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Histomonosis in Turkeys – Factors Influencing Transmission, Pathogenesis, and the Search for Prophylactic or Therapeutic Compounds

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Poultry Science

by

Thainá Landim de Barros
São Paulo State University (UNESP)
Doctor in Veterinary Medicine, 2014
São Paulo State University (UNESP)
Master of Science in Animal Science, 2017

August 2022
University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

Billy M. Hargis, Ph.D.
Dissertation Director

David Caldwell, Ph.D.
Committee Member

John R. Barta, Ph.D.
Ex-officio Member

Guillermo Tellez-Isaias, Ph.D.
Committee Member

Robert Beckstead, Ph.D
Ex-officio Member

Christine Vuong, Ph.D.
Ex-officio Member

Abstract

The incidence of histomonosis has been increasing in poultry since the ban of prophylactic and therapeutic compounds. Histomonosis is caused by the protozoa *Histomonas meleagridis*. The objective of this dissertation was to investigate factors impacting the pathogenesis and transmission of histomonosis and to evaluate compounds that could potentially prevent or reduce the severity of histomonosis in turkeys. In the first study, the effect of sodium chlorate and sodium nitrate on reducing histomonads growth was tested *in vitro* and added to a basal turkey diet. A decrease in the growth of histomonads *in vitro* was observed, but no *in vivo* effect was observed. The second study investigated the influence of *Eimeria adenoeides*, another cecal protozoan, on the pathology of histomonosis. In experiment 1, a reduction in the severity of histomonosis was observed with pre-inoculation of *E. adenoiedes* 5 days (day 15) before inoculation of *H. meleagridis* (day 20). In experiment 2, the same inoculation of *E. adenoeides* 5 days (day 14) before *H. meleagridis* (day 19) inoculation was adopted, in addition to inoculation of *E. adenoeides* 21 days before *H. meleagridis* (day 35); and inoculation of low doses of oocysts, every 2-3 days during the first three weeks, followed by inoculation of *H. meleagridis* (day 35). Histomonosis was not affected by the inoculation of *E. adenoiedes* in experiment 2. In the third study, diets with different compositions and nutritional densities and two different isolates of *H. meleagridis* and raising conditions were investigated in two pilot experiments and three validation experiments. In pilot experiment 1, one isolate of *H. meleagridis* (named Buford) was used. Turkeys were fed a low-nutrient density diet corn-soy based (LOW-CS) and raised on floor pens. In pilot experiment 2, another isolate of *H. meleagridis* was used (named PHL). Turkeys were fed a LOW diet with the addition of wheat middlings (LOW-WM) and raised on floor pens. In experiment 3, conducted on floor pens, both isolates and diets were used

in different groups. In experiment 4, turkeys were raised on battery cages; only the PHL isolate was used. Both diets (LOW-WM and LOW-CS) were used, in addition to a diet surpassing the nutritional needs of young poults (turkey started, TS). In experiment 5, conducted in battery cages, only the PHL isolate was used, and the LOW-WM and TS diets were fed to different groups. From all experiments, HT was achieved only with the PHL isolate, with a transmission rate varying depending on the experimental diets. The TS diet had the lowest transmission rate in experiments 4 and 5. Higher variability was observed in the experiments conducted on floor pens. Variation was observed between experiments and within experimental groups. The complexity and multifactorial nature of histomonosis requires further studies.

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I want to acknowledge the Poultry Health Laboratory; I have learned much from and with you. Many thanks to all professors and staff from the Center of Excellence for Poultry Science who contributed to my path, especially Prof. Susan Watkins, for assisting me without hesitation in moments of need. To Dr. Elizabeth McGill for all her contributions and effort in assisting me during some experiments. To all my friends who have made this journey lighter.

Dedication

To my mother, Angélica, my father, Ivanor, my sisters, Thamine and Lívia, my brother-in-law, Fábio, and my niece, Marina. Thank you for always holding my hand and supporting my dreams. You are my lighthouse. To my partner, Miguel, for taking me to navigate new waters, sharing his love, and inspiring me to be a better person. We have the whole world to explore together.

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List of Published Papers

Barros T.L., Vuong C. N., Tellez-Isaias G., Hargis B. M. Factors influencing histomonosis in turkeys. Submitted to *World's Poultry Science Journal* (Literature Review).

Barros, T.L., Beer, L.C., Tellez, G.; L. Fuller, Hargis, B.M., Vuong, C.N. 2020. Research Note: Evaluation of Dietary Administration of Sodium Chlorate and Sodium Nitrate for Histomonas meleagridis prophylaxis in turkeys. *Poultry Science* 99(4):1983-1987. doi: 10.1016/j.psj.2019.11.055. (Chapter 1).

Barros T.L., Beer L.C., Barta J.R., Hargis B.M., Vuong C.N. 2022. Evaluation of *Eimeria adenoeides* pre-infection on the severity of histomoniasis in turkeys. *Avian Diseases*. doi: 10.1637/21-00048. (Chapter 2).

Barros T. L., Vuong C.N., Latorre J.D., Cuesta R.S., McGill E., Rochell S.J., Tellez-Isaias G., Hargis B.M. 2022. Feed composition and isolate of *Histomonas meleagridis* alter horizontal transmission of histomonosis in turkeys. Proof of Concept. *Frontiers in Veterinary Science.* doi.org/10.3389/fvets.2022.937102. (Chapter 3).

I. INTRODUCTION

Histomonosis, caused by the protozoa *Histomonas meleagridis*, affects mainly turkeys and chickens, with outbreaks in turkey flocks leading to high mortality rates, representing a threat to animal welfare and food security (Hess *et al.* 2015; McDougald 2005). The disease has been studied since its discovery at the beginning of the last century. Disease outbreaks were controlled with the discovery of prophylactic and therapeutic drugs, such as nitroimidazoles, arsenicals, and nitrofurans, and research interest decreased (Regmi *et al.* 2016; Liebhart *et al.* 2017). In the last decades, the use of such compounds has been withdrawn, and the incidence of histomonosis has been increasing since then (Hess *et al.* 2015; Liebhart *et al.* 2017). No alternative compounds to treat or prevent the disease have been discovered until now. Studies about histomonosis have also increased; however, there are still many unanswered epidemiological aspects of the disease, bringing challenges to finding effective strategies for its prevention.

Histomonas meleagridis is a protozoon from the phylum Parabasalia (Hess and McDougald 2020). Protozoa from this phylum possess hydrogenosomes for energy metabolism instead of mitochondria, thriving in microaerophilic environments (Mazet *et al.* 2008). Another interesting characteristic of *H. meleagridis* is its pleomorphism. The protozoan possesses a rounded form in the cecal lumen, presenting one flagellum. Transitioning to tissues, it changes to an amoeboid form, presenting pseudopodia. Studies have shown the presence of a cyst-like stage; however, very little is unknown about the impact of such a morphological stage on the transmission and survival of *H. meleagridis* (Munsch *et al.* 2009; Zaragatzki *et al.* 2010; Gruber, Ganas, and Hess 2017; Hess and McDougald 2020).

Outbreaks of histomonosis in turkey flocks are typically initiated by the ingestion of embryonated eggs of *Heterakis gallinarum*, contaminated with histomonads (McDougald 2005). *Heterakis gallinarum* is the intermediate host of *H. meleagridis,* and its eggs can be brought to turkey facilities by earthworms and potentially by other mechanical vectors (Lund, Wehr, and Ellis 1966). Infected turkeys can transmit the disease to other turkeys by horizontal transmission (Hu and McDougald 2003). Replication of horizontal transmission under experimental conditions has not been consistent in the last years (Hauck and Hafez 2013). It is essential to have a reliable horizontal transmission model to search for alternative compounds or management strategies to prevent and limit outbreaks of histomonosis. Factors influencing horizontal transmission are unclear.

The presence of bacteria is essential for the protozoa to cause disease in gallinaceous birds and *in vitro* cultivation (Springer, Johnson, and Reid 1970; Ganas *et al.* 2012). The importance of such a relationship is still not understood. Some possibilities are that bacteria represent an important food source for the protozoa, can maintain a micro-environment favorable for the replication of *H. meleagridis*, and increase the expression of virulence factors essential for the invasion of tissues (Bilic and Hess 2020). The protozoa initially infect the ceca, which harbor many microorganisms and have a very complex micro-environment (Pan and Yu 2014). In turkey flocks, other intestinal protozoa and microorganisms are present in the ceca, and the influence on the pathology of histomonosis is uncertain.

Considering the lack of prophylactic and therapeutic compounds, the importance of understanding factors influencing pathogenesis and transmission of histomonosis, and the need to have a consistent and reproducible horizontal transmission model, the current dissertation consists of a literature review and three research papers investigating the previously mentioned

subjects. Section II has a literature review about factors influencing histomonosis in turkeys. Chapter I (section III) consists of a study testing two compounds, sodium chlorate and sodium nitrate, potentially preventing histomonosis in turkeys. In Chapter II (section IV), the interaction between *H. meleagridis* and *Eimeria adenoeides*, other protozoa affecting the ceca of turkeys, causing coccidiosis, was investigated. In Chapter III (section V), the horizontal transmission of histomonosis was investigated with different diets, varying in ingredients and nutritional densities, and two different isolates of *H. meleagridis*.

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II. LITERATURE REVIEW

Factors influencing histomonosis in turkeys

Barros T.L., Vuong C.N., Tellez-Isaias G., Hargis B.M.

Department of Poultry Science, University of Arkansas Division of Agriculture, Fayetteville, AR, USA, 72701

Literature review submitted to the *World's Poultry Science Journal*.

ABSTRACT

Since therapeutic and prophylactic compounds became unavailable in many countries, outbreaks of histomonosis in turkeys and chickens have been increasing. Turkeys are particularly susceptible to the disease, whereas chickens generally survive and become carriers of *Histomonas meleagridis*. Although the disease has been studied since the beginning of the last century, some epidemiological aspects remain unanswered. The cecal worm *Heterakis gallinarum* is the intermediate host, but mechanical vectors seem to play an important role in the survival, transport, and introduction of the protozoa in turkey facilities. In turkeys, the disease can be transmitted by direct contact, referred to as horizontal or lateral transmission. Replication of horizontal transmission in experimental conditions has not been consistent in the last years and factors influencing the transmission are not fully understood. The presence of bacteria is necessary for the protozoa to cause disease and be cultivated *in vitro*; however, the influence of bacteria, in the ceca and the litter, in the pathogenicity and transmission of histomonosis remains elusive. *Histomonas meleagridis* has tropism for the ceca and the liver. The cecum has a dynamic environment, presenting a large bacterial population, influencing the host's homeostasis in several ways. Genetic variability of isolates of *H. meleagridis* has been reported, but the impact of this genetic variability on the transmission of the protozoa has not been studied. Considering this complex host-protozoa-bacteria interaction, the present literature review focuses on factors that could impact the outcome of histomonosis infection and transmission.

Keywords: *Histomonas meleagridis*, protozoa, transmission, microbiota, ceca, poultry

INTRODUCTION

Histomonosis, also known as histomoniasis, blackhead disease, or enterohepatitis, is caused by the protozoa *Histomonas meleagridis*. The disease affects mainly turkeys (*Meleagris gallopavo*) and broiler chickens (*Gallus gallus*) (McDougald 2005). It severely impacted turkey production at the beginning of the last century; however, the disease was controlled with the development of therapeutic and prophylactic drugs in the 1950s (McDougald 2005; Hess *et al.* 2015). With the withdrawal of effective chemotherapeutics in the last decades, due to concerns with toxic residues to consumers, disease outbreaks started to be frequently reported, compromising animal welfare and causing severe economic losses to the poultry industry, especially to turkey producers (Hess *et al.* 2015; Liebhart *et al.* 2017; McDougald 2005). Nowadays, there are no prophylactic nor therapeutic substances available to control the disease, and consequently, there is an urgent need to search for alternatives, either pharmacological or management strategies.

The disease has been studied since the beginning of the last century (Tyzzer 1920), but the use of prophylactic drugs to control this disease made continued research on histomonosis to be of little interest. With the withdrawal of effective drugs and the re-emergence of histomonosis, studies about the disease have been published in the past few years, focusing on the etiology, diagnostic tools, development of vaccines, and testing of alternative compounds to prevent or treat the disease (Hauck and Hafez 2013; Hess *et al.* 2015; Liebhart *et al.* 2017; McDougald 2005). Some aspects of the disease remain unanswered, including parts of the life cycle of *H. meleagridis*, factors impacting transmission in turkeys, and prophylactic/therapeutic options. The present literature review focuses on important characteristics of the life cycle of *H. meleagridis*, the mechanism of action for compounds that were used to control the disease,

differences in pathogenesis and immune response between turkeys and chickens, the interaction of the protozoa with the intestinal microbiota, and factors impacting the transmission of the disease.

HISTOMONAS MELEAGRIDIS' LIFE CYCLE AND EPIDEMIOLOGY

Histomonas meleagridis is from the family Dientamoebidae, order Tritrichomonadida, class Tritrichomonadea, phylum Parabasalia (Hess and McDougald 2020). In 1920, Tizzer proposed the genus *Histomonas* since the protozoa presented unique characteristics differing from previous classifications as Amoeba, Eimeria, or Entamoeba (Tizzer 1920). Like other trichomonads, *H. meleagridis* possess an axostyle-pelta complex, a V-shaped parabasal body, and hydrogenosomes, instead of mitochondria, for energy metabolism (Gerbod *et al.* 2001; Mazet *et al.* 2008). Hydrogenosomes evolved from mitochondria to allow for the survival of eukaryotes in anaerobic environments (Lewis and Ettema 2021), producing ATP via substrate-level phosphorylation with concomitant hydrogen production (Mazet *et al.* 2008; Müller *et al.* 2012). Hydrogenosomes lack a genome, cytochromes, and citric acid cycle enzymes (BuI, Bradley, and Johnson 1996; Müller *et al.* 2012). *In vitro* growth of *H. meleagridis* is only possible in anaerobic conditions. Still, the organism can tolerate low oxygen levels in the invasion of the host's tissues; thus, it can be considered a microaerophilic organism (Mazumdar *et al.* 2017; Wei *et al.* 2020).

