phosphate	2.569	2.569	2.569	2.569	-
Salt NaCl	0.116	0.116	0.116	0.116	0.4
L-Lysine	0.3605	0.3605	0.3605	0.3605	-
DL-Methionine	0.3445	0.3445	0.3445	0.3445	0.116
L-Threonine	0.0765	0.0765	0.0765	0.0765	-
Poultry vitamin premix	0.15	0.15	0.15	0.15	0.5
Poultry mineral premix	0.2	0.2	0.2	0.2	0.1
Sodium selenate premix	0.05	0.05	0.05	0.05	-
Chlorine Chloride	0.225	0.225	0.225	0.225	-
Deflorinated Phosphate	-	-	-	-	2.228
Sand	0.204	-	-	0.204	-

MgCl	-	0.204	-	-	-
MgSO4	-	-	0.204	-	-

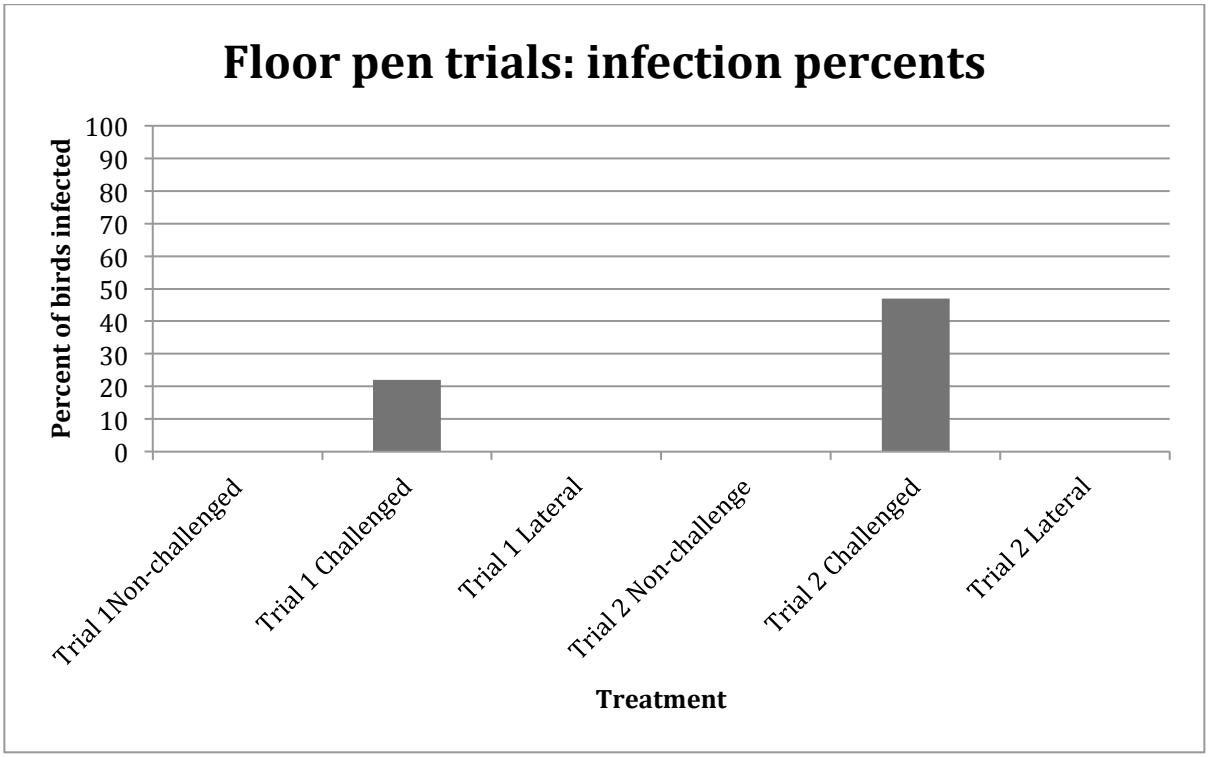

Figure 2.1. Floor pen trials: Percent of uninfected, infected and sentinel birds that showed signs of blackhead disease upon necropsy.

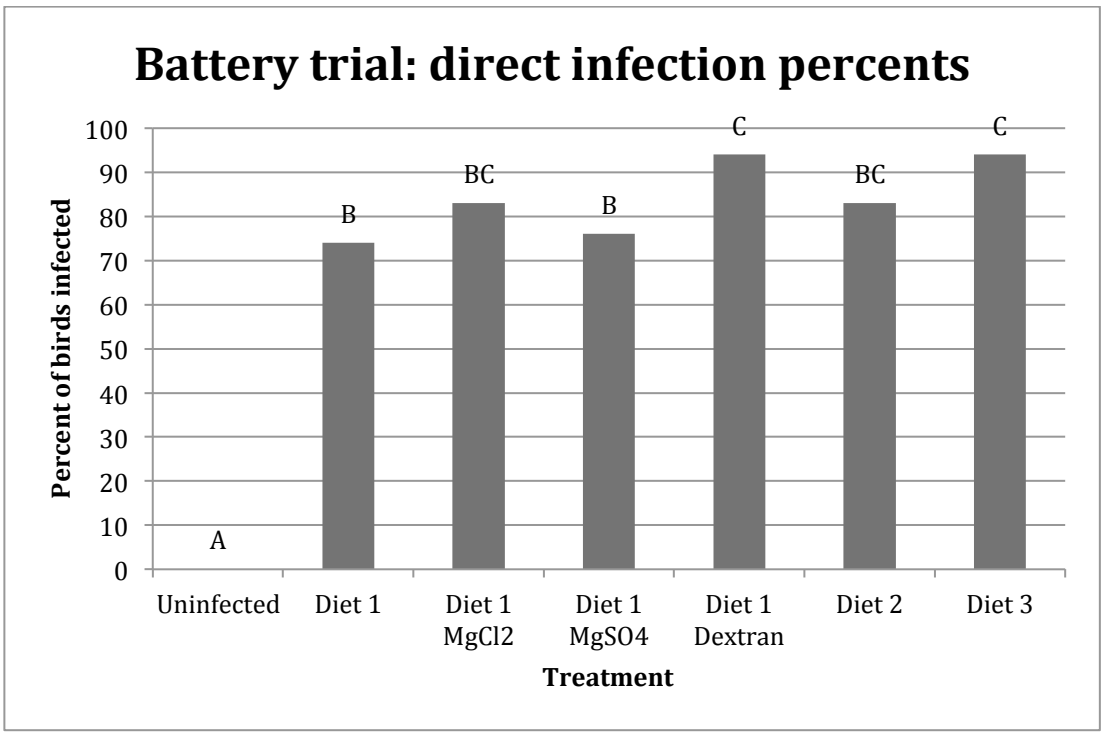

Figure 2.2. Battery trial: Percentage of birds intercloacally inoculated with *H. meleagridis* that showed signs of blackhead disease upon necropsy. Treatments not connected by the same letter are significantly different at $P<0.05$.

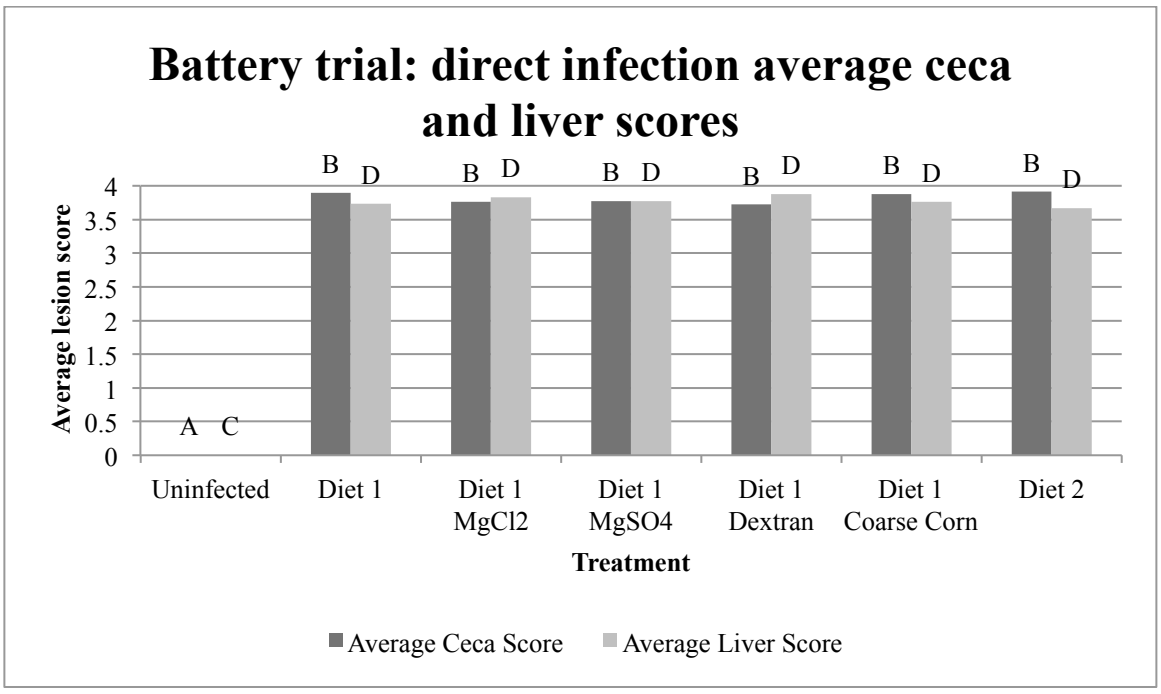

Figure 2.3. Battery trial: Average ceca and liver lesion scores. Letters A and B coorespond to ceca scores and letters C and D coorespond to liver scores. Treatments not connected by the same letter are significantly different at $P<0.05$.

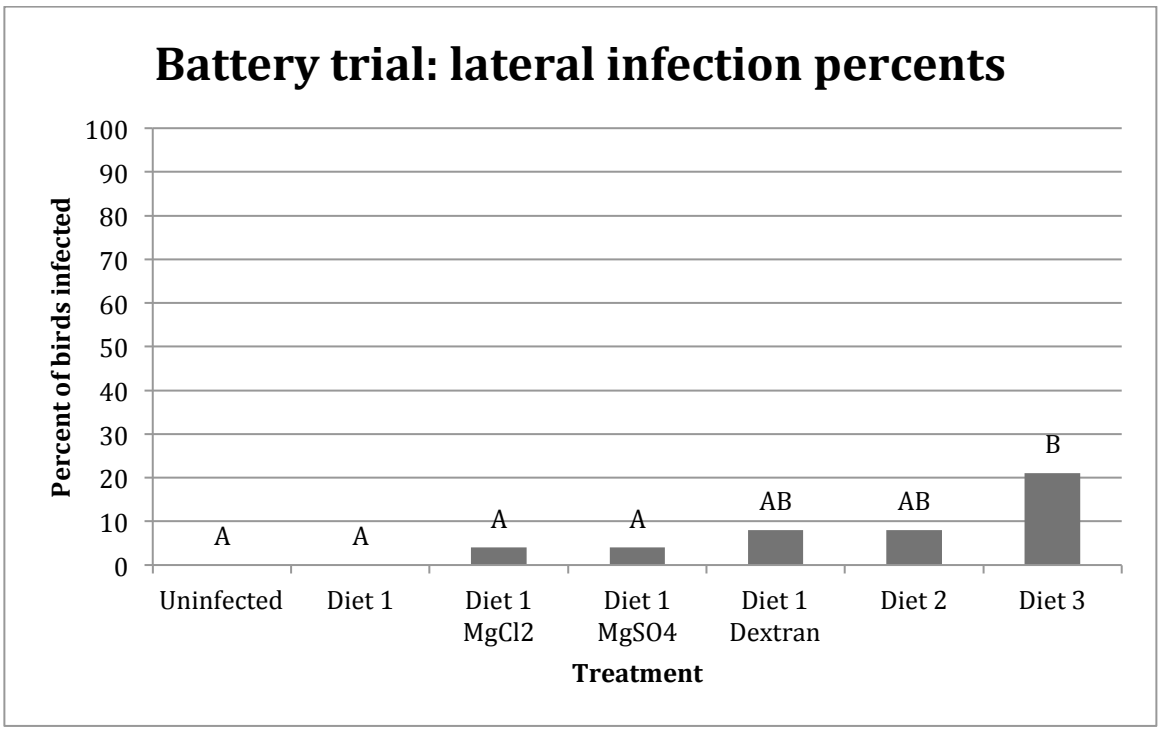

Figure 2.4. Battery trial: Percentage of sentinel birds that showed signs of blackhead after necropsy. Treatments not connected by the same letter are significantly different at p=0.05.

CHAPTER 3

EFFECT OF NATUSTAT ON *HISTOMONAS MELEAGRIDIS IN VITRO* AND *IN VIVO*[A]

[A] Jason A. Payne, Katherine Cupo and Robert B. Beckstead. To be submitted to Avian Diseases.

Abstract

Blackhead, a potentially lethal disease in turkeys, has had a reemergence of outbreaks since the 1990s coinciding with the removal of the efficacious drugs that were being used to treat the disease. With the recent removal of the last known drug for treating the disease there are currently no known approved drugs for treating or preventing blackhead outbreaks. Natustat, a proprietary plant blend previously reported to provide protection against blackhead infections, was investigated to further understand its mode of action in controlling blackhead disease outbreaks. Natustat was tested for anti-histomonal activity *in-vitro* and in a live bird model. The *in vitro* data indicates that Natustat inclusion at or above 2000ppm is able to inhibit the growth of the causative agent, *H. meleagridis*. A battery trial was performed where directly inoculated birds were caged with sentinel birds, on a control diet or a diet supplemented with Natustat at 3000ppm. Directly inoculated birds from the control and Natustat groups had 94% and 91% direct infection rates respectively, with 21% of the control and 18% of the Natustat sentinel birds that contracted blackhead disease. There was no difference between the control and Nautstat supplemented birds' average ceca or liver lesions scores associated with the disease. This data suggest that Natustat under the conditions tested is not able to provide turkeys with a protection against blackhead disease.

Introduction

Histomonas meleagridis is the causative agent of blackhead disease and is of financial concern to the turkey industry. Early research identified the cecal worm *Heterakis* gallinarum as the main vector and reservoir of the protozoan parasite (Farr, 1961; Gibbs, 1962). The initial mode of infection typically occurs when birds consume embryonated *H. gallinarum* eggs infected with *H. meleagridis*. Outbreaks in turkey facilities can quickly spread from a single infected bird to the entire flock, bypassing the host vector, through a process involving cloacal drinking (Armstrong and McDougald, 2011; Hu and McDougald, 2003; McDougald and Fuller, 2005). In turkeys, symptoms of blackhead disease include a loss of appetite, listlessness, weight loss, yellow droppings and, if left untreated, results in death, with necropsies typically revealing caseous cecal cores and severe liver lesions.

Until recently in the United States, blackhead disease was partially controlled through the addition of the drug Nitarsone, an anti-histomonal that reduced the shedding of *H. meleagridis* and essentially blocked the transmission of the disease in affected facilities (van der Heijden and Landman, 2008b). Nitarsone was used as a preventative of blackhead disease rather then a cure. However, the removal of Nitarsone by the FDA as the last treatment for blackhead disease has left the turkey industry with few options to control outbreaks (Nachman, Love, Baron, Nigra, Murko, Raber, Francesconi and Navas-Acien, 2017). *H. meleagridis in-vitro* compound screens were developed in 2002 by Calliat et al. in order to establish a methodology for identify compounds that inhibit growth of *H. meleagridis* (Callait, Granier, Chauve and Zenner, 2002). His group showed that known anti-histomonal drugs, could be added to media and maintain their anti-protozoal activity. This methodology established a means of screening compounds and

drugs that might have activity against *H. meleagridis* without having to test each compound in live bird trials.

Several reports have investigated the *in vitro* activity of plant extracts and essential oils against *H. meleagridis* (Grabensteiner, Liebhart, Arshad and Hess, 2008; Hauck and Hafez, 2007; Thofner, Liebhart, Hess, Schou, Hess, Ivarsen, Frette, Christensen, Grevsen, Engberg and Christensen, 2012; van der Heijden and Landman, 2008a; Zenner, Callait, Granier and Chauve, 2003). One such additive, Natustat, was fed to turkeys placed on used litter containing *H. gallinarium* eggs and showed similar rates of infection compared to birds fed Nitarsone. Additionally, the Natustat fed birds showed lower average cecal and liver lesion scores when compared to birds fed a control diet. A later report summarizing Natustat's efficacy in preventing blackhead disease in turkeys and chickens proposed that Natustat's "modes of action include decreasing the level of protozoa present via anti-protozoa activity, improving immune competence and aiding tissue repair in the bird following infection (Sims, Duffy, Power and Hooge, 7/10/2007)." The current study was performed to further understand the mechanisms by which Natustat may be regulating *H. meleagridis* infections.

Materials and Methods

Strain

A field-isolated strain of *H. meleagridis* was obtained from a blackhead outbreak in Buford Georgia and frozen in liquid nitrogen. For bird trials the original field-isolated strain was thawed from liquid nitrogen 7 days prior to cloacal inoculation. From this field strain a monoculture was produced and subsequently attenuated through repeated passages in media. This attenuated monoculture was used for *in vitro* compound screening.

Media

H. meleagridis was cultured in modified Dwyer's media which consist of 10.6 g/L M199 media with Hanks balanced salts supplemented with 10% horse serum, 0.35g/L NaBicarb and 0.8g/L rice powder (van der Heijdena and Landman, 2007).

Compound Screens

For the *in-vitro* trials Natustat was freshly prepared in Dwyer's media without rice powder and aliquoted into three 25cm^2 unvented culture flask per treatment. The final flask concentrations of Natustat for trial 1 were 0ppm, 50 ppm, 500 ppm and 5000 ppm. The final flask concentrations of Natustat for trial 2 were 0 ppm, 1000 ppm, 2000 ppm, 3000 ppm, 4000 ppm and 5000 ppm. All flasks were inoculated with 1X10^5 *H. meleagridis* cells per mL then incubated for 48 hours at 42^{o}C. All flasks were counted six times using a Neubauer hemocytometer.

***In vivo* experimental design**

North Carolina State University IACUC approved the animal research presented in this paper. Three dietary treatment groups were included in the study. 1)Non-challenged, non-supplemented control; 2) Challenged, non-supplemented control; 3) Challenged, Natustat supplemented (3000 ppm). The dietary formulation for this study is found in table 3.1.

Day old birds were placed in clean 2ft x 2ft x 1.5ft batteries with 9 birds per cage and 7 pens per treatment. Birds were given ad libitum access to treatment diets and water from the day of placement. Litter shavings were placed in the bottom of battery cages over a sheet of paper on day 7 to simulate floor pen conditions. On day 9, 5 birds from each challenge group pen were wing banded and inoculated intercloacally with 5x10^5 *Histomonas meleagridis* cells in 1mL of

culture medium. Birds were monitored daily for morbidity and mortality. Birds showing severe morbidity or found dead were necropsied and scored for lesions of *Histomonas meleagridis* in both the ceca and liver. 19 days post infection the study was terminated and all birds were necropsied and scored for signs of blackhead disease.

Lesion Scoring

Lesion scoring was performed as described by McDougal(McDougald and Hu, 2001). In short lesion from the ceca and liver are scored on a 0-4 scale. In the ceca scores are determined by: 0 - no signs of blackhead disease, 1- moderate thickening of the ceca wall with normal contents, 2- moderate thickening of the ceca wall with small caseous cores, 3- severe thickening of the cecal wall with caseous cores partially filling the lumen with hemorrhagic mucosa, 4- severe thickening of the cecal wall with caseous core completely filling the lumen and epithelial necrosis. In the liver scores are determined by: 0- no signs of blackhead disease, 1- one to five small foci, 2- more then five small foci, 3- numerous small foci and large foci, 4-numerous large foci and necrosis of the liver.

Statistics

Data were subjected to GLM procedures for completely randomized designs by using the general linear models procedure of SAS software. Tukeys least significant difference multiple comparisons procedure was used to determine differences among treatments.

Results and Discussion

In vitro compound screenings are a means of identifying substances that may have a deleterious effect on certain organisms or cell types (Grabensteiner, Liebhart, Arshad and Hess, 2008; Thofner, Liebhart, Hess, Schou, Hess, Ivarsen, Frette, Christensen, Grevsen, Engberg and Christensen, 2012). In order to determine whether Natustat has anti-histomonal abilities, an *in vitro* screen was performed with levels of inclusion at 50, 500 and 5000ppm with *H. meleagridis* growth being inhibited at 5000 ppm (figure 3.1A). To better delineate the concentration at which Natustat is able to inhibit *H. meleagridis* growth the *in vitro* experiment was repeated with Natustat inclusion at 1000, 2000, 3000, 4000 and 5000ppm. At 2000ppm there was a sharp decline in the growth of *H. meleagridis* with complete inhibition of growth at concentrations of 3000ppm or greater (figure 3.1B). This data suggest that between 2000 and 3000ppm that Natustat acts as an *in vitro* anti-histomonal.

Previous studies have found that *in vitro* anti-histomonal compound concentrations can correspond to the same levels of compound inclusion in feed.
In-vitro compound screens have shown that nitarsone can hinder the growth of *H. meleagridis* with as little as 50 ppm and effectively deplete cultures of the parasite at a concentration of 200 ppm (Barrios, 2015). The *in vitro* inclusion of Nitarsone at 200 ppm and its feed inclusion level at 175 ppm show a similar anti-histomonal activity. In the present Natustat compound screen, anti-histomonal activity became evident at a concentration at 2000 ppm with cultures becoming depleted at 3000ppm. Natustat has been fed to turkeys at both 2.2 lbs/ton and 3.8 lbs/ton, which correspond with concentrations of 1100 ppm and 1900 ppm respectively in the feed. Since these levels of inclusion in the feed fall below the *in*-vitro level of anti-histomonal activity, an inclusion level of Natustat at 3000 ppm (6lbs/ton) was used for this trial.

To determine the infection rate for direct and laterally infected birds fed Natustat 5 of 9 birds in each pen were intercloacally inoculated with $5x10^5$ *H. meleagridis*. Infected turkeys in both the control challenged and Natustat challenged groups contracted blackhead disease at a similar rate (figure 3.2) and showed no significant difference in their average ceca and liver lesion scores (figures 3.3). Once sentinel turkeys began to manifest signs of blackhead disease the trial was terminated and all birds were euthanize, necropsied and examined for signs of blackhead disease. 18% of the sentinel birds fed Natustat and 21% of the sentinel control birds showed signs of blackhead disease (figure 3.4). In this study there was no evidence of a reduction in disease symptoms or the lateral transmission of the disease. This indicates that Natustat was not able to provide turkeys with a protection against *H. meleagridis* under the conditions tested.

The route of infection in this study, intercloacal inoculation, differed from the previous Natustat reports, which used of litter contaminated with *H. gallinarum* eggs. As seen in figure 2 and 4, Natustat did not reduce the percent of birds that were infected and there was no difference in the number of birds that were infected through lateral transmission. It is possible that the difference seen in this study compared to previous research is the result of a higher infection rate achieved in this study. We believe that the direct inoculation of *H. meleagridis* is a better means of determining whether a product is able to have an effect due to its ability to guarantee each bird in the study is challenged with *H. meleagridis.*

The original report's use of *H. gallinarum* eggs infected with *H. meleagridis* to infect birds through an oral route possibly resulted in a lower infection dose than was delivered through the direct inoculation use in this study. This methodology may have allowed for a sufficient dose of Natustat to act as an anti-histomonal. However, we did not see a reduction in the lateral

transmission of the disease when compared to the control birds leading to the conclusion that Natustat is unable to block lateral transmission.