Distinct morphologies have been reported in *H. meleagridis*. A rounded form possessing a single flagellum is observed in the ceca of gallinaceous birds, ranging from 8-14 µm, presenting large numbers of hydrogenosomes, Golgi complex, endoplasmic reticulum, ribosomes, granules of glycogen, an extranuclear spindle, remnants of an axostyle and a pelta,

and food vacuoles with bacteria and starch (Hess and McDougald 2020; Munsch *et al.* 2009). The flagellum is lost during the invasion of the host's tissues and an amoebic form is then observed, varying in shape with a mean diameter of 10 µm. The amoebic stage possesses pseudopodia and presents all the typical organelles with fewer hydrogenosomes and Golgi complexes. Large numbers of granules of glycogen and food vacuoles with bacteria and starch can also be observed (Bishop 1938; Gruber, Ganas, and Hess 2017; Hess and McDougald 2020; Munsch *et al.* 2009; Tyzzer 1919). The last morphological form, which is still debatable, is the cyst-like stage, which is spherical, measuring from 4-14 µm, having a reduction in food vacuoles compared to the other forms, the typical cell organelles, in addition to being densely packed with glycogen granules and ribosomes. Two outer membranes are observed. The impact of these stages in the transmissibility of histomonosis (Gruber, Ganas, and Hess 2017; Munsch *et al.* 2009; Zaragatzki *et al.* 2010) and the existence of true cysts have not been confirmed so far (Liebhart and Hess 2020). Gruber, Ganas, and Hess (2017) compared the morphology of a virulent and an attenuated *H. meleagridis* originated from clonal monoxenic isolates. Amoeboid morphology was prevalent on the attenuated histomonads, while the virulent ones were predominantly rounded. Spherical cells, possibly indicating the cyst-like stage, were observed after adding antimicrobials for 24h. Factors impacting the transition to different morphological stages are not fully understood (Figure 1).

Histomonads do not persist outside the host and use the cecal nematode *Heterakis gallinarum* as the intermediate host (Tyzzer 1920; Lund, Wehr, and Ellis 1966). Embryonated eggs of *H. gallinarum* contaminated with *H. meleagridis* are the primary source of infection of the disease (McDougald 2005). It is not clear how *H. meleagridis* infects the ovaries of the female worms, but two possibilities are generally accepted: 1) adult nematodes ingest the

protozoa, which invades the gastrointestinal tract and migrate to the reproductive tract and 2) adult female nematodes acquire the protozoa during copulation (Lund and Chute 1973; Cupo and Beckstead 2019; Hess and McDougald 2020). The morphology of *H. meleagridis* in *H. gallinarum* is similar to the ones observed in birds, but with a reduction in size (Hess *et al.* 2015). Turkeys, chickens, and other gallinaceous birds become infected after ingesting contaminated eggs of *H. gallinarum* or potential mechanical hosts (McDougald 2005). *Histomonas meleagridis* emerges from the larva of *H. gallinarum* when it reaches the ceca of the susceptible host and multiplies in the lumen and mucosa (Mazumdar *et al.* 2017; Hess and McDougald 2020). Gross lesions in the ceca can vary depending on the isolate of *H. meleagridis*, as will be discussed below; however, the protozoa typically promotes inflammation and necrosis in the ceca, leading to the formation of cecal cores composed of sloughed tissue and inflammatory cells, then migrates through the hepatic portal vein to reach the liver, causing inflammation and multifocal, circular areas of necrosis, and hepatic failure (Hess *et al.* 2015; Liebhart *et al.* 2017; Powell *et al.* 2009). Other organs, including the spleen, kidney, lung, pancreas, Bursa of Fabricius, and proventriculus can also be affected (Sentíes-Cué, Chin, and Shivaprasad 2009). Clinical signs are not specific, but include depression, dehydration, self-coloured droppings, and ruffled feathers (McDougald 2005; Sentíes-Cué, Chin, and Shivaprasad 2009; Hess *et al.* 2015; Mitra *et al.* 2018). Mortality can reach 70 to 100% in turkeys, causing animal suffering and economic losses to producers.

The cecal nematode does not sufficiently reproduce in turkeys, but eggs from *H. gallinarum* contaminated with histomonads are introduced into turkey production facilities carried by earthworms and potentially by other mechanical vectors (Hauck, Balczulat, and Hafez 2010; Liebhart *et al.* 2017; Lund, Wehr, and Ellis 1966; McDougald 2005). Studies have shown

different results for the role of other mechanical vectors in histomonosis. Hauck, Balczulat, and Hafez (2010) detected DNA of *H. meleagridis* on flies but not on darkling beetles and earthworms. Huber, Gouilloud, and Zenner (2007) detected *H. meleagridis* DNA on larvae but not adult darkling beetles, suggesting that darkling beetles have a low susceptibility to infection but can act as a mechanical vector of *H. meleagridis*. Improper litter disposal, especially from chicken houses heavily populated with *H. gallinarum* in the soil, can lead to the contamination of potential mechanical vectors which could be considered one of the primary sources of spreading the disease (Chadwick, 2020).

Wild birds can also serve as carriers of *H. gallinarum*. Lund and Chute (1972) recovered great numbers of *H. gallinarum* in young guinea fowl, chickens, and ring-necked pheasants. Similarly, Greenawalt *et al.* (2020) detected the cecal worm *H. gallinarum* in ring-necked pheasants in Pennsylvania. The authors raised concerns about the potential of ring-necked pheasants working as reservoirs of *H. meleagridis* since these birds are usually resistant to histomonosis. Wild turkeys, like commercial turkeys, are susceptible to *H. meleagridis,* and *H. gallinarum* does not reproduce sufficiently on them (Lund, Chute, and Wilkins 1975; Greenawalt *et al.* 2020), not representing a potential reservoir for histomonosis. *Heterakis gallinarum* thrives in chickens, which are rarely affected by severe histomonosis pathology except under concurrent stressing conditions, representing a perfect carrier for *H. meleagridis* (McDougald 2005; Cupo and Beckstead 2019). Although intermediate and mechanical hosts are essential for the survival of *H. meleagridis* in the environment, in turkeys, the disease can be transmitted by direct contact (horizontal or lateral transmission) (Hu and McDougald 2003).

DRUGS USED TO PREVENT OR TREAT THE DISEASE

Arsenicals (nitarsone, carbarsone, roxarsone, acetarsol) were the first compounds used to prevent histomonosis (Liebhart *et al.* 2017). The mode of action of arsenicals as antiprotozoal is not clear, but arsenical compounds can inactivate enzymes involved in cellular energy metabolism and DNA replication and repair (Ratnaike 2003), representing some possible effects on *H. meleagridis*. The use of nitarsone (4-nitrophenyl-arsonic acid) in turkeys to prevent histomonosis has been correlated with increased inorganic arsenicals in humans, representing a health risk because of its carcinogenic and toxic activity (Liu *et al.* 1999; Nigra *et al.* 2017). Therefore, the use of nitarsone was withdrawn by the Food and Drug Administration in 2015 (FDA 2015).

Nitroimidazoles (dimetridazole, 1,2-dimethyl-5-nitroimidazole) have a strong antihistomonal activity and were effective prophylactic and therapeutic options (Lucas 1961; McGuire, Moeller, and Morehouse 1964). However, dimetridazole was removed from the market in the United States in 1997, and in Europe the compound was banned in the treatment of food-producing animals in 1995 and as a feed additive in 2001 (Callait *et al.* 2002). The mode of action of nitroimidazoles in *H. meleagridis* is not fully understood, but it is potentially related to the reduction of the 5-nitro group, blocking DNA synthesis (Callait *et al.* 2002; Raether and Hänel 2003; Moreno and Docampo 1985). Studies with *Tritrichomonas foetus* and *Trichomonas vaginalis*, which are other flagellated protozoa affecting cattle and humans, respectively, showed that metronidazole acts mainly in the hydrogenosomes (Benchimol 2008). A similar mode of action of dimetridazole in *H. meleagridis* should be assumed.

Nitrofurans (furazolidone, nifursol, among others) are synthetic broad-spectrum antimicrobial agents, also within the classification of nitroheterocyclic compounds (Raether and Hänel 2003). The mode of action is similar to nitroimidazoles, by redox processes mainly on the

5-nitro group of the furan ring creating reactive compounds capable of damaging proteins and DNA (Barrientos-Salcedo, Espinoza, and Soriano-Correa 2018; EFSA Panel on Contaminants in the Food Chain, 2015; Liebhart *et al.* 2017). Nitrofurans are no longer available as feed additives or therapeutic options for food animals because of their potential mutagenic and carcinogenic activity (Raether and Hänel 2003).

Other antimicrobials have shown high variability in preventing or treating histomonosis (Liebhart *et al.* 2017). Most anticoccidial drugs are not effective in reducing histomonosis lesions (Hu and McDougald 2002). Paromomycin is an aminoglycoside antibiotic, and it interferes with amino acid translation (Raether and Hänel 2003). The efficacy of paromomycin to prevent or treat histomonosis is controversial. While high doses of paromomycin have been shown to reduce morbidity and mortality related to histomonosis in turkeys by Hafez *et al.* (2010), Hu and McDougald (2002) did not observe any effect in an *in vivo* study. Reports of the effect of using paromomycin in controlling or preventing outbreaks of histomonosis have also presented variable outcomes (Liebhart *et al.* 2017). Because of the particular relationship between *H. meleagridis* and bacteria, it is difficult to understand if the antimicrobial affects the protozoa directly or indirectly by reducing the bacteria (Liebhart *et al.* 2017). In addition, overuse of paromomycin could lead to the development of resistance in some bacteria (van Duijkeren *et al.* 2019).

One possible strategy of preventing outbreaks of histomonosis is controlling the intermediate host *H. gallinarum*, but with limitations. Once an outbreak has started in turkeys, deworming drugs have little value since the disease is transmitted in the absence of vectors. In chickens, deworming programs can be an effective strategy since *H. gallinarum* thrives full life-cycle in chickens and could potentially reduce the introduction of the cecal worm into turkey

facilities. Benzimidazole agents, such as fenbendazoles, are approved to control *H. gallinarum* in chickens in the United States (Regmi *et al.* 2016).

Many alternative compounds have been tested against *H. meleagridis*. However, *in vitro* and *in vivo* studies frequently do not have the same outcome (Barros *et al.* 2020; Beer *et al.* 2020; Liebhart *et al.* 2017; van der Heijden and Landman 2008). A significant limitation in most experiments conducted to test alternative compounds is that histomonosis was induced by direct inoculation of *H. meleagridis*, mostly intra-cloacally, in turkeys or chickens. Since the major mode of transmission within turkey flocks is horizontal transmission, drugs should be tested with this infection model to decrease transmission. However, as discussed in a section below, replication of horizontal transmission under experimental conditions has not been reliable in the last years.

Currently, the only strategies producers can rely on to prevent histomonosis are farm management techniques, including cleaning and disinfection, changing litter after an outbreak, physical barriers within a house, raising turkeys and chickens separately, and adequate biosecurity, although these measures are not consistently effective (Regmi *et al.* 2016).

PATHOLOGY AND IMMUNE RESPONSE IN TURKEYS AND BROILER CHICKENS

The pathogenesis of the disease is reported to be different between turkeys and chickens (McDougald 2005; Mitra *et al.* 2018). In turkeys, severe typhlitis and hepatitis are frequently fatal, whereas chickens usually have only the ceca affected and recover from the disease (Sigmon *et al.* 2019; Chadwick *et al.* 2020; Lagler *et al.* 2021). However, studies reported a drop in egg production and weight gain in laying hens (Liebhart *et al.* 2013; Liebhart and Hess 2020), reduction in body weight in broiler breeders (Chadwick *et al.* 2020), and predisposition to

colibacillosis in laying hens (Abdelhamid *et al.* 2020), showing that even though histomonosis is less severe in chickens it can still represent a substantial economic loss for producers and increase susceptibility to other infectious diseases.

Since chickens frequently do not manifest severe clinical signs and can harbour large numbers of the cecal nematode *H. gallinarum* (Cupo and Beckstead 2019), they can serve as carriers for *H. meleagridis*. Outbreaks in turkey flocks have been reported more frequently in production facilities which are geographically close to broiler breeder facilities (Jones *et al.* 2020) and *H. meleagridis* have been detected in broiler breeders one year after inoculation (Chadwick *et al.* 2020). Studies have reported a relationship between resistance to histomonosis and increased ability to reproduce *H. gallinarum* eggs (Daş *et al.* 2011; 2021; Lund 1967).

One of the explanations for the difference in pathogenesis between turkeys and chickens is the immune response (Powell *et al.* 2009; Kidane *et al.* 2018; Mitra *et al.* 2018; Lagler *et al.* 2021). Studies suggest that broiler chickens can build an effective innate immune response in the ceca, decreasing the number of protozoa reaching the liver, whereas turkeys are not able to do the same (Powell *et al.* 2009; Mitra *et al.* 2021). Powell *et al.* (2009) showed a greater migration of histomonads to the liver of turkeys compared to chickens. The authors also evaluated the mRNA expression levels of specific cytokines and chemokines in the liver and ceca of chickens and turkeys. An early innate immune response in the ceca of chickens was detected, possibly explaining the decrease of protozoa reaching the liver. The same was not observed in turkeys, which had an exacerbated immune response in the liver in later stages of the infection. In agreement with the previous study, Lagler *et al.* (2021) compared the immune response of chickens and turkeys vaccinated with an attenuated isolate of *H. meleagridis* and challenged with a wild-type isolate. An absence of IFN-γ production by intrahepatic lymphocytes in chickens was

reported; in contrast, IFN-γ production was observed in both the liver and spleen in turkeys. This suggests that even using an attenuated isolate of *H. meleagridis*, chickens can limit histomonads to the ceca with an immediate local immune response while turkeys produce a more systemic immune response. IFN-γ is a primary activator of macrophages, related to a Th1-mediated immune response (Erf 2004). Although *H. meleagridis* is an extracellular pathogen, it can be phagocytized by macrophages and giant cells, leading to activation of cellular and humoral immune responses (Kidane *et al.* 2018). Interestingly, Kidane *et al.* (2018) reported that experimentally infected chickens had a higher production of IFN-γ in the ceca than infected turkeys, showing a physiological difference between species. Vaccinated and infected turkeys also had a higher production of IFN-γ not observed in the only infected turkeys, suggesting that higher production of IFN-γ can be related to resistance to infection (Kidane *et al.* 2018).