The diet used in this study was formulated to induce flushing by causing an amino acid imbalance and providing an excess of sodium. This diet leads to excess drinking and a loosening of the stool. Recent research in our laboratory has shown that transmission of blackhead disease is directly related to flushing (data not shown). In previous studies, lateral transmission was blocked when birds were fed this same diet supplemented with Nitarsone after intercloacal challenges of this same strain of *H. meleagridis* (Abraham, McDougald and Beckstead, 2014). This suggests that anti-histomonals can reduce the level of lateral transmission even when flushing occurs. However, in the current study there was no evidence of a reduction in disease transmission indicating that Natustat may not be acting as an anti-histomonal in live birds. It is possible that Natustat is acting to promote intestinal health to reduce flushing in turkeys. Natustat's reported ability to protect broilers against coccidia (Duffy, Mathis and Power, 2005) may directly transfer over to turkey intestinal health and lead to reduced instances of flushing. This could possibly result in turkeys that are less vulnerable to blackhead disease infections and lateral transmission.

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Tables and Figures

Table 3.1. Dietary formulations

Ingredient/supplements%	Control Diet	Natustat Diet
Corn	54.3	54.3
Soy bean meal	39.5	39.5
Limestone	0.4	0.4
Salt	0.4	0.4
Defluor phosphate	2.2	2.2
Trace minerals	0.1	0.1
Vitamins	0.5	0.5
D.L. Methionine	0.1	0.1
Soy bean oil	2.4	2.4
Natustat	-	0.3

Figure 3.1 A

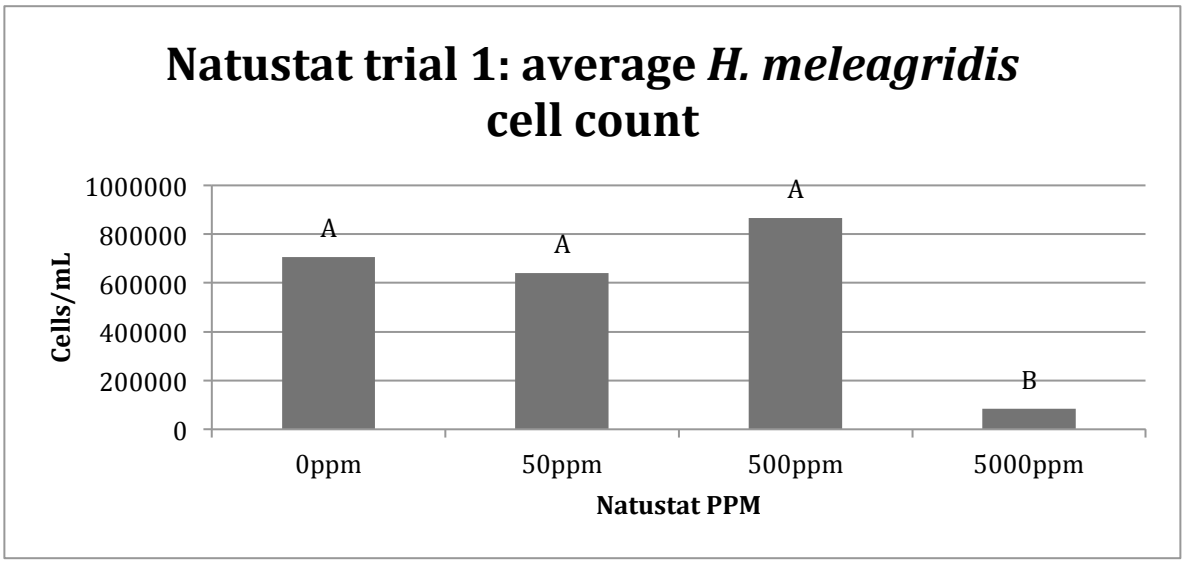

Figure 3.1 B

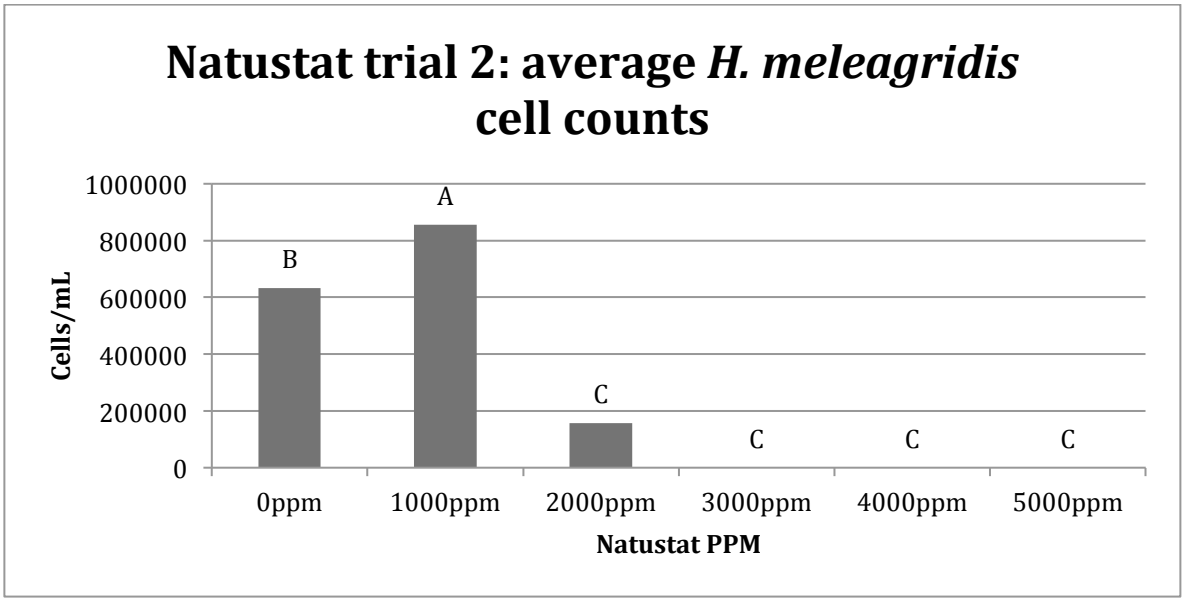

Figure 3.1. Average *H. meleagridis* cell counts after 48 hours in the presence of different Natustat concentrations. Trial 1 and 2 were performed on different days. Treatments not connected by the same letter are significantly different at $P < 0.05$.

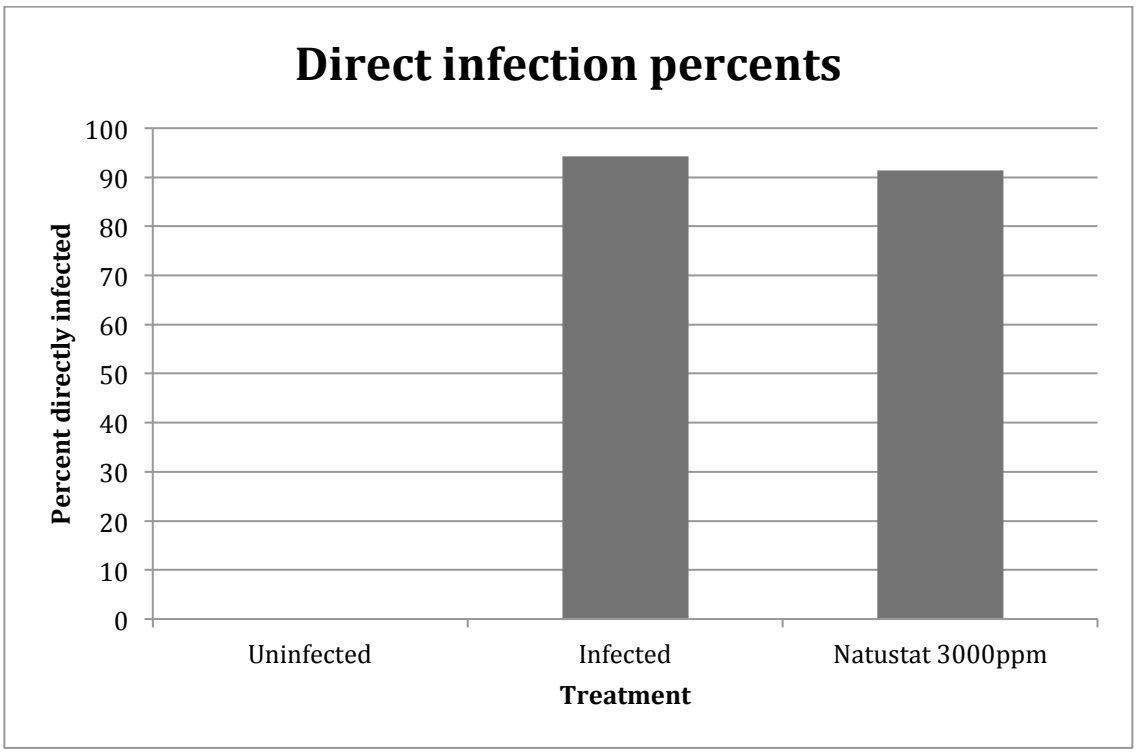

Figure 3.2. Graphical representation of the percentage of birds directly inoculated with *H. meleagridis.* Any birds showing signs of blackhead disease were considered positive.

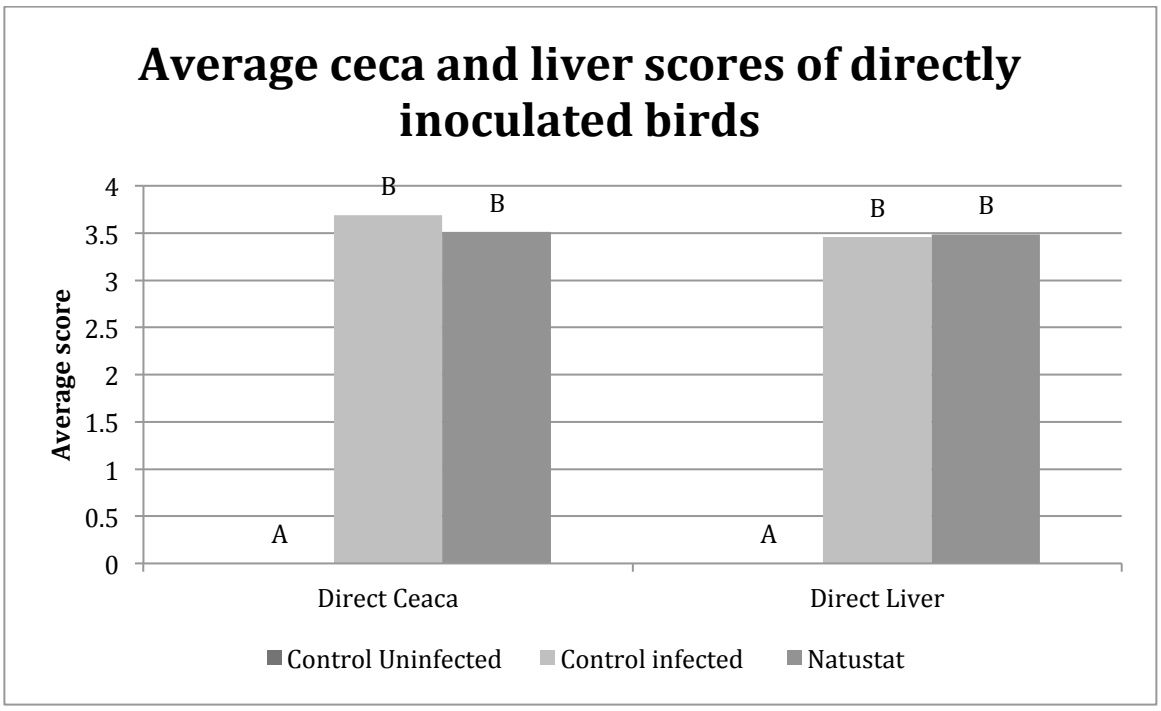

Figure 3.3. Graphical representation of *H. meleagridis* average cecal and liver lesion scores based in diet. Treatments not connected by the same letter are significantly different at $P < 0.05$.