THE MICROBIOTA-PROTOZOA-HOST INTERACTION AND ITS INFLUENCE ON DISEASE OUTCOME AND TRANSMISSION

Host-microbiota-protozoa interplay

Histomonas meleagridis becomes fully virulent in the ceca only in the presence of cecal bacteria. *In vitro* cultivation of *H. meleagridis* is only possible in the presence of bacteria, in xenic or monoxenic conditions (Ganas *et al.* 2012; Springer, Johnson, and Reid 1970). Studies trying to induce the disease in gnotobiotic turkeys and chickens were unsuccessful (Doll and Franker 1963; Franker and Doll 1964; Springer, Johnson, and Reid 1970). Franker and Doll (1964) inoculated *H. meleagridis* in gnotobiotic turkeys with an established cecal monoflora, utilizing different bacteria (*Lactobacillus fermenti*, *Bacillus cereus*, *Streptococcus faecalis*,

Escherichia intermedia). The disease was not induced in turkeys that received only *L. fermenti* or *B. cereus*; whereas, with inoculation of *S. faecalis*, one out of eight turkeys developed histomonosis. Turkeys that received *L. fermenti* or *B. cereus* associated with *E. intermedia* were able to develop lesions similar to those observed in turkeys with conventional microflora. Similarly, Springer, Johnson, and Reid (1970) administered 18 different bacteria isolates with *H. meleagridis* in gnotobiotic chickens and could not induce the disease, but the disease could be induced by administering only *E. coli* and *Clostridium perfringens* to gnotobiotic turkeys, suggesting more complex pathogenesis in chickens. Adding more complexity to the disease, studies suggest that the intermediate host *H. gallinarum* also has a particular interaction with the host microbiota (Doll and Franker 1963; Bilic and Hess 2020).

It is unknown how the protozoa-bacteria relationship modulates disease manifestation and transmission. Some studies suggest that bacteria can serve as a food source or provide specific compounds necessary for *H. meleagridis* survival, including providing an anaerobic environment (Bilic and Hess 2020; Ganas *et al.* 2012; Mazumdar *et al.* 2017). Most of the time, *in vitro* cultures of *H. meleagridis* are xenic (Mitra *et al.* 2018). The bacteria required to produce the disease is not well defined; however, *E. coli* is frequently isolated with *H. meleagridis* in histomonosis outbreaks and it is also reported to best support the protozoa growth *in vitro* (Bradley and Reid 1966; Franker and Doll 1964; Ganas *et al.* 2012; Springer, Johnson, and Reid 1970). Ganas *et al.* (2012) cultivated *H. meleagridis* with defined bacteria species, demonstrating that each bacterium affects the protozoa growth differently. *Escherichia coli* and *Salmonella* Typhimurium provided the best support to *H. meleagridis* growth because of the high growth rate of these bacteria, potentially because these bacteria provided more food for the protozoa and

consumed the oxygen within the media, establishing an anaerobic environment favourable to *H. meleagridis* growth.

As previously mentioned, the protozoa *H. meleagridis* initially colonize the ceca. The avian ceca have important functions, including fermentation, digestion, osmoregulation, and immune response (Clench and Mathias 1995; Svihus, Choct, and Classen 2013; Braun 2015). The ceca are densely populated by microorganisms (Pan and Yu 2014). The commensal microbiota provides a barrier against pathogens, known as “colonization resistance” (Thaiss *et al.* 2016), and are also able to produce important compounds for the host, such as short-chain fatty acids (Pan and Yu 2014). The interaction between the host and the microbiota is vital to keep homeostasis, having a direct impact on the host’s immune system and metabolism (Peterson and Artis 2014; Thaiss *et al.* 2016).

Pathogens with a tropism for the ceca encounter a very dynamic environment, dealing with the intestinal content and urine entering the ceca, local mucus production, the commensal microbiota, and competition with other pathogens (Clench and Mathias 1995). For example, *Eimeria adenoiedes*, *E. meleagridis,* and *E. gallopavonis* are protozoa causing coccidiosis in turkeys, and *E. tenella* causes coccidiosis in chickens. *Eimeria* species also cause lesions in the ceca of turkeys and chickens. It has been shown that infection with *E. tenella* can increase the severity of histomonosis in chickens (McDougald and Hu 2001). Studies with *Eimeria* species in turkeys are less conclusive. Only interaction with *E. adenoeides* has been investigated, and the results are variable depending on the virulence, day of infection, and dose of *E. adenoeides* (Barros *et al.* 2022; Chadwick and Beckstead 2020; McDougald and Fuller 2005). In commercial flocks, it is important to consider that many *Eimeria* species are probably present, together with

other microorganisms, adding complexity to the cecal environment and the outcome of infections.

Factors influencing the transmission of histomonosis in turkeys

Turkeys become infected by ingesting contaminated embryonated eggs of *H. gallinarum*, but once a turkey is infected, the transmission to other turkeys can happen in the absence of the intermediate host, by direct contact, referred to as horizontal or lateral transmission (Hu and McDougald 2003). Horizontal transmission of histomonosis without *H. gallinarum* was not achieved in chickens (Hu *et al.* 2006). Although horizontal transmission of histomonosis has been replicated in experimental conditions, considerable variability has been reported, and some research groups have not been able to experimentally reproduce it (personal communication, March 9, 2020; Hauck and Hafez 2013). A summary of potential factors influencing horizontal transmission in turkeys is present in Figure 2.

Some studies were able to achieve horizontal transmission, but with variations in the results. The rate of transmission of histomonosis in turkeys exposed to directly inoculated turkeys (referred to as seeders) was higher compared to turkeys exposed to contaminated cages where directly inoculated turkeys were previously present (Armstrong and McDougald 2011). The infection rate was also compared between turkeys raised on bare-wire cage floors, paper-covered cage floors, or pine shavings-covered floors. There were no differences in the transmission rate between groups with birds on cages covered with paper or pine shavings, while a lower infection rate was observed on turkeys on bare-wire cage floors. From three experiments with seeder poults in paper-covered cage floors conducted under similar conditions, infection of contacts was not observed in one experiment, even though the seeders developed severe lesions

of histomonosis. The authors did not provide information about the diet nor explain the variability. McDougald and Fuller (2005) achieved similar results to the previously mentioned studies, with turkeys exposed to seeder turkeys getting infected and presenting lesions/mortality due to histomonosis, but in only one experiment. Two other experiments in floor pens could replicate horizontal transmission, but only one experiment was reported by each study (Hess, Grabensteiner, and Liebhart 2006; Landman *et al.* 2015).

In five experiments where the horizontal transmission of histomonosis was compared in floor pens covered with pine shavings or battery cages with the floor covered with paper, in addition to the influence of different diets, varying in ingredients and nutritional densities, and using two isolates of *H. meleagridis* in different groups of turkeys was conducted by Barros *et al.* (Forthcoming). An impact of diet and a possible effect of litter conditions in the transmission of histomonosis was observed. Intriguingly, there was variability within treatments, wherein in some cages presented no horizontal transmission even though the seeder turkeys had severe cecal and hepatic lesions. Horizontal transmission of histomonosis was achieved with only one isolate of *H. meleagridis*. The other isolate was successfully transmitted to other turkeys using horizontal transmission models in previous experiments conducted by another research group (personal communication, March 19, 2020); however, it seems that this isolate lost the ability of causing disease by direct contact.

Disease manifestation has been reported in only one sex in turkey houses with mixed flocks, separated by a wire mesh (Liebhart *et al.* 2017; Sulejmanović *et al.* 2019). Differences in outbreaks in turkey breeders and commercial productions have also been reported (Callait-Cardinal *et al.* 2007). One study showed that although not manifesting severe disease, female turkeys were infected by *H. meleagridis*, demonstrated by the presence of antibodies detected by

ELISA (Sulejmanović *et al.* 2019). At the same house, separated by wire mesh, males were severely affected by clinical histomonosis. The presence of histomonads was detected in dust samples by PCR, confirming the spread of the protozoa throughout the whole house. The authors hypothesized that the gut microbiota and a variation in the immune response between males and females could be responsible for the difference in the disease outcome. The stocking density was not reported in the two compartments of the house, nor the composition of the diets, nor if the diets were different among males and females.

Genetic variability

Since there is variability of disease manifestation in different outbreaks of histomonosis, it is crucial to take into consideration the genetic variability of isolates of *H. meleagridis* and their influence on disease transmissibility and pathogenesis. *Histomonas meleagridis* has a close phylogenetic relationship with *Dientamoeba fragilis* and *Tritrichomonas foetus*, demonstrated by the comparison of small subunit rRNA sequences (Gerbod *et al.* 2001) and the partial sequence of three genes, including the GAPDH, enolase, and α-tubulin β-tubulin gene (Hauck and Hafez 2010). *Dientamoeba fragilis* causes diarrhoea in humans, affecting mainly the colon, but it is not a well-studied protozoan and is even considered neglected in terms of research focus (Stark *et al.* 2016; Garcia 2016). *Tritrichomonas foetus* affects the reproductive tract of cattle and the gastrointestinal tract of cats, also mainly the colon (Tolbert and Gookin 2016).

In a study by van der Heijden *et al.* (2006), *H. meleagridis* was subtyped using the Internal Transcribed Spacer-1 (ITS1) C-profiling method. Two C-profiles were identified, types I and II. The authors speculated about the existence of a third type; however, it was not confirmed. The third ITS1 type was more similar to *D. fragilis* than to *H. meleagridis* and now the

microorganism is identified as *Parahistomonas wenrichi* (Mantini *et al.* 2009). Later on, Hauck, Balczulat, and Hafez (2010) also used the C-profiling method tailored to be more specific for *H. meleagridis*, and four C-profiles of the ITS1 region were identified, differing from the profiles found by van der Heijden *et al.* (2006). The four C-profiles were identified as A, B, C, and D; A and B were detected similarly in chickens and turkeys, while type C was found mainly in turkeys. Type D was rarely detected. The previous study analysed samples from outbreaks and two clonal cultures. Interestingly, they reported variation within the clonal cultures, suggesting an intragenomic variation of the ITS1 region. Lollis *et al.* (2011) sequenced the ITS1-5.8S rRNA-ITS2 region and the 5.8S region alone. In that study, a correlation between geographic location, host species, and genetic sequence of isolates was investigated, but significant correlations were not achieved. However, like Hauck, Balczulat, and Hafez (2010), the authors detected variation in the sequencing, possibly because of the presence of multiple isolates. Considering the high variability within a clonal isolate, sequencing the ITS1-5.8S rRNA-ITS2 region does not seem to be ideal to characterize *H. meleagridis* genetic profiles (Bilic *et al.* 2014).

The existence of two genotypes was indicated by a multilocus sequence approach comparing phylogenetic data of partial 18S rRNA, α-actinin1, and rpb1 genes (Bilic *et al.* 2014). The pathology caused by isolates from the two genotypes is different, with severe typhlitis, perforation of the cecal wall with development of peritonitis, and absence of the typical circular multi-focal areas of necrosis in the liver within genotype 2 (Bilic *et al.* 2014; Grafl *et al.* 2015). Isolates within genotype 1 were more frequently isolated than within genotype 2 (Bilic *et al.* 2014). It is unknown if there are differences in transmission rate between isolates.

Virulence factors

The establishment of a clonal monoxenic *H. meleagridis* culture, derived from the same single cell and cultivated with a specific serotype of *E. coli*, was developed by a research group and had been assisting with the molecular characterization of *H. meleagridis* (Hess *et al.* 2006; Mazumdar *et al.* 2019; Monoyios *et al.* 2018; Palmieri *et al.* 2021). Two markedly different phenotypes were produced, one being attenuated by *in vitro* passaging and the other being fully virulent (wild type).

Studies have compared *de novo* transcriptome sequencing (Mazumdar *et al.* 2017), the proteome (Monoyios *et al.* 2018), the exoproteome (Mazumdar *et al.* 2019) and the genomes (Palmieri *et al.* 2021) of attenuated and wild type isolates of *H. meleagridis*. Both isolates share similar genetic characteristics (Palmieri *et al.* 2021). In summary, small GTPases, protein kinases, membrane proteins, and peptidases which are related to attachment and invasion of host tissues by protozoa were identified in both isolates. Palmieri *et al.* (2021) compared the attenuated and wild-type isolates' genomes and observed deletions and truncations of genes and proteins in the attenuated isolate. One of these genes is potentially involved in cell-surface adhesion.

Serine proteases, metalloproteases, and cysteine proteases which are associated with adhesion and killing of host cells by protozoa were also identified (Mazumdar *et al.* 2017; 2019; Palmieri *et al.* 2021). Since *H. meleagridis* is an extracellular pathogen, virulence factors associated with adherence to host cells are essential (Hinderfeld and Simoes-Barbosa 2020). Actin was identified as the major component of the proteome and a reduction in expression was observed in the attenuated isolate (Monoyios *et al.* 2018). In addition, upregulation of enolase, a protein involved in energy metabolism, and an increase in bacterial protein expression in the

virulent isolate was reported (Monoyios *et al.* 2018). Wei *et al.* (2020) compared the expression of virulence factors of three isolates of *H. meleagridis* from various locations and two attenuated isolates. Agreeing with previous studies, the authors identified differences in the expression levels of some cysteine proteases genes, with a reduced expression in highly passaged isolates of *H. meleagridis*.

Considering the life cycle of the protozoa, mechanisms to allow adaptation and survival to different environmental conditions would be necessary, including varying levels of oxygen and reactive oxygen species (Mazumdar *et al.* 2017). The enzyme superoxide dismutase has been identified as a potential virulence factor, allowing the protozoa to survive low oxygen levels during the host infection. Heat shock proteins and chaperonin subunits have also been identified, indicating a possible mechanism of adaptation (Mazumdar *et al.* 2017; Wei *et al.* 2020).

Comparison with other extracellular protozoa

A similar interaction between bacteria and protozoa is reported in other extracellular protozoa, including *Entamoeba histolytica* and *Trichomonas vaginalis* (Bär *et al.* 2015; Burgess and Petri 2016; Burgess *et al.* 2017; Varet *et al.* 2018). Studies investigating virulence factors of *H. meleagridis* typically compare with the mentioned protozoa since they have been investigated and molecularly characterized (Klodnicki, McDougald, and Beckstead 2013; Palmieri *et al.* 2021; Wei *et al.* 2020). The enteric microbiota has been shown to be an essential determinant of the virulence of *E. histolytica*, a protozoon from the phylum Rhizopoda and the causative agent of amoebiasis in humans (Marie and Petri 2014). Not only that, studies have shown that the host-bacteria-protozoa interaction can modulate the virulence of *E. histolytica* and the response of the intestinal epithelial cells towards the protozoa (Galván-Moroyoqui *et al.* 2008; Burgess *et al.*

2017; Leon-Coria, Kumar, and Chadee 2020). The production of intestinal mucus can be altered by *E. histolytica* in a dysbiotic environment, altering the enteric microbiota and the host immune response, and increasing the pathogenicity of *E. histolytica* (Leon-Coria, Kumar, and Chadee 2020). Dysbiosis can be caused by a diverse range of factors, including diet, water quality, antimicrobial usage, and diseases (Leon-Coria, Kumar, and Chadee 2020).

Trichomonas vaginalis, also from the phylum Parabasalia, causes trichomoniasis in humans, the most common nonviral sexually-transmitted infection globally (Burgess *et al.* 2017). Trophozoites of *T. vaginalis* are the infective stage and there is a transition to an amoeboid form when contacting and invading the host tissue (Hirt 2013), similar to *H. meleagridis* in the ceca. Brotman *et al.* (2012) reported a relationship between the vaginal bacterial community and higher incidence of *T. vaginalis* in women, with women having a vaginal microbiota with a low abundance of Lactobacilli tending to infection. Adhesion to human vaginal cells by *T. vaginalis* has been decreased when associated with *Lactobacillus* spp. (Phukan *et al.* 2013) and vaginal dysbiotic bacteria increased the cytoadhesion of *T. vaginalis* to ectocervical cells, also increasing the pathogenicity of the protozoa (Hinderfeld and Simoes-Barbosa 2020).

Asymptomatic infections are commonly reported with *E. histolytica* and *T. vaginalis*. In the case of human amoebiasis, for example, the acute intestinal or extra-intestinal disease will occur in only 10% of infected people. This variation has been partially linked to the composition of the host's gut microbiota (Marie and Petri 2014; Varet *et al.* 2018). Based on the impact of the vaginal or intestinal microbiota on the virulence of *T. vaginalis*/*E. histolytica* and disease outcome and genetic similarities between *H. meleagridis* and *T. vaginalis*, and some similarity with *E. histolytica* (Klodnicki, McDougald, and Beckstead 2013), together with the importance

of bacteria on *H. meleagridis* pathogenesis and *in vitro* growth, it is plausible to consider that a similar interaction occurs in histomonosis.

Environmental impact

It has been shown that histomonads can survive up to 9 h in fresh faeces and un-chlorinated water, raising the possibility that transmission may occur by contacting faeces contaminated with histomonads in the litter (Lotfi, Abdelwhab, and Hafez 2012). The litter is a combination of bedding material, excreta, feathers, water, and dust. Microbial communities in the litter are influenced by a range of conditions, from ventilation, bedding materials, management practices, feed, and age of the birds (Pan and Yu 2014). There is a direct correlation between gut and litter microbiota, and both are the reflection of the interaction between feed, ventilation, air quality, water quality, gender, among other factors (Adhikari *et al.* 2020; Bindari *et al.* 2021; Dumas *et al.* 2011; Pan and Yu 2014). The litter microbiome has been gaining visibility on influencing infectious diseases (Thépault *et al.* 2020), but more studies are needed to investigate the role of litter management and quality on disease outcomes. Considering the relationship of *H. meleagridis* and bacteria, it is also important to consider the microbiota present in the litter, which can represent a challenging environment for *H. meleagridis*, and not only the cecal microbiota. Callait-Cardinal *et al.* (2010) evaluated factors impacting the incidence and severity of histomonosis in free-range turkey flocks in France and the authors observed an interaction between hygiene and litter quality to the presence and severity of histomonosis. The authors hypothesized that higher levels of moisture in the building, caused by diarrhoea, poor hygiene, and wet litter could increase the contact between turkeys and their excreta.

Considering the influence of the microbiota on the ability of *T. vaginalis* and *E. histolytica* to adhere to the host mucosa and cause disease, one possible hypothesis is that depending on the bacteria and consequent by-products of bacterial fermentation present in the litter and ceca, the ability of *H. meleagridis* to adhere and invade host cells can be impaired. Under challenging conditions, the shape and behaviour of histomonads can change to a cyst-like stage (Gruber, Ganas, and Hess 2017). It is unknown the ability of this morphological stage to adhere and infect a tissue.

CONCLUSION

Although histomonosis is not a new disease and multiple investigations have been conducted over the last century to better understand this disease, at the present time, multiple aspects of transmission and factors influencing disease outcome are not fully understood. The complex dynamism between *H. meleagridis*, the host immune system, and the microbiota in the ceca and the litter makes prediction and understanding of the pathogenicity and transmission very difficult. Studies evaluating the impact of the litter and the ceca microbiota on influencing the morphology and adhesion of *H. meleagridis* would help to further understand the disease manifestation and possible interventions.

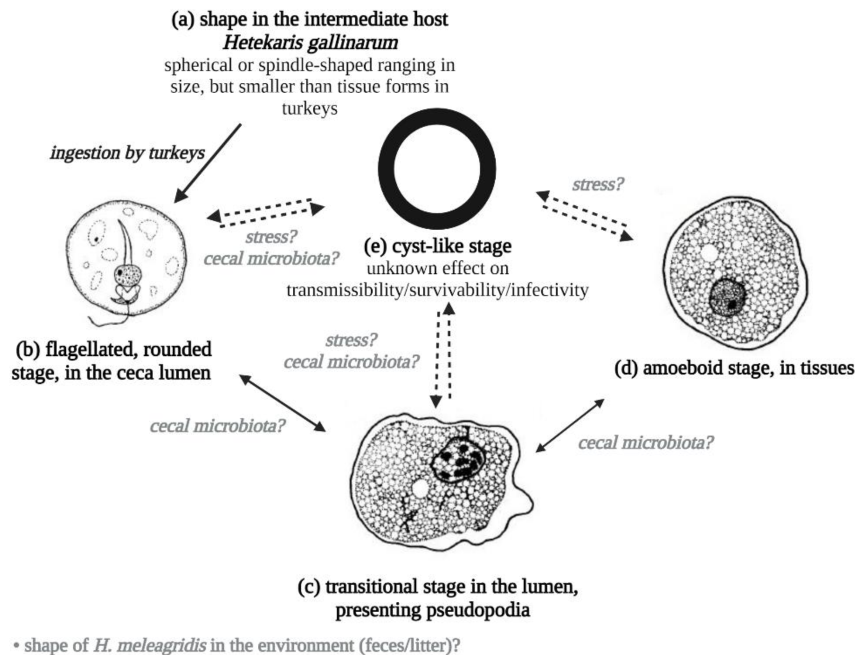

Figure 1. Morphological stages of Histomonas meleagridis. Histomonads are pleomorphic and very little is known about factors influencing the transition to different morphological stages. The dashed lines and gray/italics writings are speculations. Turkeys ingest embryonated eggs of Heterakis gallinarum in which histomonads are smaller than in turkeys (a). In the ceca lumen, histomonads are rounded, presenting one flagellum (b). To start the tissue penetration, histomonads start to change to a transitional stage, presenting pseudopodia (c). In tissues, histomonads are amoeboid (d). Experiments have shown that under stressing conditions, histomonads can change to a cyst-like stage (e). The impact of the cyst-like stage on transmissibility, survivability, and infectivity is unknown. Images (b), (c), and (d) obtained from book Diseases of Poultry (reuse granted by John Wiley and Sons). Created with BioRender.com.

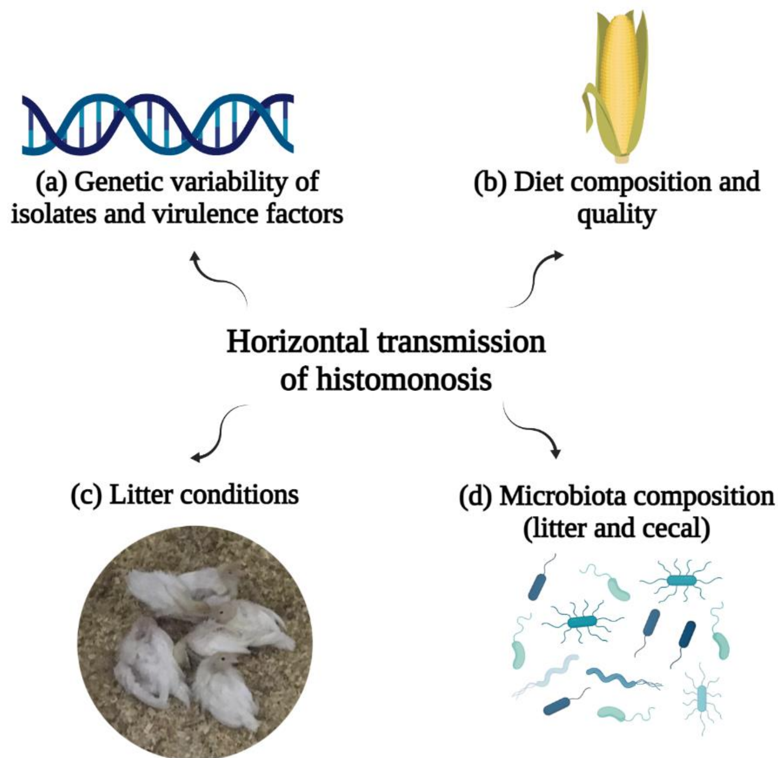

Figure 2. Factors potentially influencing the horizontal transmission of histomonosis. Turkeys become infected by *Histomonas meleagridis* by ingesting contaminated eggs of *Heterakis gallinarum*. However, once a turkey is infected, histomonosis can be transmitted by direct contact (horizontal transmission). Replication of horizontal transmission under laboratory conditions has not been consistent. We speculate that the genetic varialibity of *H. meleagridis* isolate, the quality and composition of poultry diets, litter conditions, and the microbiota composition of the ceca and the litter can influence horizontal transmission of histomonosis. Created with BioRender.com

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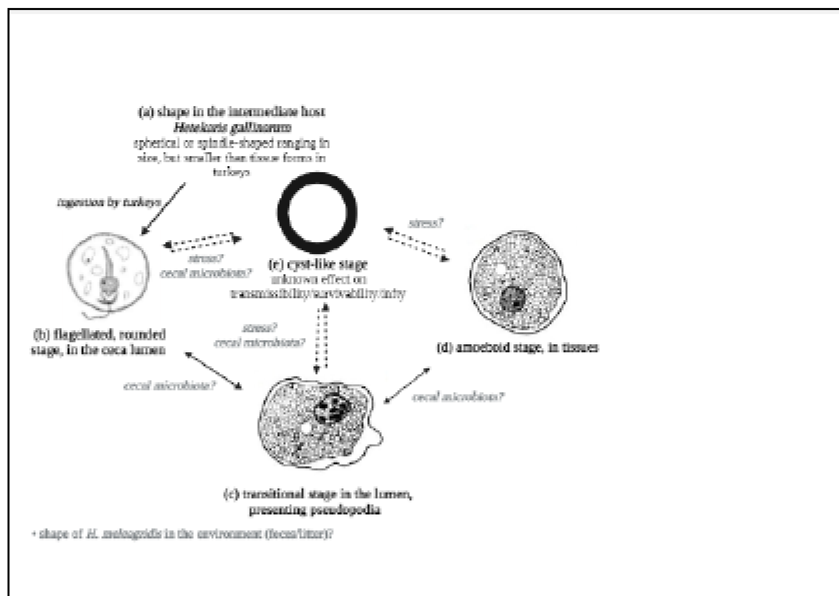