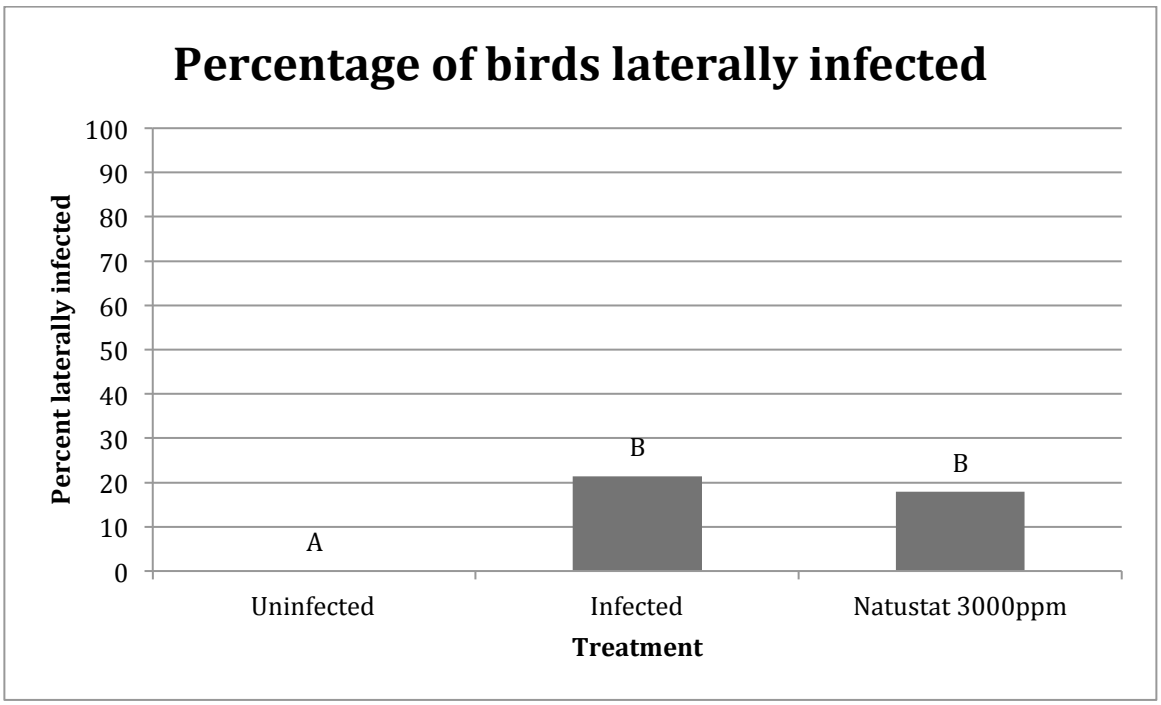

Figure 3.4. Graphical representation of the percentage of birds laterally infected with *H. meleagridis.* Any birds showing signs of blackhead disease were considered positive.

CHAPTER 4

A YEAST FERMENTATION BY-PRODUCT FAILS TO PROTECT TURKEYS AND CHICKENS FROM *HISTOMONAS MELEAGRIDIS* INFECTION[1]

[1] Jason A. Payne and Robert Beckstead. To be submitted to Poultry Science

Abstract

The present study was performed to determine whether yeast fermentation by-product (FBP) could boost or alter the innate immune response of turkeys and broilers when challenge with *H. meleagridis* the causative agent of blackhead disease. In turkeys, a direct infection and a lateral transmission trials were performed using the FBP at 1250 ppm and 2000 ppm in the feed to determine whether the FBP could alter the turkey's ability to fight off a *H. meleagridis* infection. The FBP did not provide turkeys with protection against infection, reduce the severity of cecal or liver lesions, or reduce the rate at which blackhead disease spread from infected to uninfected birds. Analysis of the turkeys' innate immune response indicated that the FBP was not able to significantly alter the transcription of key innate immune response genes. In the broiler trial, there were no significant differences in performance parameters between the uninfected and infected groups following an intercloacal inoculation of *H. meleagridis,* regardless of FBP supplementation. The supplementation of the FBP at 2000 ppm significantly increased the average bird weight at 35 days of age. This data suggest that the FBP used did not provide turkeys with a protection against *H. meleagridis* and that broiler may not be as susceptible to *H. meleagridis* infections as other types of chickens.

Introduction

H. meleagridis is the causative agent of blackhead disease that has led to financial losses in both turkey and chicken facilities. In chickens, mortality up to ~30% and performance related issues have been reported, while infections in turkey facilities can result in the loss of entire flocks. The difference in levels of mortality between chickens and turkeys is believed to be the result of the turkeys' insufficient immune response to *H. meleagridis* infections (Powell, Rothwell, Clarkson and Kaiser, 2009). Until recently, blackhead disease was managed with the feed additive Histostat 50, which reduced the transmission of blackhead disease within flocks. However, its removal was mandated by the FDA in 2016 due to the arsenic contained within the compound (Nachman, Love, Baron, Nigra, Murko, Raber, Francesconi and Navas-Acien, 2017). With no known approved feed additives or drugs to treat, prevent or reduce lateral transmission of blackhead disease, there is a need to identify treatments that may provide poultry with some level of protection when infected with *H. meleagridis*.

Various strategies including the addition of sub-therapeutic levels of antibiotics, prebiotics, probiotics and fermentation by-products (FBP) to the feed have been employed to encourage gut health (Ahmed, Mun, Islam, Kim, Hwang, Kim and Yang, 2014; Bednarczyk, Stadnicka, Kozlowska, Abiuso, Tavaniello, Dankowiakowska, Slawinska and Maiorano, 2016; Chou, Park, Carey, McIntyre and Berghman, 2017; Forkus, Ritter, Vlysidis, Geldart and Kaznessis, 2017; Gusils, Perez Chaia, Gonzalez and Oliver, 1999; Parks, Grimes and Ferket, 2005). Of importance to the research herein is the use of FBP due their potential immunomodulatory properties. FBP derivatives, in particular from yeast, contain compounds such as B-glucan, mannan oligosaccharides and nucleotides. B-glucans are known to activate macrophage's phagocytosis, production of cytokines and eicosanoids (Abel and Czop, 1992;

Doita, Rasmussen, Seljelid and Lipsky, 1991; Gao, Zhang, Yu, Wu, Yoon, Quigley, Gao and Qi, 2008). Mannan oligosaccharides can stimulate turkey's serum IgG, bile IgA antibody levels and bind gram negative pathogenic bacteria (Ferket, Parks and Grimes, 2002; SAVAGE, COTTER and ZAKRZEWSKA, 1996). Nucleotides can enhance spleen cell proliferation and the production of cytokines IL-2 and IFN-y (Adjei, Jones, Enriquez and Yamamoto, 1999; Carver, 1994; Gil, 2002). FBP have been reported to also encourage the maturation of T cells as evidenced through increased CD3+, CD4+ and CD8+ T lymphocyte expression (Gao, Zhang, Wu, Yu, Yoon, Moore, Gao, Yan and Qi, 2009). This data suggests that FBP may be a means of boosting the immune response of poultry and potentially allow for some of the health-related issues to be alleviated. The following studies were performed in order to test a hypothesis that a FBP could boost the innate immune response of turkeys and broilers when challenged with *H. meleagridis* and help mitigate mortality and performance losses.

Materials and Methods

Strain

The *H. meleagridis* strain used in these studies was isolated from field outbreaks in Buford, GA. This isolate was cultured and frozen in liquid nitrogen. Cultures were resuscitated and cultured at 42^{o}C in Dwyers media for experiments.

Treatments diets

A standard starter corn-soybean meal ration was used without antibiotics or coccidiostats as the basal ration for all treatment diet preparations. All diets (tables 4.1 and 4.2) were formulated to meet or exceed nutrient concentrations recommended by the NRC (1994). Treatment diets were

either a control diet, a diet supplemented with Nitarsone at 187ppm or a diet supplemented FBP at 1250 ppm or 2000 ppm. Treatments consisted of both infected and uninfected groups on each diet.

Animal Care and experimental design

All turkeys were raised following protocols established by The University of Georgia Institution of Animal Care and Use Committee. Experiments were conducted using day-old male poults (Aviagen, Lewisburg, WV). Poults were maintained on a 14 h lighting schedule in a thermostatically controlled room with *ad libitum* access to treatment diets and water starting at day 1.

For the direct inoculation trial, poults were placed in custom design battery cages with 6 cages per treatment and 7 poults per cage. On day 18, poults were challenged intracloacally with 20,000 histomonads/bird using a blunt-tipped pipette inserted about 3 cm into the cloaca. Body weights and feed weights were recorded on infection day as well as upon termination for growth performance data. Deceased birds were removed as necessary and necropsied. Ten days post infection, birds were euthanized by cervical dislocation and necropsied to determine cecal and liver lesions.

For the lateral transfer trial, poults were placed 3 pens per treatment with each pen containing 30 poults housed in steam-sterilized floor pens (1.85 m2) with fresh litter. On day 10, poults were weighed resorted within treatments so that each pen has similar body weights. On day 18, five out of the 30 turkeys per inoculated treatment (Treatment 1, 3, 5 and 7) were intracloacally infected with 1 mL of *H. meleagridis* culture for a total of 100,000 histomonads per bird, while the others remained uninoculated, but free to interact with the infected birds. The

trial was terminated when approximately 80% total mortality was reached in the infected control birds and remaining birds were euthanized by cervical dislocation and necropsied to determine cecal and liver lesions. Tissue samples of the ceca and liver of 5 birds per treatment were collected for gene expression analysis at day 5 and day 10 post infection and stored at -80C in RNAlater (see RNA Extraction and isolation below).

For the broiler uniformity trial 788 Cobb 500 by-product male chicks were obtained from a local hatchery wing banded, weighed, and placed in floor pens with fresh pinewood shavings. Each of the 6 experimental diets (table 4.7) was replicated with 4 pens each containing 32 chicks. Chicks were maintained on an 18 hours light and 6 hours dark schedule in a thermostatically controlled room with temperatures set based on the Cobb broiler management guidelines. Chicks had *ad libitum* access to treatment diets and water starting on day 1. Birds were on a starter diet from days 0-14, a grower diet from days 14-28, and a finisher diet from days 28-35. Birds were weighed individually on days 14, 28, and 35. On day 14, chicks were inoculated intracloacally with 30,000 histomonads/bird. At termination, broilers were individually weighed to calculate growth parameters and necropsied for ceca and livers lesions. Feed was weighed throughout the study for calculation of feed conversion.

Lesion scoring

Cecal lesions were scored as: 0: none**,** 1: moderate thickening of the cecal wall and normal cecal content**,** 2: moderate thickening of the cecal wall and caseous core partially filling the lumen and slightly hemorrhagic mucosa**,** 3: severe thickening of cecal wall and caseous core partially filling the lumen and hemorrhagic mucosa**,** and 4: severe thickening of the cecal wall and caseous core totally filling the lumen and epithelial necrosis of the mucosa. The liver lesions were scored as:

0: none, 1: 1 - 5 small foci, 2: more than five small foci, 3: numerous small and large foci, and 4: numerous large foci and extended necrosis. Turkeys were considered infected if they had a cecal or liver score of 1 or greater.

RNA isolation and gene expression analysis

RNA was extracted using TRIzol reagent (Invitrogen, Grand Island, New York, USA). Gene expression of CXCLi2, IFN-γ, Il1B, IL-10, Il13, and IL4 (tables 4.3-4.6) was determined using the TaqMan RNA-to-Ct 1-Step Kit (Fermentas Life Sciences, Clen Burnie, Maryland, USA) on a StepOneTM real-time PCR instrument (Applied Biosystems, Carlabad, California, USA). Primer and probe sequences were described by Powell et al. 2009 (Powell, Rothwell, Clarkson and Kaiser, 2009)

Statistics

Data were subjected to GLM procedures for completely randomized designs by using the general linear models procedure of SAS software. For live bird trials, the Tukey least significant difference multiple comparisons procedure was used to determine differences among treatments. Significance was determined at $P \leq 0.05$. For rtPCR analysis logarithmic transformation was performed before being analyzed by the Student's T test. Standard deviation was calculated using the fold change values of three replicates from each gene measured.

Results and Discussion

In order to determine whether a yeast FBP could boost the innate immune response of turkeys and thereby enable the bird to mount an effective immune response against an infection of *H. meleagridis*, a direct infection study was performed. Poults were raised in batteries for 18 days prior to infection with *H. meleagridis*. Turkeys on the yeast FBP diet at 1250 ppm and 2000 ppm had 100% and 94% infection rate respectively. Yeast FBP infection rates did not differ significantly from infection rates in control birds (95%) (figure 4.1). There was no difference seen in average ceca and liver lesions scores between any of the treatment groups (figure 4.2).

A direct dose of 20,000 *H. meleagridis* in the turkey leads to high infection rates. It is possible that with this high level of infection, that the turkey is unable to mount an immune response prior to the parasite causing signs of blackhead disease. Lateral transmission studies correlate more closely with the natural transmission and infectious dose of the disease in the turkey flock (Armstrong and McDougald, 2011; Hu and McDougald, 2003; Liebhart, Grabensteiner and Hess, 2008; McDougald and Fuller, 2005). To test if the yeast FBP could provide protection under these conditions, a lateral transmission trial was performed where uninfected birds were placed in pen with turkeys infected with *H. meleagridis*. Histostat 50 (nitarsone) was used as a positive control for reduced transmission of *H. meleagridis*. As seen in figure 3, two of the 3 infected pens on the control diet had lateral transmission rates of blackhead disease at 65% and 71%, while none of the pens containing Histostat50 had lateral mortality above 8%. 1 out of the 3 pens of turkeys fed a diet of 1250 ppm of the yeast FBP had lateral mortality of 92%, while 2 out of the 3 pens of turkeys fed a 2000 ppm of the yeast FBP had lateral mortality of 92% and 96% (figure 4.3). Lateral transmission of blackhead disease is dependent upon the parasite, the density of the birds, the behavior of the birds, and condition of

the litter (Armstrong and McDougald, 2011; Liebhart, Grabensteiner and Hess, 2008; McDougald and Fuller, 2005). Thus, lateral transmission is not always observed under experimental conditions. We conclude that inclusion of the yeast FBP did not inhibit lateral transmission in that blackhead as evidenced by high lateral mortality in 1 or more pens in the treatment groups.

Since the yeast FBP did not improve the turkey's outcome to H. meleagridis infections, further analysis was performed to determine whether the yeast FBP was able to modulate innate immune response genes in the turkey. Gene expression analysis for key immune genes Cxcli2, IL10, IFN-g, IL1B and IL13 was performed on RNA samples taken from the ceca and liver of all turkey from all treatment groups at days 5 and 10 post challenge. As shown in tables 4.3-4.6. No significant difference in gene expression was observed in uninfected and infected turkeys grown on diets that contained a yeast FBP. Based on these results, it is not surprising that birds on the yeast FBP had similar direct and lateral infection rates compared to control birds. Thus, it is possible, that a product that boost the immune response of the turkey, could improve the response of the turkey to *H. meleagridis* infection.

Changes in gene expression profiles for Cxcli2, IFN-g and IL1B in the liver of turkeys infected with *H. meleagridis* in this study differed with a previously published report (Powell, Rothwell, Clarkson and Kaiser, 2009). A sufficient innate immune response to a parasitic infection would involve Cxcli2, IFN-g, IL1B and IL13 being up regulated early on during an infection with IL10 initially being suppressed followed by increased levels to regulate the immune response (Parkin and Cohen, 2001). As indicated in tables 4.3 and 4.4 turkeys did not elicit a sufficient immune response in the ceca. Cxcli2 up-regulation is associated with an inflammatory response that is accompanied by the infiltration of heterophil and macrophages. In

our study, Cxcli2 is highly upregulated in the ceca of infected poults at both days 5 and 10 indicating that the turkeys recognize the infection of *H. meleagridis* in the ceca and respond through inflammatory mediators. In the ceca IL13 is an inducer of glycoprotein hypersecretion that is meant to make the gut unsuitable for invading organisms. At both days 5 and 10 the cecal expression of IL13 is highly upregulated indicating that the ceca is responding to the infection by trying to flush the *H. meleagridis* out of the organ, however, it's more then 10 fold increase from day 5 to 10 indicates a continued infection. A persistent parasitic infection can also be indicated by a lack of IFN-g expression, with an upregulation indicative of the clearing of a parasite (Guo, Stroup and Houpt, 2008). IFN-g was not detected in the ceca at day 5, but was detected in infected birds at day 10. IL10 in the ceca at both days 5 and 10, is present at similar or lower levels then the uninfected control birds, indicating that turkeys are not able to regulated the immune response and mitigate the damage cause by excessive inflammation and hypersecretions. Additionally, IL1B, which is an activator of adaptive immune response, is only slightly elevated in the infected groups. This indicates that there may only be a moderate induction of the adaptive immune response.

In the liver, the immune response to *H. meleagridis* infection suggest that the parasite is actively suppressing the immune response. In control infected livers, IL10 is expressed at 306 times the level of noninfected birds (table 4.5). At this time, only a few parasites would have migrated to the liver, but IL10 levels suggest that the immune response is already being shut down in the liver of infected turkeys. At 10 days post infection, IL10 expression is still 157 times higher in the liver of infected birds compared to noninfected controls (table 4.6). Day 10 levels of expression of IFN-g, Cxcli2, IL13 genes are similar between infected and noninfected livers, indicating that the immune response in the liver is not active, even though severe infection

is evident. The immune expression data suggest that infection of the parasite in the ceca promotes inflammation allowing the parasite to enter the blood stream and migrate to the liver. Once in the liver, not only does the turkey fail to mount an immune response, but it appears that the immune response is suppressed by high levels of IL10 expression. In both the ceca and the liver, the immune system appears to promote *H. meleagridis* destruction of the tissues that ultimately results in the death of the turkey.

H. meleagridis has been shown to effects performance in chickens (AAAP Disease Report 1983). To establish whether *H. meleagridis* infection effects production parameters in broilers and determine whether a yeast FBP can rescue these changes, broilers were challenged on day 14 with *H. meleagridis*. Birds were monitored weekly for weight gain and signs of blackhead disease. No difference in weight gain, feed conversion ratio, or coefficient of variation where observed between control and *H. meleagridis* infected broilers. Additionally, no clinical signs of blackhead disease were observed in the infected and control birds. As opposed to other strains of chickens, broilers may not be as susceptible to *H. meleagridis* infections.

The inclusion of the yeast FBP in the broiler trial at 1250 ppm and 2000 ppm did not significantly improve feed conversion ratio for either the control or FBP diets (table 8). Inclusion of the FBP was positively correlated with a decrease in coefficient of variation (CV) at days 14 ($P = 0.04$) and 28 ($P = 0.05$) indicating that the inclusion of the FBP increased the uniformity of the flock at those time points. At day 35 there was no significant difference in CV among any of the treatment groups (table 4.9). There was a significant difference of mean weight gain in uninfected birds that are fed the FBP at 2.5 lb/ton post infection from days 14-28 and a significant difference in the uninfected FBP 2.5lb/ton day 28 mean treatment weight (tables 4.11 and 4.12). At day 35 the uninfected FBP at 4 lb/ton showed a significant difference in weight

gain from days 0-35 as well as from days 28-35 when compared to other groups (tables 4.11 and 4.12). This data suggests that supplementation with a yeast FBP in broilers may be advantageous to help boost broiler weight. Because infection with *H. meleagridis* did not alter growth parameters, the activity of this yeast FBP in regards to blackhead disease in broilers, its potential benefit in controlling blackhead disease in other chickens needs to be evaluated.

In summary, the inclusion of FBPs in the feed does not sufficiently alter or boost the innate immune response of turkeys and allow them to respond to *H. meleagridis* infections in a timely manner. Despite reports that chickens suffer performance losses after *H. meleagridis* infections, broiler performance data suggest that they may not be as susceptible to infections as other poultry breeds. Additionally, performance data coincides with previous reports that yeast FBP can increase performance parameters of broilers, however, there was no difference seen in feed conversion ratio as previously reported. This data suggests that a yeast FBP alone may not be a viable means of controlling blackhead disease outbreaks although yeast FBP may aide in increasing bird performance and overall bird health.

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Tables and Figures

Table 4.1. Treatment identification and fermentation by-product concentrations used in the direct challenge trial.

Treatment ID	Treatment	Fermentation By-Product Concentration
1	Uninoculated Control	N/A
2	Inoculated Control	N/A
3	Uninoculated FBP	1250ppm
4	Inoculated FBP	1250ppm
5	Uninoculated FBP	2000ppm
6	Inoculated FBP	2000ppm

Fermentation by product (FBP).

Table 4.2. Treatment identification, Nitarsone concentrations and fermentation by-product concentrations used in the lateral transmission trial.

Treatment ID	Treatment	Nitarsone Concentration	Fermentation By-Product Concentration
1	Uninfected Control	N/A	N/A
2	Infected Control	N/A	N/A
3	Uninfected FBP	N/A	1250ppm
4	Infected FBP	N/A	1250ppm
5	Uninfected FBP	N/A	2000ppm
6	Infected FBP	N/A	2000ppm
7	Uninfected Nitarsone	187.5 ppm	N/A
8	Infected Nitarsone	187.5 ppm	N/A

Fermentation by product (FBP).