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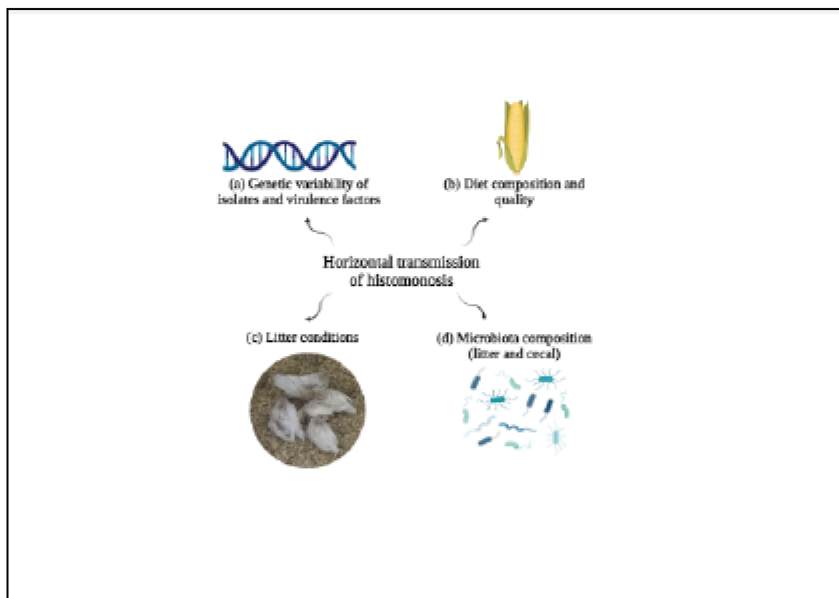

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III. CHAPTER I

Research Note: Evaluation of Dietary Administration of Sodium Chlorate and Sodium Nitrate for *Histomonas meleagridis* prophylaxis in turkeys

Barros T.L.[1], Beer L.C.[1], Tellez G.[1], Fuller L.[2], Hargis B.M.[1], Vuong C.N.[1]

[1]Department of Poultry Science, University of Arkansas, Fayetteville, AR, USA, 72701

[2]Department of Poultry Science, University of Georgia, Athens, GA, USA, 30602

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ABSTRACT

Histomoniasis is currently a re-emerging disease of major significance for many commercial turkey and broiler breeder production companies due to the unavailability of drugs or vaccines. The protozoa *Histomonas meleagridis* (HM) requires the presence of enteric microflora to promote the disease. The objectives of this research note were to evaluate the effect of dietary administration of sodium chlorate (**SC**) and sodium nitrate (**SN**) *in vitro* and *in vivo* for *Histomonas meleagridis* prophylaxis in poults. A total of 128 day-of-hatch female poults obtained from a commercial hatchery were wing-tagged and randomly assigned into one of four experimental groups: negative control (NC), positive control (PC), dietary inclusion of SC (3,200 ppm), SN (500 ppm). Poults from groups SC and SN started on their respective diets on day 12. All groups, except the NC, were challenged with 2 x 10^5 HM on day 19. Controls were fed a basal diet, identical to the treatment diets but not supplemented with SC or SN. Body weight gain (BWG) was determined weekly, starting on day 1 until day 28, and post-challenge morbidity and mortality were recorded. On day 28 of age, all surviving poults were lesion scored for hepatic and cecal lesions. Ceca and distal ileum were collected on day 28 for bacterial recovery on selective media for total aerobic, lactic acid bacteria, or Gram-negative bacteria. The addition of SC and SN in the *in vitro* growth of HM greatly reduced the growth of the protozoa after 20 h of incubation when compared with the control non-treated group ($P < 0.05$). However, dietary supplementation of SC and SN had no effect against HM *in vivo*, as was demonstrated by BWG, the severity of lesions in the liver and ceca, or bacterial recovery of treated poults when compared with the positive control group.

Keywords: sodium chlorate, sodium nitrate, *Histomonas meleagridis,* prophylaxis, turkey poults

INTRODUCTION

Histomoniasis is a re-emerging disease of major significance for many commercial turkey and broiler breeder production companies due to the unavailability of drugs or vaccines (Clark and Kimminau 2017). The protozoa *Histomonas meleagridis* (HM) requires the presence of enteric microflora to promote the disease (Doll and Franker 1963; Franker and Doll 1964), with evidence for preference of *Enterobacteriaceae* (Ganas *et al.* 2012). However, the relationship between *H. meleagridis* and bacteria is not completely understood (Hauck, Armstrong, and McDougald 2010; McDougald 2005). Interestingly, there are two factors regarding the immune response towards Histomoniasis: 1) a high production of IFN-γ, the main cytokine representing a Th1 response, has been associated with providing resistance to the disease (Kidane *et al.* 2018) and 2) unlike turkeys, broiler chickens are able to mount an efficient innate immune response restraining the disease (Powell *et al.* 2009).

The present research note describes a preliminary evaluation of 1) the dependency of the HM protozoa on the cecal microbiota to promote disease and 2) stimulating the host innate immune response to fight the infection. Previous studies have shown that sodium chlorate (SC) has a marked antimicrobial effect against *Salmonella* in the ceca of chickens and turkeys (Byrd *et al.* 2003; McReynolds *et al.* 2004; Moore *et al.* 2006). Sodium nitrate (SN) has been reported to have antimicrobial activity and acts to stimulate the innate immune response by increasing the production of nitric oxide (Ascenzi, Bocedi, and Gradoni 2004; Tiso and Schechter 2015). Hence, the objectives of this research note were to evaluate the effect of dietary administration of SC and SN as a HM prophylaxis in poults.

MATERIALS AND METHODS

***Anti-histomonal activity* in vitro**

Histomonads from a wild-type HM isolated from a field break of histomoniasis in chickens (layer pullets) from the southern United States previously used by (Hauck, Armstrong, and McDougald (2010) were cultured in modified Dwyer's medium (MDM) and 250 µL of original culture containing 1.5 x 10^5 histomonads were added to 700 µL of new MDM medium enriched with rice. Treatments of sodium chlorate (Science Company, CAS: 7775-09-9; SC) or sodium nitrate (Science Company, CAS: 7631-99-4; SN) were reconstituted in water and added to the histomonads at 0.5 mg in 50 µL, whereas controls received 50 µL of the vehicle; final concentration of tested products was 0.5 mg/mL. Each treatment was completed with three replicates. After 20 h, the histomonads were enumerated using a hemacytometer, in duplicate.

***Evaluation of anti-histomonal activity* in vivo**

A total of 128 day-of-hatch female poults were obtained from a commercial hatchery (Cargill, Gentry, AR, USA). Poults were wing-tagged and randomly assigned to one of four experimental groups: negative control (NC), positive control (PC), dietary inclusion of SC (3,200 ppm), SN (500 ppm). Poults were provided *ad libitum* access to water and a balanced, unmedicated corn and soybean diet meeting the nutritional requirements for turkey poults recommended by the NRC. Controls were fed a basal diet for the duration of the study. Poults from groups SC and SN were fed the basal diet until day 11; after which they received their respective dietary treatments from day 12 forward. All groups, except the NC, were intracloacally-challenged with 2 x 10^5 histomonads (divided administration, 1h apart) on day 14

of age with the same wild-type HM described in the *in vitro* essay. This isolate was deliberately kept at a very low passage in culture to maintain virulence/avoid culture-induced attenuation. Body weight gain (BWG) was determined weekly, starting on day 1 until day 28 of age, and final mortality were recorded. On day 28, all surviving poults were lesion scored for hepatic and cecal lesions on a 0-3 scale as described by (Beer *et al.* 2020) (ceca: 0= no macroscopic alterations; 1= detectable thickening of the ceca and small lesions on the mucosa, but normal architecture and cecal content; 2= meaningful cecal wall thickening with some areas of the mucosa presenting hemorrhages and erosions, abnormal architecture of some portions of the ceca, fluid and yellowish cecal content; 3= classic typhilitis with thickened cecal walls, severe inflammation, erosions, and total loss of the normal cecal architecture, presence of caseous cores; liver: 0= no macroscopic alterations; 1= few localized necrotic areas; 2= inflammation and presence of circular necrotic areas in some regions of the liver; 3= severe inflammation and circular necrotic areas approaching confluency on the surface of the liver consistent with the classic "target-like" lesions characteristic of Histomoniasis). Ceca and distal ileum were collected on day 28 for bacterial recovery on Man Rogosa Sharpe (Sigma, Rogosa SL Agar, Cat. No. R1148, St. Louis, MO, 63178, USA) and MacConkey (Becton, Dickinson and Company, BBL™ MacConkey Agar, Cat. No. 211387, Sparks, MD 21152, USA) agar plates. All animal handling procedures were in compliance with the University of Arkansas, Institutional Animal Care and Use Committee (IACUC; protocol number 19118).

Statistical analysis

Data from BWG were subjected to multi-way analysis of variance for the randomized design using the General Linear Models procedure in SAS (version 9.1, SAS Institute Inc., Cary,

NC, USA). Means were separated with Tukey's multiple-range test and considered significant at $P < 0.05$. Data were reported as mean ± SE. A PROC MIXED, ANOVA program was used to test statistical significance for lesion scores. For BWG, each of the replicate pens was considered as the experimental unit (n = 4/treatment); for lesion scores, each bird was the experimental unit (n = 32/treatment); for bacterial recovery, three birds were randomly selected from all replicates of each group (n = 12/group). Mortality was compared with all possible combinations using the chi-square test of independence to determine significance ($P < 0.05$).

RESULTS AND DISCUSSION

The addition of SC and SN to *in vitro* culture of HM significantly ($P < 0.05$) reduced the growth of the protozoa after 20 h of incubation when compared with the non-treated control group (Figure 1). However, dietary supplementation of SC and SN had no effect against HM in the host (Table 2). Turkeys fed SC had a lower BW compared to the other groups (Table 1), suggesting that higher levels of SC would not be practical; in addition, the mortality for the SC group reached 30% on day 25, and the remaining birds were euthanized on the same day due to IACUC protocol requirements. The selected dose of SC was based on experiments where SC was administered in the drinking water, considering the approximate proportion of feed to water consumption (3x the water concentration). Feed consumption was not measured in the present experiment but it is possible that the birds rejected the feed, leading to weaker birds more susceptible to the disease.

The conversion of nitrate to nitric oxide involves complex pathways requiring the participation of different bacteria and enzymes (Tiso and Schechter 2015). Previous researchers reported that turkeys are more sensitive to nitrate toxicity than chickens (Adams, Emerick, and

Carlson 1966). Although in the present study, until the turkeys started showing clinical signs related to histomoniasis, the poults receiving SN did not exhibit suppression in body weight (Table 1), higher concentrations could be detrimental to the bird (Marrett and Sunde 1968).

Bacterial recovery was not significantly different between groups (Table 2), although a tendency of higher Gram-negative and lower lactic-acid bacteria recovery was observed in the birds inoculated with HM, which can be explained by a dysbacteriosis caused by the disease.

No mortalities or lesions were observed in the NC group and mortalities ranged from 18.8 to 31.3% ($P > 0.05$) in all challenged groups. Similarly, lesion scores were not markedly different between treatments for either cecal or hepatic lesions (Table 2).

Previous researchers also showed a discrepancy between *in vitro* and *in vivo* results (Thøfner *et al.*, 2012). The bioavailability of the compounds was not evaluated; it is possible that the compounds did not reach the ceca, not impacting the protozoa.

These data are not encouraging for these candidate approaches for controlling HM as no beneficial effects of these selected treatments and time frames were observed. Higher dietary concentrations of SC and SN are not likely candidates for evaluation due to known negative effects (SC and SN) in turkeys.

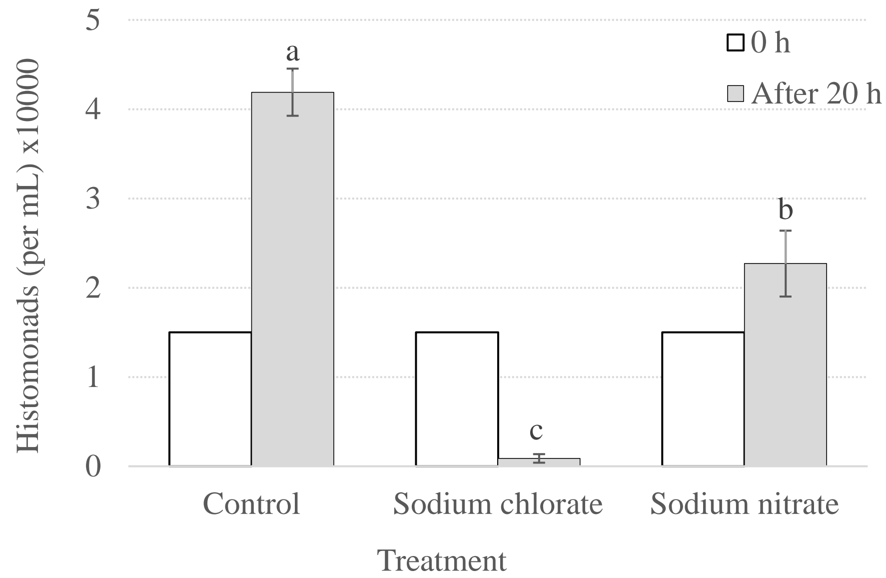

Figure 1. *Histomonas meleagridis* response to *in vitro* treatment with sodium chlorate or sodium nitrate. Histomonads (150,000 cells seeding density) were treated with 0.5 mg sodium chlorate or sodium nitrate (0.5 mg/mL); sterile water treatment served as a negative control. Histomonads proliferation/density enumerated after 20 hours of treatment. Statistical significance (P>0.05) indicated with non-matching letters.