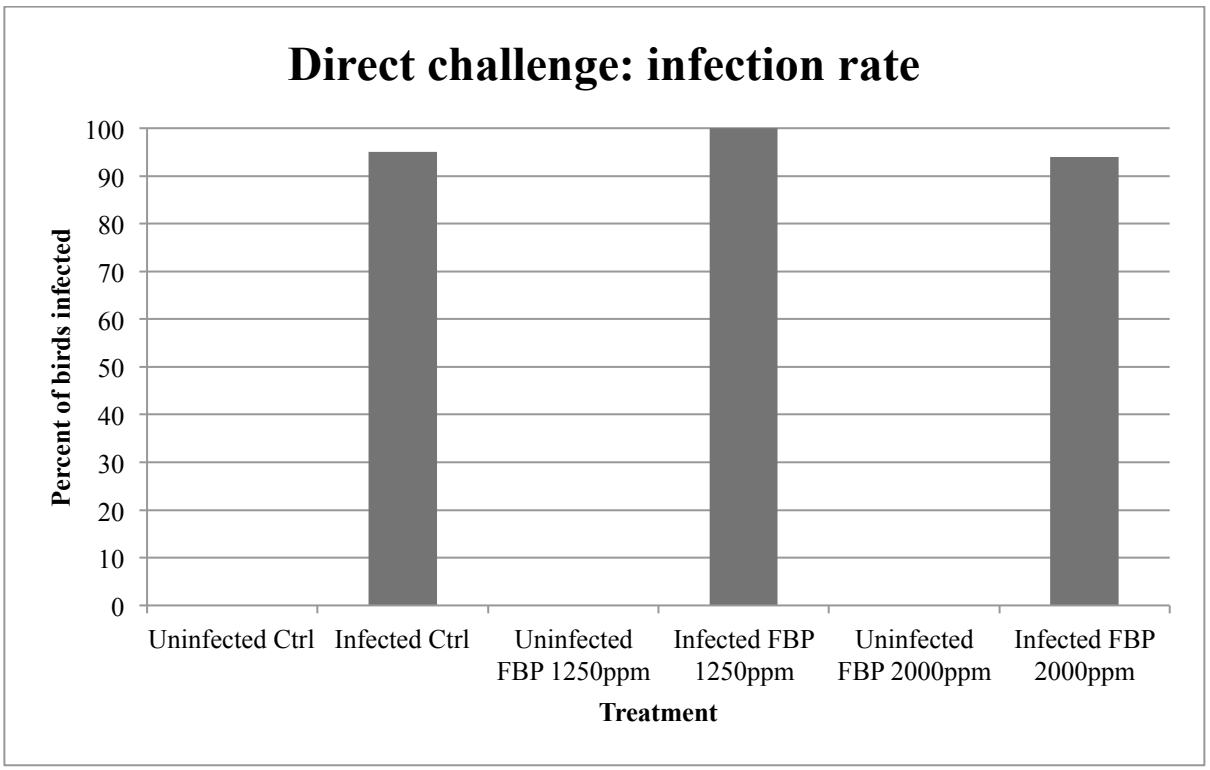

Figure 4.1. Graphical representation of infection rate for directly challenged poults by treatment. Poults that showed any signs of blackhead disease in the ceca or liver were considered positive. Fermentation by product (FBP).

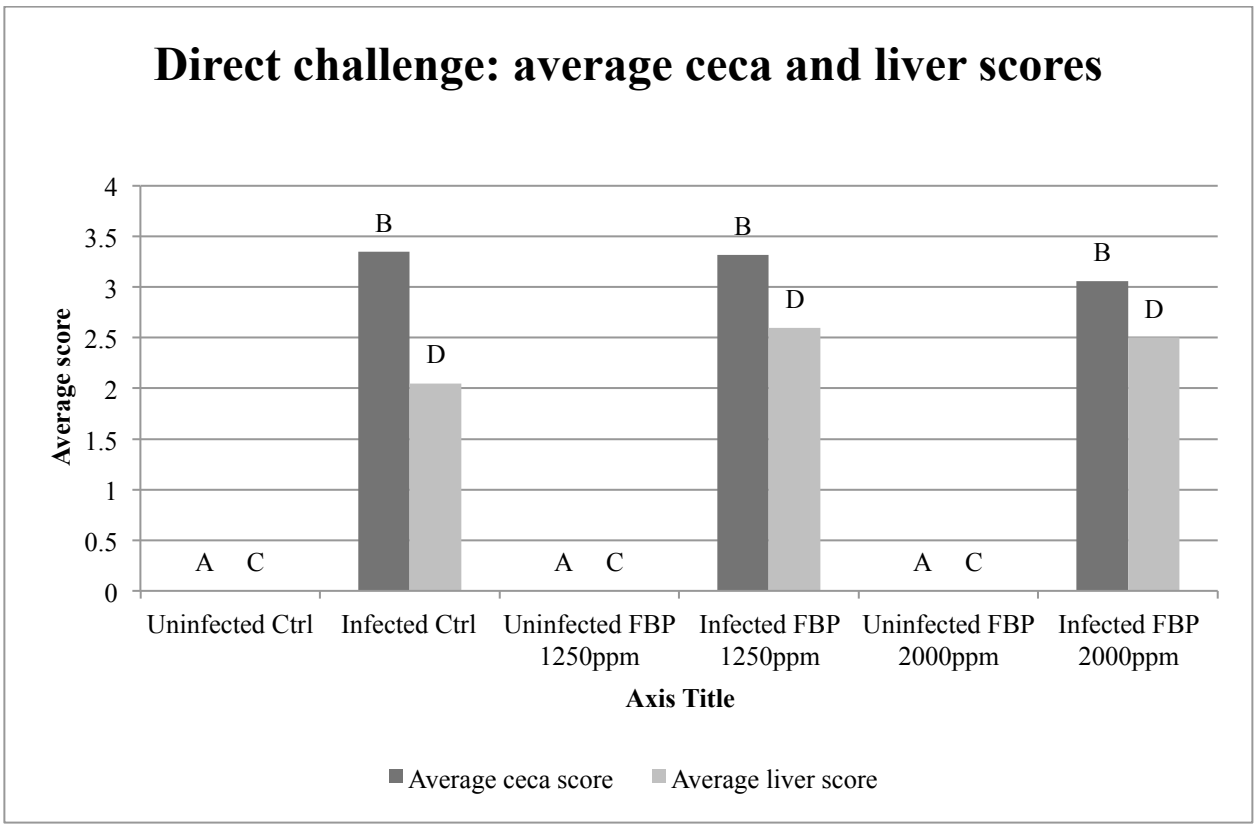

Figure 4.2. Graphical representation of average ceca and liver scores by treatment. Letters A and B coorespond to ceca scores and letters C and D coorespond to liver scores. Treatments not connected by the same letter are significantly different at $P<0.05$. Fermentation by product (FBP).

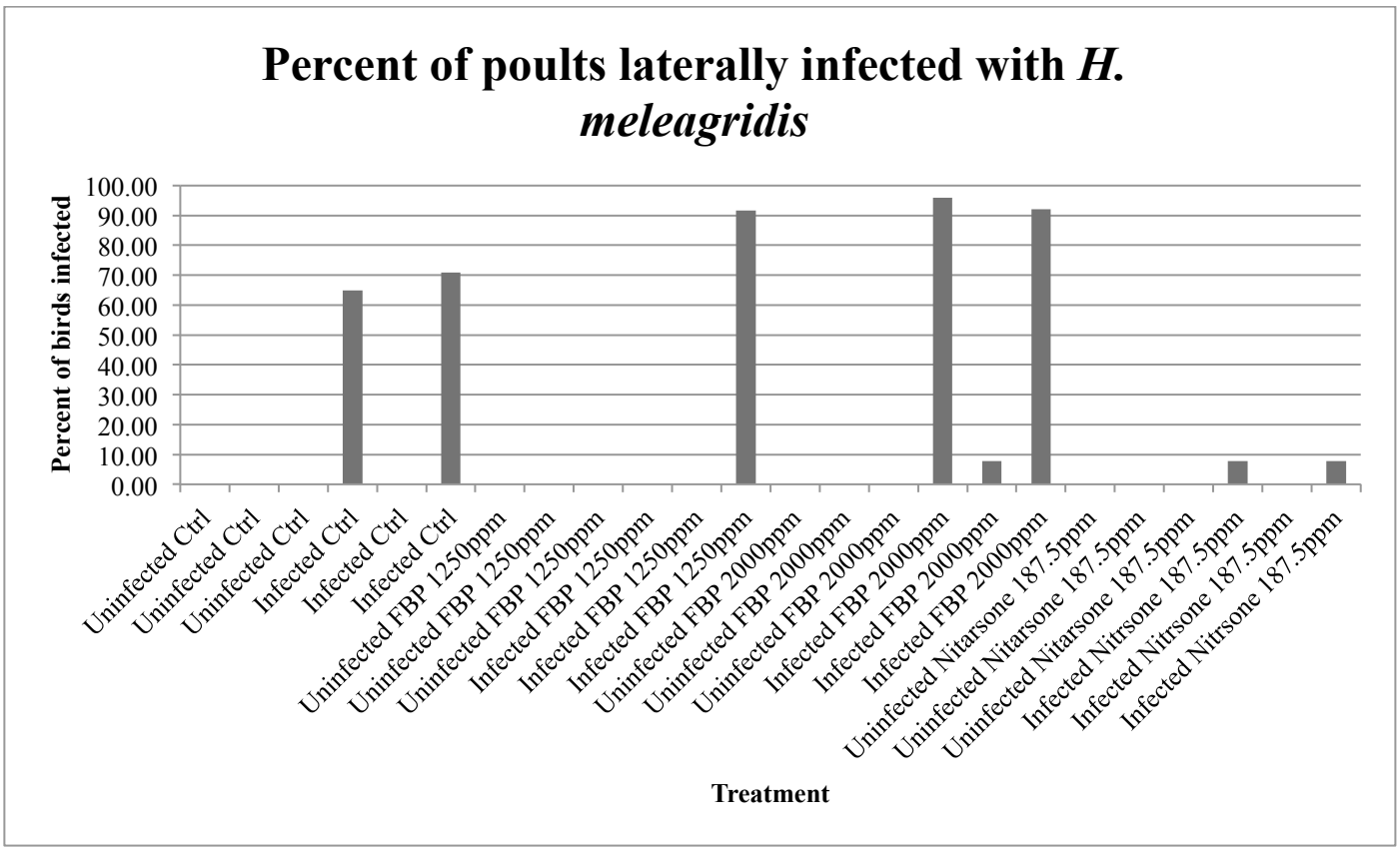

Figure 4.3. Graphical representation of lateral transmission of *H. meleagridis* by treatment for each pen. Poults that showed any signs of Blackhead disease in the ceca or liver were considered positive. Fermentation by product (FBP).

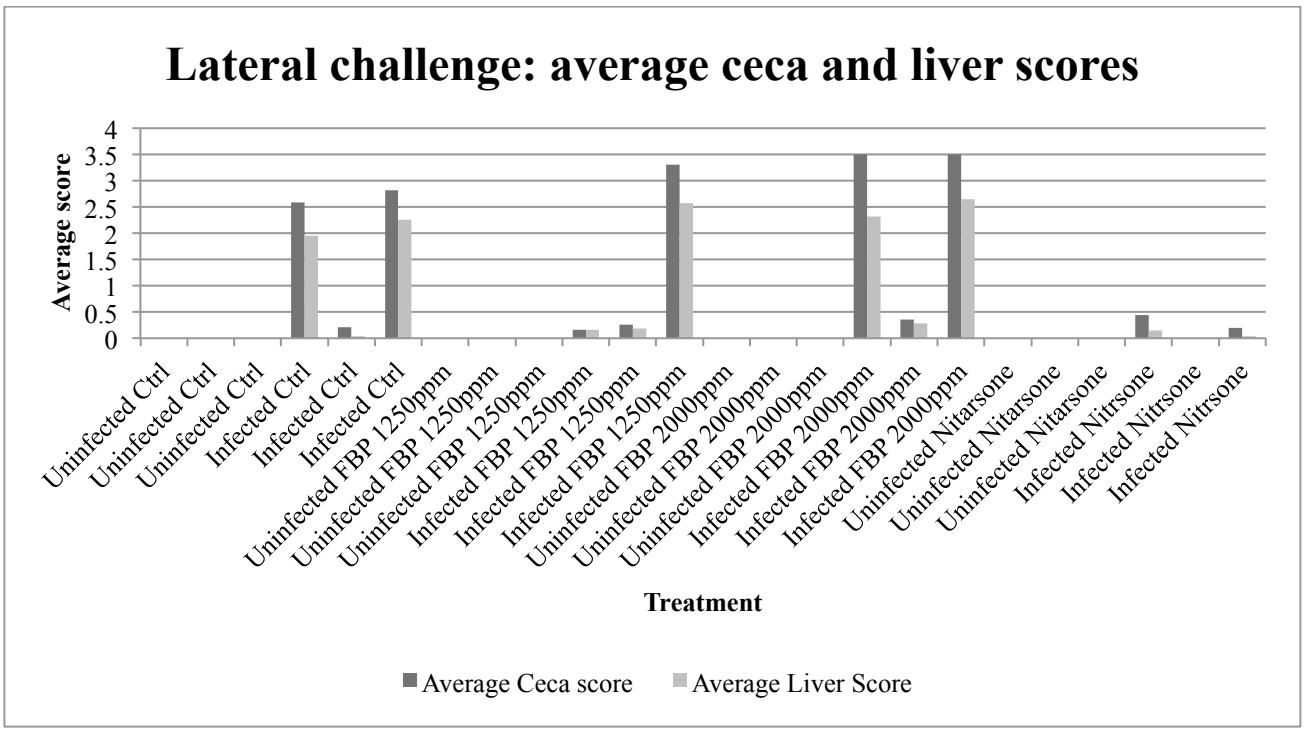

Figure 4.4. Graphical representation of average ceca and livers scores by pen. Fermentation by product (FBP).