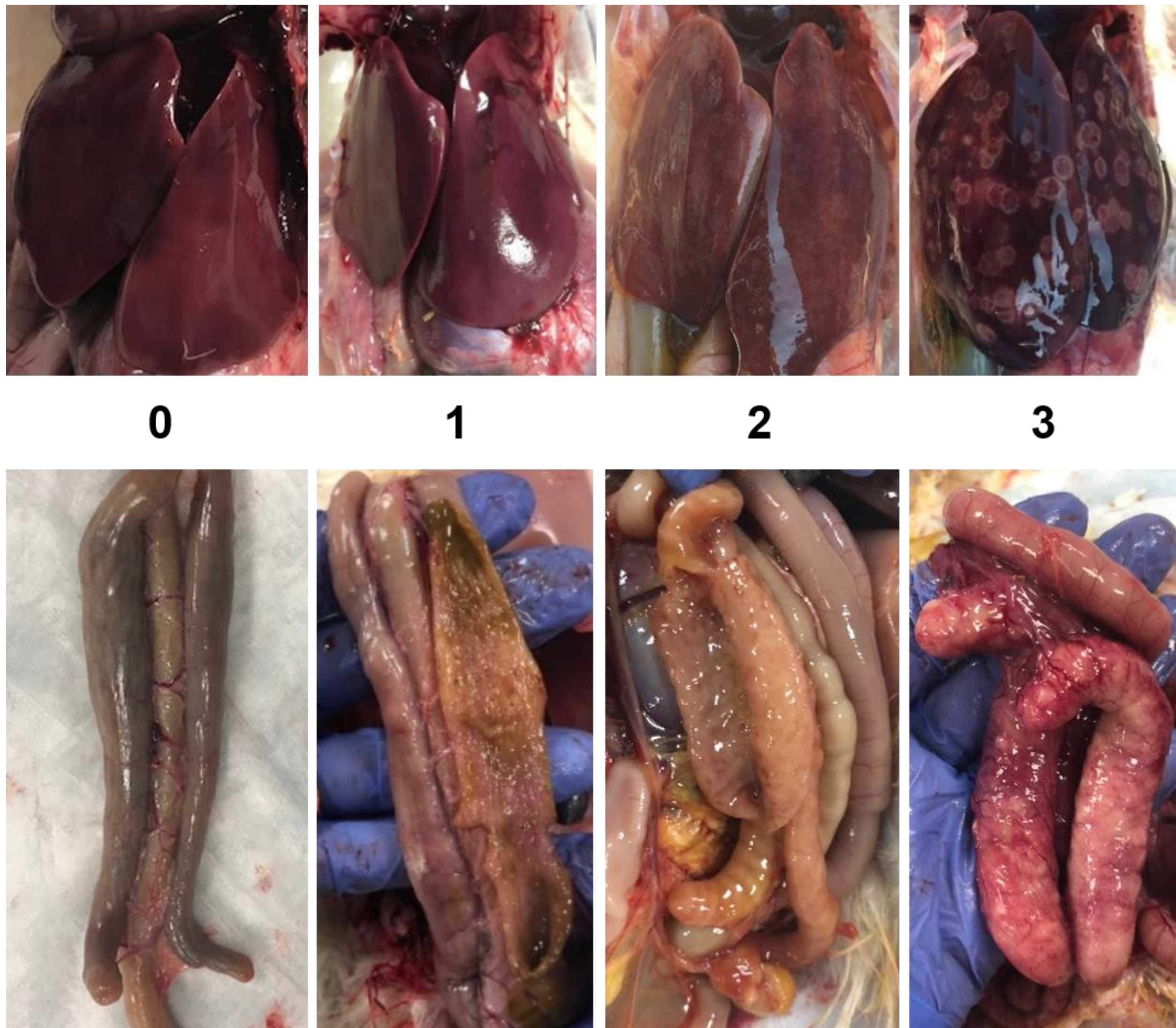

Figure 2. Pictures of the lesion scores according a 0-3 scale presented by Beer *et al.* (2019), where in the ceca 0 = no macroscopic alterations; 1 = detectable thickening of the ceca and small lesions on the mucosa, but normal architecture and cecal content; 2= meaningful cecal wall thickening with some areas of the mucosa presenting hemorrhages and erosions, abnormal architecture of some portions of the ceca, fluid and yellowish cecal content; 3= classic typhilitis with thickened cecal walls, severe inflammation, erosions, and total loss of the normal cecal architecture, presence of caseous cores; and in the liver: 0= no macroscopic alterations; 1= few localized necrotic areas; 2= inflammation and presence of circular necrotic areas in some regions of the liver; 3= severe inflammation and circular necrotic areas approaching confluency on the surface of the liver consistent with the classic "target-like" lesions characteristic of histomoniasis.

Table 1. Body weight (BW) and body weight gain (BWG) of turkeys receiving sodium chlorate (SC) and sodium nitrate (SN) in feed.

Treatment	**NC**	**PC**	**SC**	**SN**	***P*-value**
BW, g/poult					
d 0	57.88 ± 1.33	58.16 ± 1.15	58.81 ± 0.37	59.53 ± 0.76	0.6451
d 7	161.78 ± 3.53	166.06 ± 2.27	161.78 ± 2.83	164.94 ± 2.97	0.6519
d 14	363.47 ±16.29	381.84 ± 5.94	356.88 ± 6.33	385.47 ± 6.23	0.1626
d 21	560.63 ± 39.35	569.19 ± 13.31	494.41 ± 3.73	582.25 ± 14.68	0.0653
d 28	816.44 ± 62.87 [a]	704.62 ± 44.35 [ab]	578.26 ± 21.68 [b]	641.32 ± 36.24 [ab]	0.0142
BWG, g/poult					
d 0 to 14	305.59 ± 17.14	323.69 ±6.51	298.06 ± 6.33	325.94 ± 5.91	0.1984
d 14 to 28	452.97 ± 47.54 [a]	318.99 ± 48.25 [ab]	227.87 ± 25.27 [b]	257.67 ± 40.60 [b]	0.0109
d 0 to 28	758.56 ± 63.89 [a]	646.40 ± 44.26 [ab]	518.74 ± 21.61[b]	581.97 ± 36.01[ab]	0.0141

Data are expressed as the mean ± SEM.

[a-c]Values within rows with different superscripts differ significantly ($P < 0.05$).

NC= negative control; PC= positive control; SC= dietary sodium chlorate (3,200 ppm); SN= dietary sodium nitrate (500 ppm).

Poults from groups NC and PC were fed a basal diet; poults from groups SC and SN started to be fed with the respective compounds on day 12.

Poults from groups PC, SC and SN were intracloacally-challenged with 2 x 10^5 histomonads on day 14.

Table 2. Lesion scores (liver and cecal) and total aerobic, presumptive Gram-negative, and lactic acid bacteria (cecal and lower ileum) of turkeys receiving dietary sodium chlorate (SC) or sodium nitrate (SN) challenged with *Histomonas meleagridis*.

Treatment	NC	PC	SC	SN	*P*-value
Lesion Score Liver (0-3)	0.00 ± 0.00 [b]	2.00 ± 0.23 [a]	2.38 ± 0.19 [a]	2.31 ± 0.21 [a]	<.0001
Lesion Score Ceca (0-3)	0 ± 0.00 [b]	2.13 ± 0.22 [a]	2.31 ± 0.18 [a]	2.38 ± 0.21 [a]	<.0001
Recoverable gram-negative bacteria (Log10 CFU/g) [1]	6.34 ± 0.20	6.81 ± 0.19	6.79 ± 0.16	6.97 ± 0.17	0.0989
Recoverable lactic-acid bacteria (Log10 CFU/g) [2]	7.16 ± 0.18	6.42 ± 0.17	6.65 ± 0.25	6.78 ± 0.20	0.0793
Mortality (%)	0 [b]	18.8 [a]	31.3 [a,1]	18.8 [a]	<0.05

Data are expressed as the mean ± SEM.

[a-c]Values within rows with different superscripts differ significantly ($P < 0.05$).

NC= negative control; PC= positive control; SC= dietary sodium chlorate (3,200 ppm); SN= dietary sodium nitrate (500 ppm).

Poults from groups NC and PC were fed a basal diet; poults from groups SC and SN started to be fed with the respective compounds on day 12.

Poults from groups PC, SC and SN were intracloacally-challenged with 2 x 10^5 histomonads on day 14.

[1]Birds from group SC were euthanized on day 25 because mortality reached 30% as required in IACUC protocol.

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IV. CHAPTER II

Evaluation of *Eimeria adenoeides* pre-infection on the severity of histomoniasis in turkeys.

Barros T.L.[1], Beer L.C.[1], Barta J.R.[2], Hargis B.M.[1], Vuong C.N.[1]

[1]Department of Poultry Science, University of Arkansas Division of Agriculture, Fayetteville, AR, USA, 72701

[2]Ontario Veterinary College, Department of Pathobiology, University of Guelph, Guelph, ON, Canada.

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ABSTRACT

The objectives of this study were to evaluate whether a pre-infection of EAD or ET could impact the severity of subsequent histomoniasis in turkeys (experiment 1) and if previous exposure to EAD infection, when a single or multiple inoculation of EAD were administered with sufficient time for complete cecal recovery, would impact severity of HM incidence and lesions (experiment 2). In experiment 1, 200 poults were assigned to one of five groups: unchallenged negative control; positive challenge control inoculated with 105 HM; EAD at 500 oocysts/bird and HM; EAD at 2,500 oocysts/bird and HM; or ET at 9x106 oocysts/bird and HM. ET and EAD were inoculated on day 15 and HM on day 20. In experiment 2, the trial consisted of two difference challenge ages to evaluate short- or long-term EAD effects before HM challenge. Poults (n= 260) were either assigned to an early HM challenged groups: HM on day 19 challenge control; or EAD at 2,500 oocysts/bird on day 14 with HM on day 19; or the late HM-challenged groups: HM on day 35 challenge control; EAD at 2,500 oocysts/bird on day 14 and HM on day 35; or EAD at 100 oocysts/bird every 2-3 days during the first 3 weeks and HM on day 35. An unchallenged negative control group was utilized for both the early and late challenge phases in experiment 2. Mortalities were recorded and surviving poults were scored for histomoniasis-related hepatic and cecal lesions. In experiment 1, pre-infection with both doses of EAD reduced the mortality, as well as the cecal and hepatic lesions caused by histomoniasis. In experiment 2, neither short- nor long-term pre-infection with EAD had an effect on histomoniasis-related mortality or lesions. Differences between experiments 1 and 2 may be due to the level of infection caused by the pre-challenge with EAD, and the resulting destruction of cecal tissue.

Keywords: histomoniasis; coccidiosis; turkey; cecal protozoa

INTRODUCTION

After the withdraw of arsenicals and nitroheterocyclic compounds, outbreaks of histomoniasis caused by *Histomonas meleagridis* have been frequently reported in turkeys, in addition to broiler breeders and pullets (Liebhart and Hess 2020; McDougald 2005; Powell *et al.* 2009). The search for alternative prophylactic and therapeutic substances has grown in the last few years; however, no consistently effective alternative has been found (Barros *et al.* 2020; Mitra *et al.* 2021). Histomoniasis is primarily lethal in turkeys while chickens usually recover from the disease (Powell *et al.* 2009; McDougald 2005). The varied pathogenesis between turkeys and chickens may be explained by differences in immune responses or pathogen adaptation to chickens. It is hypothesized that broiler chickens can build an effective innate immune response in the ceca, limiting the number of protozoa that will reach the liver, whereas turkeys are unable to do the same and allow an exacerbated number of *H. meleagridis* to enter the liver. Consequently, turkeys mount excessive cytokine production in the liver in response to the protozoa, leading to an uncontrolled immune response (Powell *et al.* 2009; Beer *et al.* 2020).

Coccidiosis, another protozoal disease, is caused by the genus *Eimeria* and is one of the most economically important diseases affecting the poultry industry. *H. meleagridis* and some *Eimeria* species, including *E. adenoeides* in turkeys and *E. tenella* in chickens, mainly affect the ceca of poultry (El-Sherry *et al.* 2014; Gadde *et al.* 2020; McDougald and Fuller 2005; Chadwick and Beckstead 2020) and, frequently, *Eimeria* spp. and *H. meleagridis* parasitize the ceca of poultry at the same time (Chadwick and Beckstead 2020). Previous studies have shown that inoculation of *E. adenoeides* in day-of-hatch poults and indirect exposure to *H. meleagridis*, through contact with inoculated birds, decreased the mortality and cecal lesions caused by *H. meleagridis* (McDougald and Fuller 2005). The mentioned study had a short duration (only 7

days) and the fact that the birds were indirectly infected by *H. meleagridis* does not provide precision regarding infection mechanics; however, these findings suggested that *E. adenoeides* could decrease the severity of *H. meleagridis* infection. Of additional interest, *E. tenella*, a chicken-specific *Eimeria*, can invade and cause inflammation in the ceca of turkeys (Augustine and Danforth 1995). McDougald and Hu (2001) reported that inoculation of *E. tenella* oocysts and *H. meleagridis* in broiler breeders led to increased histomoniasis severity.

In commercial conditions, it is common to have more than one *Eimeria* species, as well as other intestinal protozoa and microorganisms. The influence of this complex microbial community in aggravating histomoniasis is still unknown. Chadwick and Beckstead (2020) reported two outbreaks of histomoniasis, where the authors related the severity of the outbreaks to poor poult quality and coccidiosis. However, the species of *Eimeria* spp. causing coccidiosis were not identified and since coccidiosis was not the only problem, it is not possible to associate the severity of the histomoniasis outbreaks with previous infection by *Eimeria* spp., but the case report illustrates the complexity of the interaction between pathogens in commercial production.

With the limitations and contradictions reported by previous studies, we aimed to evaluate whether pre-infection with either *E. adenoeides* or *E. tenella* could influence the severity of subsequent histomoniasis in turkeys in the first experiment and in the second experiment, we aimed to evaluate if single or multiple challenges with EAD (simulating *Eimeria* cycling in vaccinated or naturally infected commercial turkeys), followed by an early (5 days later) or late (21 days later) HM challenge, would similarly affect lesions and severity of histomoniasis.

MATERIALS AND METHODS

Pathogen culture and challenge

Single-oocyst derived isolates of *E. adenoeides* Guelph strain (El-Sherry *et al.* 2014) and *E. tenella* Guelph strain were propagated *in vivo* in coccidia-free turkeys or chickens, respectively, and held in the Central Animal Facility's Isolation Unit, University of Guelph. Oocysts of *E. adenoeides* and *E. tenella* were isolated from cecal scrapings and fecal material by flotation using saturated sodium chloride (sat. NaCl, aqueous), washed with distilled water, and stored in 2.5% potassium dichromate (w/v, aqueous) at 4°C, as previously described by El-Sherry *et al.* (2014). Prior to use as a challenge stock, oocysts were washed in 0.9% sterile saline, enumerated using a McMaster counting chamber, and then gavaged orally using a 1mL syringe without a fitted needle. For both experiments, a wild-type *H. meleagridis* (HM) isolate originating from a field outbreak of histomoniasis in layer pullets in the southern United States (Hauck, Armstrong, and McDougald 2010) was cultured in Medium 199 (Lonza®, Walkersville, Maryland) supplemented with 10% horse serum (Gibco®, Penrose, Auckland, New Zealand)) and 1.6 mg/mL rice powder (Arrowhead Mills®, Hereford, Texas), following previously described procedures (Beer *et al.* 2020). A 1mL vial of cryogenically stored HM isolate was thawed and cultured in T-25 cell culture flasks (12.5mL culture volumes) approximately one week before challenge. Cultures were incubated anaerobically at 40°C and passaged every 2 to 3 days. On the challenge administration date for each experiment, histomonads were counted with a hemocytometer and the inoculum adjusted to 10^5 histomonads/dose using unsupplemented medium. Challenge volume was 250µL or 500µL per poult for each experiment and was administered cloacally.

Humane care of animals

All animal handling procedures complied with the University of Arkansas Institutional Animal Care and Use Committee (Animal Use Protocol #19118) or the University of Guelph Animal Care Committee (Animal Use Protocol #4314), as applicable. Experiments were terminated once positive control groups reached 30% mortality to comply with animal welfare guidelines; thus, termination dates vary for each experiment (day 31 for experiment 1; days 41 and 48 for early and late phases, respectively, for experiment 2).

Experiment 1

A total of 200 day-of-hatch female poults were obtained from a commercial hatchery (Cargill, Gentry, AR, USA). Poults were wing-tagged, weighed, and 8 poults were randomly placed per battery cage, with a total of 5 battery cages/group (n= 40/group). Poults were assigned to one of five experimental groups: 1) unchallenged negative control (NC), 2) positive challenge control inoculated with the wild-type HM strain (HM) on day 20, 3) *E. adenoeides* at 500 oocysts/bird on day 15 and subsequent HM on day 20 (EADlow + HM), 4) *E. adenoeides* at 2,500 oocysts/bird on day 15 and subsequent HM on day 20 (EADhigh + HM), or 5) *E. tenella* at 9×10^6 oocysts/bird on day 15 and subsequent HM on day 20 (ET + HM). Poults were raised in battery cages and provided *ad libitum* access to water and a balanced, unmedicated corn-soybean diet meeting the recommended nutritional requirements for turkeys (14). *Eimeria* inoculations were orally administered on day 15. *H. meleagridis* challenge was intracloacally administered on day 20 at 10^5 histomonads/bird. Body weight (BW) was measured on days 15, 20, and 31 in order to calculate body weight gain (BWG). Mortality was recorded throughout the trial and lesions evaluated. On day 31, all surviving poults were evaluated for hepatic and cecal lesions related to histomoniasis based on a 0-3 scale, as previously described (Beer *et al.* 2020).

Experiment 2

For the second experiment, only *E. adenoeides* was used in the pre-infection. A preliminary *E. adenoeides* dose titration trial was performed to select the most appropriate dose of *E. adenoeides* for pre-infection: not causing severe lesions nor reductions in body weight. For this pre-trial, the oocyst dose selected should cause an infection similar to those achieved with commercial vaccines. The doses tested were 0; 2,500; 25,000; or 50,000 oocysts/mL per bird. Poults were weighed on the day of hatch, day 14, and day 19. The final dose selected was 2,500 oocysts/bird based on an average lesion score of 2.8 ± 0.26 and no difference in BWG from the negative control (data not shown).

The second experiment was divided in two phases: an early *H. meleagridis* challenge, in which turkeys received *H. meleagridis* 5 d.p. *E. adenoeides* inoculation and a late *H. meleagridis* challenge, in which turkeys received *H. meleagridis* approximately 21 days after *E. adenoeides* challenge. The experiment was conducted in floor pens. The experiment timeline is shown in Figure 1. Female poults (n= 260) were obtained from a commercial hatchery (Cargill, Gentry, AR, USA) and were randomly assigned to one of the following experimental groups: 1) unchallenged negative control (NC, n= 48) to serve as negative control for both early and late challenge phases; for the early challenge phase groups: 2) *E. adenoeides* at 2,500 oocysts/bird on day 14 and HM on day 19 (EAD + HM1, n= 40), 3) HM only (HM1, n= 41) on day 19; or for the late challenge phase groups: 4) *E. adenoeides* at 2,500 oocysts/bird on day 14 and HM on day 35 (EAD + HM2, n= 44), 5) inoculation of HM only on day 35 (HM2, n= 43), or 6) *E. adenoeides* at 100 oocysts/ bird every 2-3 days during the first 3 weeks of life, to assure simulated cycle exposure, and HM inoculation on day 35 (multiple EAD + HM2, n= 44). On day 19, five days post the *Eimeria* inoculation, 10 turkeys in total from groups EAD + HM1 (n= 6) and EAD +

HM2 (n= 4) were humanely euthanized and cecal lesions specific to *E. adenoeides* were evaluated and scored (Gadde *et al.* 2020).

Treatment groups not receiving *E. adenoeides* (NC, HM1, and HM2) were fed a diet containing the anticoccidial (Clinacox®), since Hu and McDougald (2004) reported no impact of diclazuril on histomoniasis. Mortalities were recorded and all surviving poults (22 days post HM1 for early-challenged groups or 13 days post HM2 inoculation for late-challenged groups) were evaluated for histomoniasis-related hepatic and cecal lesions, as described above.

Statistical analysis

Data from BWG were subjected to multi-way analysis of variance for the randomized design using the General Linear Models procedure in SAS (version 9.1, SAS Institute Inc., Cary, NC, USA). Means were separated with Duncan's multiple-range test and considered significant at $P < 0.05$. Data were reported as mean ± SEM. A PROC MIXED procedure was used to determine statistical significance for lesion scores. Mortality was compared with all possible combinations using the chi-square test of independence to determine significance ($P < 0.05$).

RESULTS

The BWG, lesion score, and mortality data for experiment 1 are presented in Table 1. BWG between the day of HM inoculation (day 20) until the end of the experiment (day 31) was lower for poults receiving HM only (126.7 ± 28.07), compared to the NC (451.3 ± 15.39), ET + HM (261.3 ± 39.35), EADlow + HM (267.9 ± 52.16), and EADhigh + HM (298.5 ± 54.38) groups ($p < 0.01$). Poults receiving only HM presented with 33% mortality, not differing from the group ET + HM (15%). Both EADlow + HM and EADhigh + HM groups experienced 2.5%

mortality, differing from HM and ET + HM ($p < 0.01$). Cecal and hepatic lesions were less severe in the poults that received either doses of EAD and HM, with cecal scores of 2.0 ± 0.21 (EADlow + HM) and 1.8 ± 0.21 (EADhigh + HM) being different from ET + HM (2.5 ± 0.16) and HM (2.8 ± 0.11) ($p < 0.01$). Hepatic lesions had scores of 1.8 ± 0.24 (EADlow + HM), 1.7 ± 0.23 (EADhigh + HM), 2.5 ± 0.18 (ET + HM), and 2.8 ± 0.12 (HM) ($p < 0.01$).

Lesion scores and mortality data for experiment 2 are presented in Table 2. For the first phase of the experiment, turkeys that received a single dose of *E. adenoeides* (on day 14) possessed lesion scores of 1.6 ± 0.16. The hepatic lesions in the HM1 challenge control group averaged 1.2 ± 0.28, exhibiting with no significant differences from the single dose EAD + HM1 treatment group (0.9 ± 0.26). Likewise, significant differences were not observed for cecal lesions (HM1: 1.1 ± 0.27, EAD + HM1: 0.8 ± 0.22). The mortality 22 days post HM inoculation in the HM1 group (day 41) was 36% and was 21% for the EAD + HM1 group, which were not statistically different. In the second phase of the experiment, terminating at day 48, the hepatic lesions averaged 2.5 ± 0.32 in the HM2 positive challenge control group, 1.8 ± 0.27 in the EAD + HM2 group, and 2.1 ± 0.27 in the multiple EAD + HM2 group. Similarly, no differences in cecal lesions were observed (HM2: 2.5 ± 0.32, EAD + HM2: 1.8 ± 0.27, and multiple EAD + HM2: 1.9 ± 0.27). Mortality was 72% in the HM2 group, 41% in EAD + HM2, and 44% in the multiple EAD + HM2.

DISCUSSION

In this study, the potential of *E. adenoeides* infection to exacerbate or reduce histomoniasis severity was evaluated in two experiments. In neither experiment was additive or synergistic effects observed, suggesting that neither prior nor concurrent *E. adenoeides* infection

are significant risk factors for increasing severity of histomoniasis in turkeys. In experiment 1, *E. adenoeides*-induced typhlitis was approximately at peak lesions at the time of HM inoculation (5 days-post *E. adenoeides* inoculation). The BWG for the NC and HM groups should have been identical for the 15-to-20-day period (Table 1) since no challenge had been introduced for either group but, perhaps due to pen effects, a reduction in the BWG for the HM was observed. However, the group challenged with ET during this period were observed to have a BWG which was statistically equivalent to the NC group, which would be expected, as ET is not able to replicate in turkeys. In comparison, those birds challenged with either high or low dose EAD were significantly lower than either the NC or the ET challenged birds during this period, suggesting that EAD challenge, as expected, would reduce performance during this period.

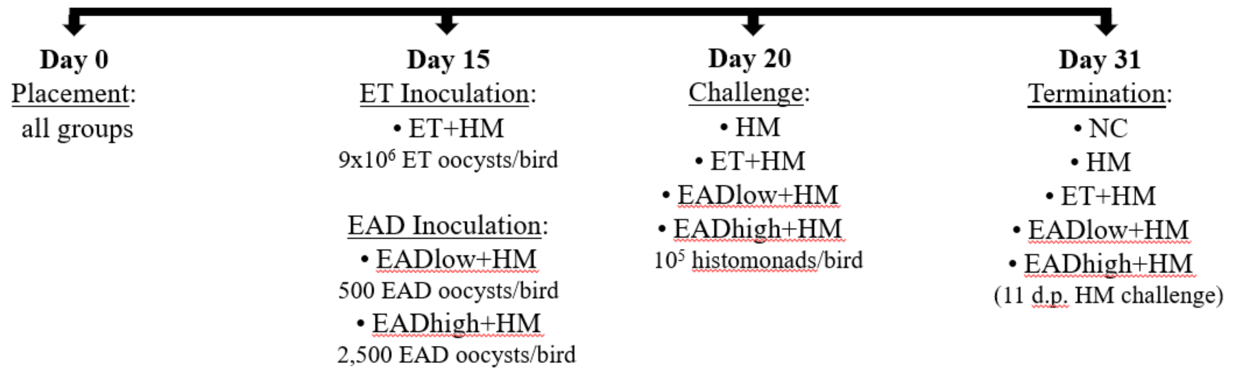
In addition to the reduction in BWG, the formation of cecal cores was observed post-mortem in two birds 5 days-post-*E. adenoeides* inoculation. It is possible that this severe and contemporary *E. adenoeides* challenge and associated innate immune response in the ceca and/or damage to the cecal epithelium, may have prevented retrograde transport into the ceca following HM challenge or sufficiently destroyed the cecal tissue, which effectively partially reduced histomoniasis incidence and severity.

Contraditory results were also observed in *E. tenella* and *H. meleagridis* in broiler chickens. Chappel (1973) could not induce histomoniasis when inoculating oocysts of *E. tenella* (10^4 oocysts) in broiler chickens four or five days before *H. meleagridis*. The author reported that broiler chickens from those groups developed only coccidiosis-related lesions in the ceca, suggesting that histomonads were not able to penetrate the cecal epithelium. In the same study, however, with inoculation of *E. tenella* one, two, three- or four-days post *H. meleagridis* inoculation, broiler chickens had coccidiosis and histomoniasis-related lesions or only

histomoniasis-related lesions in the ceca. Different results were observed by McDougald and Hu (2001) who reported that a light infection with *E. tenella* (10^3 or 10^4), given 4 days before *H. meleagridis* or on the same day, increased the severity of histomoniasis in broiler chickens.

It is important to point out that even though the same dose was used in both trials, the oocysts of *E. adenoeides* used in experiments 1 and 2 are from different amplification batches. This may explain the differences in *E. adenoeides*-induced pathology between trials. Based on the current experimental results, as well as those of previous studies (McDougald and Fuller 2005; McDougald and Hu 2001; Chappel 1973), the effect of pre- or co-infection with cecal *Eimeria* spp. may be variable and dependent on the severity of the *Eimeria*-induced pathology. In the present study, it is concluded that neither mild nor severe pre-infection with *E. adenoeides* exacerbated lesions of histomoniasis in turkeys.