Table 4.3. Ceca day 5 relative gene expression

Treatment ID	Cxcli2	IFN-γ	IL1B	IL10	IL13
Uninfected Control	1.00^{ab}+/-0.44	ND	1.00^{a}+/-0.61	1.00^{a}+/-0.68	1.00^{a}+/-0.29
Infected Control	19.25^{ab}+/-7.15	ND	2.97^{a}+/-0.27	1.24^{a}+/-0.34	117.7^{b}+/-2007
Uninfected FBP 1250ppm	0.37^{a}+/-0.28	ND	0.49^{a}+/-0.34	0.15^{a}+/-0.15	0.98^{a}+/-0.58
Infected FBP 1250ppm	9.37^{b}+/-23.3	ND	6.69^{a}+/-10.14	0.33^{a}+/-0.57	26.53^{ab}+/-8.99
Uninfected FBP 2000ppm	3.43^{ab}+/-1.36	ND	1.14^{a}+/-0.18	2.05^{a}+/-0.55	6.91^{a}+/-16.6
Infected FBP 2000ppm	3.87^{ab}+/-0.37	ND	2.28^{a}+/-4.26	0.39^{a}+/-0.16	2760^{b}+/-833

ND indicates that the sample was not detected in the assay. Fermentation by product (FBP).

Table 4.4. Ceca day 10 relative gene expression

Treatment ID	Cxcli2	IFN-γ	IL1B	IL10	IL13
Uninfected Control	1.00^{a}+/-0.54	1.00^{abc}+/-0.83	1.00^{a}+/-0.82	1.00^{a}+/-0.23	1.00^{a}+/-0.63
Infected Control	9.33^{a}+/-13.5	10.63^{bc}+/-14.5	2.73^{a}+/-0.29	0.32^{a}+/-0.33	38843^{b}+/-22134
Uninfected FBP 1250ppm	0.30^{a}+/-0.10	0.03^{ab}+/-0.02	0.40^{a}+/-0.34	0.20^{a}+/-0.02	2.19^{a}+/-0.58
Infected FBP 1250ppm	6.89^{a}+/-11.9	5.03^{abc}+/-6.10	3.29^{a}+/-0.88	0.15^{a}+/-0.09	20259^{b}+/-26021
Uninfected FBP 2000ppm	0.27^{a}+/-0.58	0.02^{a}+/-0.66	0.34^{a}+/-0.25	0.16^{a}+/-0.46	4.55^{a}+/-5.34
Infected FBP 2000ppm	10.32^{a}+/-9.09	11.84^{c}+/-2.41	3.26^{a}+/-0.98	0.26^{a}+/-0.11	2896^{b}+/-2187

Fermentation by product (FBP).

Table 4.5. Liver day 5 relative gene expression

Treatment ID	Cxcli2	IFN-γ	IL1B	IL10	IL13
Uninfected Control	1.00^{b}+/- 0.53	ND	1.00^{b}+/-0.66	1.00^{ab}+/-0.94	ND
Infected Control	0.16^{a}+/-0.04	ND	0.37^{ab}+/-0.10	305.95^{c}+/-21.9	ND
Uninfected FBP 1250ppm	0.30^{ab}+/-0.06	ND	0.32^{ab}+/-0.04	0.28^{a}+/-0.03	ND
Infected FBP 1250ppm	0.83^{ab}+/-0.43	ND	0.56^{ab}+/-0.49	127.23^{bc}+/-22.4	ND
Uninfected FBP 2000ppm	1.00^{b}+/-0.11	ND	0.28^{ab}+/-0.08	42.06^{abc}+/-27.2	ND
Infected FBP 2000ppm	0.27^{ab}+/-0.12	ND	0.19^{a}+/-0.04	8.50^{abc}+/-6.38	ND

ND indicates that the sample was not detected in the assay. Fermentation by product (FBP).

Table 4.6. Liver day 10 relative gene expression

Treatment ID	Cxcli2	IFN-γ	IL1B	IL10	IL13
Uninfected Control	1.00^{ab}+/-0.54	ND	$1.0O^{a}$+/-0.39	1.00^{a}+/-0.70	ND
Infected Control	3.85^{b}+/-0.44	D	2.51^{a}+/-0.64	156.79^{b}+/-31.4	D
Uninfected FBP 1250ppm	1.04^{ab}+/-0.15	ND	2.62^{a}+/-0.29	0.79^{a}+/-0.40	ND
Infected FBP 1250ppm	0.71^{ab}+/-0.95	D	1.26^{a}+/-0.69	151.35^{b}+/-26.0	D
Uninfected FBP 2000ppm	0.27^{a}+/-0.05	ND	1.01^{a}+/-0.15	2.12^{a}+/-0.48	ND
Infected FBP 2000ppm	2.11^{ab}+/-0.99	D	1.14^{a}+/-0.27	100.42^{b}+/-1.95	D

ND indicates that the sample was not detected in the assay. D indicates that the sample was detected but relative gene expression was not able to be determined. Fermentation by product (FBP).

Table 4.7. Treatment identification and fermentation by-product levels in a broiler breeder uniformity model with an infection with *H. meleagridis*.

Treatment ID	Treatment	Fermentation by-product
1	Uninoculated Control	N/A
2	Inoculated Control	N/A
3	Uninoculated FBP 1250	1250ppm
4	Inoculated FBP 1250	1250ppm
5	Uninoculated FBP 2000	2000ppm
6	Inoculated FBP 2000	2000ppm

Fermentation by product (FBP).

Table 4.8. Feed conversion ratios (FCR) in a broiler breeder uniformity model utilizing a diet with and without Fermentation by-product and infection with or without *H. meleagridis*.

Treatment ID	FCR 0-14d	FCR 14-28d	FCR 28-35d	FCR 0-35d	Adj FCR 0-35d
Uninfected Control	1.215	1.672	2.007	1.617	1.613
Infected Control	1.215	1.631	1.877	1.608	1.616
Uninfected FBP 1250ppm	1.204	1.636	2.066	1.623	1.614
Infected FBP 1250ppm	1.217	1.642	1.844	1.606	1.622
Uninfected FBP 2000ppm	1.196	1.644	1.751	1.574	1.552
Infected FBP 2000ppm	1.200	1.653	1.884	1.615	1.615

At $P < 0.05$ there was no statistical difference between the feed conversion ratio of the different diets. Fermentation by product (FBP).

Table 4.9. Coefficient of variation (CV) for body weights in a broiler breeder uniformity model utilizing a diet with and without fermentation by-product and infection with or without *H. meleagridis.*

Treatment ID	Day 14 CV	Day 28 CV	Day 35 CV
Uninfected Control	12.21	10.55	8.95
Infected Control	9.57	8.83	9.83
Uninfected FBP 1250ppm	8.37	11.24	10.68
Infected FBP 1250ppm	9.71	7.27	9.79
Uninfected FBP 2000ppm	8.95	8.47	9.48
Infected FBP 2000ppm	6.28	6.15	7.06

Fermentation by product (FBP).

Table 4.10. Broiler weight gain by diet prior to infection.

Treatment ID	Mean	Standard Error
Control	553.56	3.37
FBP 1250ppm	558.08	3.39
FBP 2000ppm	564.09	3.36

At $P < 0.05$ there was no statistical difference between the average diet weigh gain from 0-14 days prior to infection with *H. meleagridis*. Fermentation by product (FBP).

Table 4.11. Average broiler weight gainse

Treatment ID	Weight Gain (g) 14-28d	Weight Gain (g) 14-35d	Weight Gain (g) 28-35d	Weight Gain (g) 0-35d
Uninfected Control	1015[b]	1837[abc]	822[ab]	2356[bc]
Infected Control	1035[ab]	1799[bc]	763[bc]	2308[bc]
Uninfected FBP 1250ppm	1073[a]	1864[ab]	790[bc]	2390[ab]
Infected FBP 1250ppm	1016[b]	1762[c]	746[c]	2271[c]
Uninfected FBP 2000ppm	1044[ab]	1915[a]	871[a]	2444[a]
Infected FBP 2000ppm	1031[ab]	1821[bc]	790[bc]	2337[bc]

Treatments not connected by the same letter are significantly different at $P < 0.05$. Fermentation by product (FBP).

Table 4.12. Average broiler weights on 28 and 35 days of age.

Treatment ID	Day 28 Weights (g)	Day 35 Weights (g)
Uninfected Control	1575[b]	2398[bc]
Infected Control	1586[b]	2351[bc]
Uninfected FBP 1250ppm	1643[a]	2334[ab]
Infected FBP 1250ppm	1567[b]	2313[c]
Uninfected FBP 2000ppm	1615[ab]	2486[a]
Infected FBP 2000ppm	1590[ab]	2379[bc]

Treatments not connected by the same letter are significantly different at $P < 0.05$. Fermentation by product (FBP).

CHAPTER 5

CONCLUSIONS

In the decades following the first case of blackhead disease (1893), turkey production in the United States had a sharp decline due to severe mortality. Research identified *H. meleagridis* as the causative agent, focused on the epidemiology and found treatments for blackhead disease, which aided in a renewal of turkey production. However, the banning of efficacious drugs has led to increased mortality associated with blackhead outbreaks. Variations in flock mortality associated with blackhead disease outbreaks led to an investigation of factors that play a role in the lateral transmission of *H. meleagridis* from bird to bird. The research presented here discovered that the rate of *H. meleagridis* transmission increased when reverse peristalsis was stimulated, fecal dropping was loosened or intestinal damage was induced. These results lead to the supposition that blackhead disease transmission is directly correlated with intestinal health and function.

To further investigate drug alternatives that protect against blackhead outbreaks, the plant derivative Natustat, previously reported to act as an anti-histomonal, was tested *in vitro* and *in vivo*. It was found that Natustat can inhibit the *in vitro* growth of *H. meleagridis* but does not protect turkeys against direct inoculations of the parasite or prevent its transmission. This suggests that Natustat may not be an efficacious treatment for blackhead disease; however, its continued use may still be merited if it can provide a benefit to intestinal health and integrity. Additionally, the immunomodulatory properties of a yeast fermentation by-product were

assessed in turkeys as a deterrent of mortality and in broilers as a means of ameliorating performance losses. The yeast fermentation by-product proved ineffective in enhancing the turkey's immune response against a *H. meleagridis* infection, while broilers did not appear to suffer performance losses regardless of the yeast fermentation by-product being supplemented. This leads to the deduction that the supplementation of a yeast fermentation by-product alone is not sufficient to alter the immune response of turkeys and provide a protection against blackhead outbreaks, although it may aide in increasing overall bird health and growth.

This research is of importance to turkey producers, growers, veterinarians and researchers because it reveals novel factors that contribute to greater levels of blackhead disease transmission and establishes a model in which future studies can be performed. While investigators continue to find alternative compounds for controlling blackhead outbreaks, a focus on intestinal health may help ameliorate the severity of some outbreaks, mitigate financial losses and improve bird welfare. Future research is still required to better understand the relationship between intestinal health and the transmission of blackhead disease.

Appendix 1

IDENTIFICANTON OF EARLY EMBRYONIC DEFORMITIES IN THE ATHENS CANADIAN RANDOM BRED LINE MAINTAINED AT THE UNIVERSITY OF GEORGIA[2]

[2] Jason A. Payne and Robert Beckstead. To be submitted to Poultry Science

Abstract

The Athens Canadian Random Bred (ACRB) chicken line maintained at the University of Georgia was first established in 1958 to preserve the genetics of the 1950s broiler. Though randomly bred to prevent directional selection, the lack of new genetics in the breeding process can allow for the accumulation of recessive genomic mutations within the flock. The current study was aimed at determining whether there are recessive mutations within the ACRB flock that manifest deleterious phenotypes during the first few days of embryogenesis. Pedigree information from the 2014 ACRB flock allowed 215 potential sibling crosses to be performed with an average yield of 9 embryos produced per mating. 13 of the 215 crosses manifested embryonic abnormalities in Mendelian ratios, including face/head (1), brain (1), hind limb (1), eye (1), spine (2), trunk (3), or developmental delay (4). Two of the crosses were used to produce F1 birds, where one of the two phenotypes was recovered. This data suggests that there are embryonic lethal mutations present in the ACRB flock maintained at the University of Georgia and that as the flock becomes more inbred there is a potential that these mutations can reduce the fitness of the line.

Introduction

Advances in poultry breeding, selection and production over the past century have made substantial changes to the way food is produced to sustain a rapidly growing world population. Most of these improvements in production traits can be directly attributed to the genetic selection (Marquez, Siegel and Lewis, 2010). In the 1950s, the Southern Regional Breeding Project was tasked with the creation of several control flocks that would not undergo genetic selection (Quisenberry, 1959). These flocks maintain valuable genetic diversity and allow researchers the ability to monitor changes associated with modern genetic breeds. The Poultry Science Department at the University of Georgia maintains one of these control flocks, the Athens Canadian Random Bred Line (ACRB)(Quisenberry, 1959).

The ACRB chicken line is currently the oldest pedigreed flock and even today closely resembles the genetic make-up of the 1950s broiler (Collins, Marks, Aggrey, Lacy and Wilson, 2016). The ACRB is a derivative of the Ottawa meat control strain that was originally derived from the White Wyandotte and three synthetic populations that were made up of wide genetic backgrounds from several common breeds and varieties (Somes, 1988). Through random mating, the ACRB flock has been propagated since 1958 at the University of Georgia. The closed genetics of this population over the past 6 decades may have reduced the genetic diversity of this flock and lead to accumulation of deleterious recessive alleles (Loewe and Hill, 2010). The following study was performed to determine whether there are deleterious alleles that cause early embryonic lethality present in the ACRB flock and what the frequency of these mutations is within the population.

Materials and Methods

The University of Georgia IACUC approved the animal research presented in this paper.

Crosses and embryonic screening

Pedigree information maintained on the 2014 ACRB line was used to identify 214 potential siblings crosses within the flock. Matings were performed biweekly for three weeks by means of artificial insemination. Eggs produced from the crosses were incubated at 99.5 F and 50% humidity for 5 days. Embryos were removed from extra embryonic tissues, rinsed in phosphate buffered saline, fixed in 10% formalin and screened under a dissecting microscope for abnormalities.

Generation and screening of F1 flock

Two of the sires from the original screen that manifested a 1:4 ratio of offspring phenotypes were crossed to the same potential sibling that manifested the observed embryonic phenotype or outcrossed to a layer line in order to generate a F1 line. F1 offspring were raised to sexual maturity. Siblings were crossed as described above and embryos were screened on day 3 and 5 of incubation for their corresponding phenotype.

Results and Discussion

Although the ACRB chicken line is valuable as a model to compare and contrast its genetics with our modern day broiler, there may be limitations for this chicken line as a long-term model. In order to determine whether there are deleterious alleles within the ACBR line,

pedigree information from the 2014 flock was used to perform 214 potential sibling crosses. Direct siblings could not be determined due to the methodology of pooling rooster semen for artificial insemination when the ACRB flock is regenerated each year. For each cross, an average of 9 fertile embryos were obtained and screened under a dissecting microscope. The low number of embryos in this experiment was due to a high number of infertile eggs. Of the 215 crosses, 13 manifested a Mendelian ratio of embryos exhibiting abnormalities (figure A.1) including those to the face/head (1), brain (1), hind limb (1), eye (1), spine (2), dwarfing of the trunk (3), or severe developmental delay (4). This data demonstrates that there are several recessive mutations found within the ACRB flock.

To further understand the genetics of these phenotypes, birds that when crossed produced a scoliosis phenotype and a severe head deformity phenotype were selected to generate F1 progeny. 3 males and 20 females from these two F1 flocks were mated to produce embryos that were screened for the original phenotype associated with the original cross. The scoliosis phenotype could not be rescued from the F1 offspring. 3 hens in the F1 flock when mated to a sibling sire had embryos that manifested the head phenotype at a ratio of 1:8-1:16 (figure A.2). This genetic ratio suggests that the phenotype observed could be associated with recessive mutations in 2 genes. It is also possible that there is low penetrance of the phenotype.

In our study we found that 13 of the 215 potential sibling crosses (6%) manifested severe phenotypes that were embryonic lethal. Due to low embryo numbers cause by fertility, time constraints and the age of the birds, and the inability to know which birds were actually siblings from the same sire, we acknowledge that we did not identify all of the allelic mutations in this flock that result in early embryonic lethality. Since this study only focused on phenotypes that can be visualized at day 5 of incubation, it did not identify mutations that could result in late

stage mortality and deformities. Thus, the number of embryonic mutations within this flock may be higher than the 6% reported. Over time the accumulation of these alleles and likely several others may hinder future research relating to the ACRB flock. However, the identification of the genetic mutations that result in early embryonic malformations provides a valuable resource to identify genes that are critical for early developmental events.

Though the propagation of mutations within the ACRB flock is not surprising considering the lack of new genetics over several decades, it does raise a concern as to the long-term efficacy of maintaining the ACRB flock. Genetic drift can cause a loss of genetic diversity, resulting in the loss of less frequent alleles that were present in the population and allowing for the accumulation of recessive lethal alleles (Loewe and Hill, 2010). It is possible ACRB maintained today at the University of Georgia has undergone a loss of genetic diversity. In addition, genetic drift over long periods of time can eventually fix certain alleles within a population (Loewe and Hill, 2010; Wakeley, 2005). Without the reintroduction of genetic diversity into the ACRB flock there may come a time where this valuable genetic resource may become lost due to potential health and welfare concerns. A potential solution for ensuring the future health and genetic diversity of the ACRB flock may be the pooling and redistribution of several of the original flocks that have the genetic background of 1950 and 1960 broilers. While this methodology would alter the genetic profile of the current ACRB flock, it may provide the gained benefit maintaining genetic diversity.

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Figures

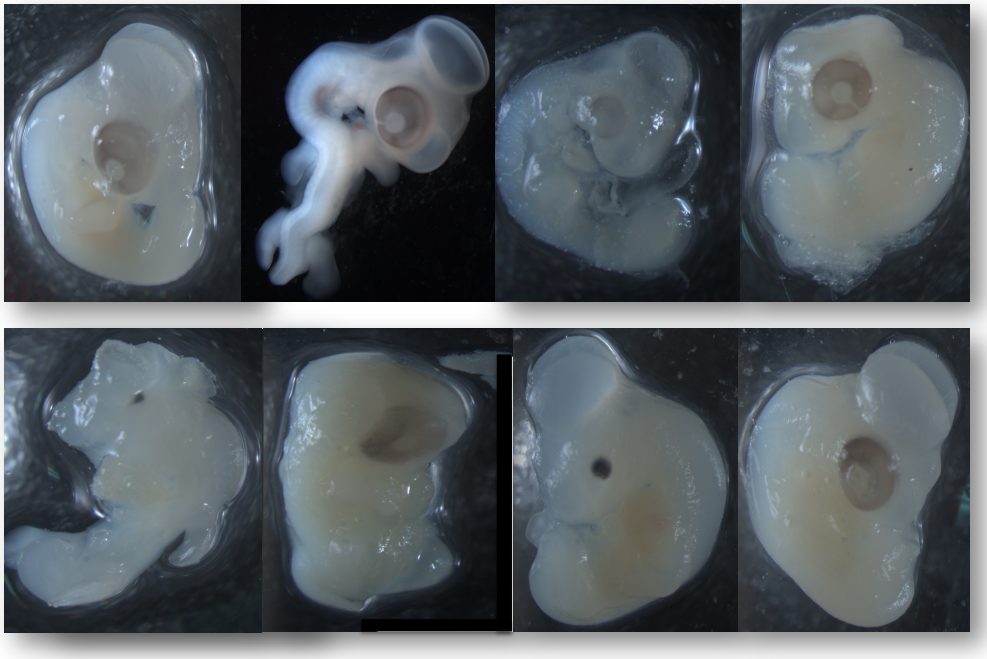

Figure A.1. Several F1 embryos manifesting abnormalities at day 5. F1 embryos manifesting phenotypes in a Mendelian ratio at 5 days of incubation. From top left to bottom right: Normal, scoliosis, missing hind limb, brain, head, dwarfing, eye (embryos as seen from both sides).

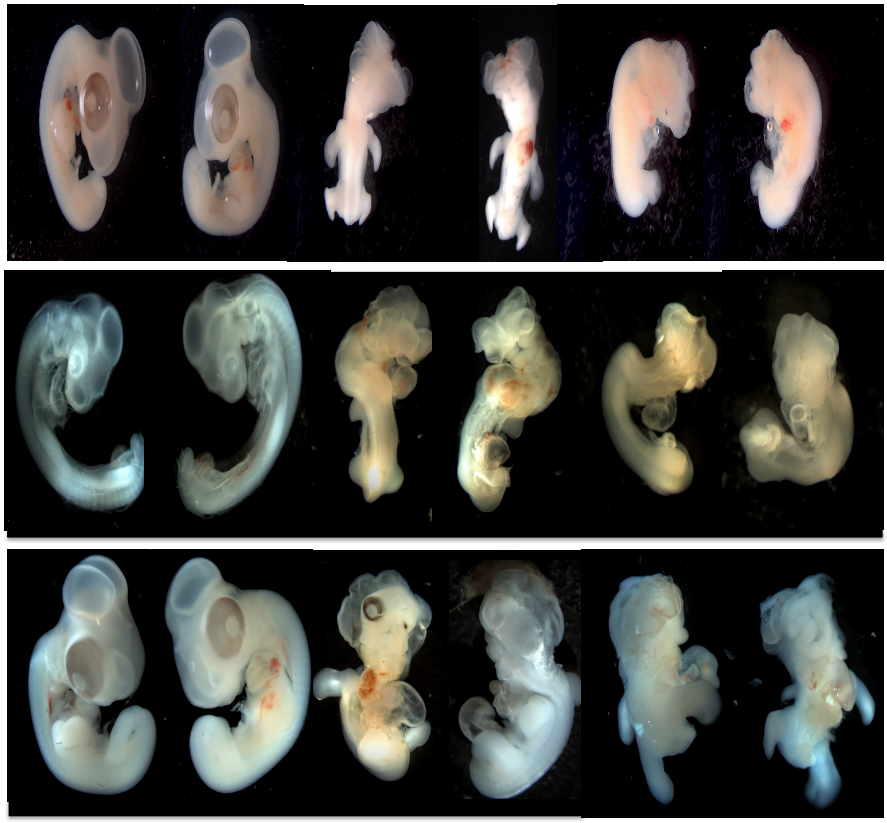

Figure A.2. Images of head phenotype in both F1 and F2 generation embryos. Top panel are F1 embryos collected at day 5. Middle and bottom panels are F2 embryos collected at days 3 and 5 respectively. Images are of the same embryo as seen from different sides. Left: Normal embryo. Center and right: severe head phenotype embryos.