Experiment 1



Experiment 2

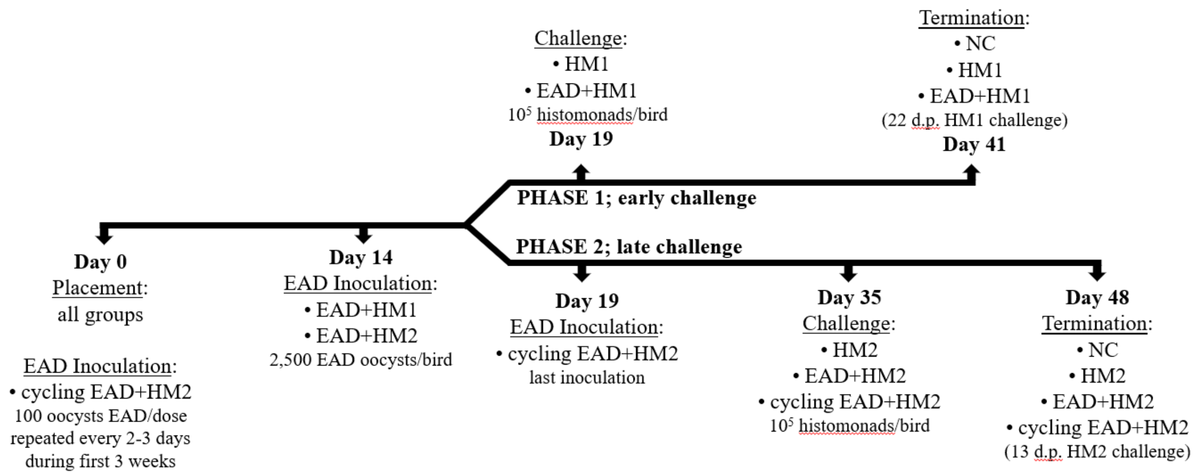


Figure 1. Experimental timelines.

Table 1. Experiment 1: Lesion, mortality, and body weight gain data after *Eimeria tenella* or *E. adenoeides* pre-infection and subsequent *Histomonas meleagridis* challenge.

Groups	**LS Liver**	**LS Ceca**	**Mortality (%)**	**BWG, g/poult**	
				15 to 20 days	20 to 31 days
NC (n= 40)	0^{c}	0^{c}	0^{b}	161.1 ± 5.25^{a}	451.3 ± 15.39^{a}
HM (n=	2.8 ± 0.12^{a}	2.8 ± 0.11^{a}	33^{a}	136.6 ± 8.81^{b}	126.7 ± 28.07^{c}
ET + HM	2.5 ± 0.18^{a}	2.5 ± 0.16^{a}	15^{a}	159.3 ± 2.50^{a}	261.3 ± 39.35^{b}
EADlow + HM	1.8 ± 0.24^{b}	2.0 ± 0.21^{b}	2.5^{b}	121.8 ± 3.95^{b}	267.9 ± 52.16^{b}
EADhigh + HM	1.7 ± 0.23^{b}	1.8 ± 0.21^{b}	2.5^{b}	118.8 ± 10.27^{b}	298.5 ± 54.38^{b}
***P*-value**	<.0001	<.0001	<.0001	0.0003	0.0005

Data are expressed as mean ± SEM.
Lesion scores are the average of mortalities and evaluation on termination (day 31).
LS= lesion score for *H. meleagridis* specific lesions, based on a scale of 0-3.
$^{a-c}$Values within columns with different superscripts differ significantly ($P < 0.05$).
NC: unchallenged negative control,
HM: challenge control administered at 10^5 HM on day 20,
ET+HM: 9×10^6 oocysts/bird *E. tenella* on day 15 and 1×10^5 histomonads on day 20,
EADlow+HM: 500 oocysts/bird *E. adenoeides* on day 15 and 1×10^5 histomonads on day 20, and
EADhigh+HM: 2,500 oocysts/bird *E. adenoeides* on day 15 and 1×10^5 histomonads on day 20.

Table 2. Experiment 2: Lesion and mortality data after *Eimeria adenoeides* pre-infection and subsequent *Histomonas meleagridis* challenge.

Groups	LS Liver	LS Ceca	Mortality (%)
Phase 1 (Early Challenge): HM1 challenge day 19			
NC	n/a	n/a	0 [b]
EAD + HM1	0.9 ± 0.26	0.8 ± 0.22	21 [a]
HM1	1.2 ± 0.28	1.1 ± 0.27	36 [a]
***P*-value**	0.4064	0.3597	<0.05
Phase 2 (Late Challenge): HM2 challenge day 35			
NC	0 [y]	0 [y]	0 [y]
EAD + HM2	1.8 ± 0.27 [x]	1.8 ± 0.27 [x]	41 [x]
Multiple EAD + HM2	2.1 ± 0.27 [x]	1.9 ± 0.27 [x]	44 [x]
HM2	2.5 ± 0.32 [x]	2.5 ± 0.32 [x]	72 [x]
***P*-value**	>0.05	>0.05	<0.05

Data are expressed as mean ± SEM.
Lesion scores are the average of mortalities and evaluation on termination (day 41 - phase 1 and day 48 - phase 2).
LS= lesion score, for specific histomoniasis lesions based on a scale of 0-3.
[a-c, x-y]Values within columns with different superscripts differ significantly ($P < 0.05$).
NC: negative unchallenged control,
HM1: challenge control administered at 10^5 HM on day 19,
EAD+HM1: 2,500 oocysts/bird *E. adenoeides* on day 14 and then challenged with 10^5 HM on day 19,
HM2: late-age challenge control at 10^5 HM only on day 35,
EAD+HM2: 2,500 oocysts/bird *E. adenoeides* on day 14 and 10^5 HM on day 35;
Multiple EAD+HM2: 100 oocysts/bird of *E. adenoeides* every 2-3 days during the first 3 weeks of life and HM on day 35.

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V. CHAPTER III

Feed composition and isolate of *Histomonas meleagridis* alter horizontal transmission of histomonosis in turkeys. Proof of concept

Barros T.L.[1], Vuong C.N.[1], Latorre J.D.[1], Cuesta R.S.[1], McGill E.[2], Rochell S.J.[1], Tellez-Isaias G.[1], Hargis B.M.[1]

[1]Center of Excellence for Poultry Science, University of Arkansas, Fayetteville, AR, United States

[2]Cargill Turkeys LLC, Springdale, AR, United States

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ABSTRACT

Outbreaks of histomonosis in turkeys are typically initiated by the ingestion of contaminated embryonated eggs of *Heterakis gallinarum*, potentially present in earthworms and mechanical vectors. Once an outbreak is started, infected turkeys can transmit the disease by horizontal transmission. Factors influencing horizontal transmission of histomonosis are poorly understood. Replication of horizontal transmission in experimental conditions has not been consistent, presenting an obstacle in searching for alternatives to prevent or treat the disease. Two pilot experiments and three validation experiments were conducted in the present study. In pilot experiment 1, one isolate of *Histomonas meleagridis* (named Buford) was used. Turkeys were fed a low-nutrient density diet corn-soy based (LOW-CS) and raised in floor pens. In pilot experiment 2, another isolate of *H. meleagridis* was used (named PHL). Turkeys were fed a low-nutrient density diet with the addition of wheat middlings (LOW-WM) and raised in floor pens. In experiment 3, conducted on floor pens, both isolates and diets were used in different groups. In experiment 4, turkeys were raised on battery cages and only the PHL isolate was used. Both diets (LOW-WM and LOW-CS) were used, in addition to a diet surpassing the nutritional needs of young poults (turkey starter, TS). In experiment 5, conducted in battery cages, only the PHL isolate was used, and the LOW-WM and TS diets were in different groups. The horizontal transmission was achieved only with the PHL isolate from all experiments. The transmission rate varied among experimental diets, with the TS diet having the lowest transmission rate in experiments 4 and 5. Variation was observed between experiments and within experimental groups.

Keywords: intestinal health, enterohepatitis, protozoa, ceca, epidemiology

INTRODUCTION

The incidence of histomonosis, caused by the protozoa *Histomonas meleagridis*, has been increasing after the ban of nitroimidazoles and arsenicals as preventative and therapeutic measures (Regmi *et al.* 2016). Outbreaks can lead to high mortality rates in turkey flocks (McDougald 2005, Hess *et al.* 2015, Liebhart *et al.* 2017). Turkeys become infected by ingesting contaminated *Heterakis gallinarum* eggs, but once a turkey is infected, the transmission to other turkeys can happen in the absence of *H. gallinarum* by direct contact (Hu and McDougald 2003), referred to in the present paper as horizontal transmission.

In the last decade, there has been an increase in research about histomonosis (McDougald 2005, Hess *et al.* 2015, Hauck and Hafez 2003); however, some epidemiologic aspects remain unanswered, such as risk factors associated with the transmission of histomonosis and the lack of a model of infection by horizontal transmission. The replication of horizontal transmission of histomonosis has not been consistent in many research groups in the last years (Hauck and Hafez 2003). One explanation is the duration of the trials, with experiments ending before turkeys could show lesions (Hauck and Hafez 2003). In outbreaks of histomonosis in turkey flocks, it is common to have some variability, whereas, in some outbreaks, only females or only males were affected in a mixed flock house, typically separated by a wire mesh. Factors behind the variability in disease manifestation and incidence are unknown. Previous studies have reported the existence of two distinct clusters of *H. meleagridis* differing in prevalence in geographical locations and pathology (Bilic *et al*. 2014) and variation in the expression of virulence factors and pathogenicity of different field isolates (Wei *et al.* 2020). Nevertheless, as far as we are aware, the impact of different isolates of *H. meleagridis* or the feed composition of the diet in the horizontal transmission of the disease has not been investigated. Hence, the purpose of this study

was to evaluate the feed composition and isolates of *H. meleagridis* on the transmissibility of histomonosis in floor pens or battery cages.

MATERIALS AND METHODS

Bioethics

All animal handling procedures complied with the University of Arkansas Agricultural Institutional Animal Care and Use Committee (Animal Use Protocol #19118). Following inoculation with *H. meleagridis*, turkeys were monitored at least twice daily to evaluate and potentially euthanize terminally moribund animals. Severe clinical morbidity, with evidence of inability to ambulate, were euthanized and considered as mortalities.

Pathogen culture and challenge

The Buford isolate consists of a wild-type *H. meleagridis* from a field outbreak of histomonosis in layer pullets in the southern United States (Hauck, Armstrong, and McDougald 2010). The PHL isolate is also a wild-type *H. meleagridis*, isolated in 2017 from turkeys in the Northwest Arkansas, USA. Both isolates were cultured in Medium 199 (Lonza®, Walkersville, Maryland) supplemented with 10% horse serum (Gibco®, Penrose, Auckland, New Zealand)) and 1.6 mg/mL rice powder (Arrowhead Mills®, Hereford, Texas), following previously described procedures (Beer *et al.* 2020). The 1mL vial cryogenically stored from each isolate was thawed and cultured in T-25 cell culture flasks (12.5mL culture volumes) approximately one week (Buford isolate) and two weeks (PHL isolate) before the inoculation. Cultures were incubated anaerobically at 40°C and passaged every 2 to 3 days. On the inoculation day for each

experiment, histomonads were counted with a hemocytometer, adjusting the inoculum to 10^5 histomonads/dose using unsupplemented media. The volume of the inoculum varied between 250-500 µL between the trials, always administered via the intra-cloacal route, holding turkeys in an inverted position for one minute after the inoculation to reduce the possibility that the turkeys would expel the material.

Animal source and husbandry

In all experiments, turkeys had *ad libitum* access to feed and water. All turkeys were female and were obtained from a commercial hatchery (Cargill, Gentry, AR, USA) for experiments 1, 2, and 3 and from another commercial hatchery (Butterball, Goldsboro, NC, USA) for experiments 4 and 5.

Experimental design

Pilot studies

In the present study, two pilot experiments were conducted with the intention of assessing the horizontal transmission of two different isolates of *H. meleagridis* and two diet compositions in turkeys.

In the first pilot study (Experiment 1), the inoculation of a typical isolate of *H. meleagridis* (Buford) and the use of a low-nutrient density diet with a reduction in the crude protein level would cause transmission of histomonosis in turkeys raised on floor pens. Pilot experiment 1 was conducted on floor pens (4.5 m^2), with wood shavings as the bedding material. Fifty day-of-hatch poults were randomly distributed to either a non-challenged control (NC) or horizontal transmission group (HT), both being fed a mashed low-nutrient density (LOW) diet,

corn-soy based (CS) diets divided into two phases: the first CS diet (CS1) from day 10 to day 21 and the second CS diet (CS2) from day 21 to 45. The diets were formulated based on requirements for broilers (broiler starter and developer), with a lower protein content compared to a turkey diet commonly used in trials with turkeys conducted by our group, which surpasses the nutritional needs of young turkeys, referred to as turkey starter (TS). The composition of the diets is available in Table 1. The turkey starter diet was fed during the first ten days of the poults' life. Seven out of twenty-five turkeys were directly inoculated on day 14 with the Buford isolate (10^5 histomonads/turkey), referred to as seeders. The experiment was terminated on day 45; mortality was monitored daily, and hepatic and cecal lesions were evaluated on a scale of 0-3 (Beer *et al.* 2020). The following experiments followed the same criteria of evaluation.

In the second pilot study (Experiment 2), we used a different isolate of *H. meleagridis*, named PHL, and a low-nutrient density diet with the addition of 20.5% wheat middlings provided by a commercial company that frequently reports outbreaks of histomonosis in pre-reproductive turkey hens. Sixty-eight day-of-hatch poults were randomly distributed to either a non-challenged control group (n=34) or a horizontal transmission group (n=34). Ten of the 34 poults in the HT group were directly inoculated (seeders) on day 18 with only the PHL isolate (105 histomonads/turkey). Poults were fed the mashed turkey starter diet for the first 14 days, and on day 15, a pelleted LOW diet containing 20.5% wheat-middlings (WM) was introduced until the end of the experiment (day 52). The experiment was conducted on floor pens. The composition of the diet is available in Table 1. The experiment was terminated on day 52; mortality was monitored daily, with an evaluation of the lesions.

Validation studies

Based on the findings of the pilot studies, three experiments were conducted to validate the preliminary data as described below.

In Experiment 3, also conducted in floor pens, two LOW diets (CS and WM) with the same formulation used in the first two experiments were introduced after the first week of the poults' lives: WM diet (WM, d7-38) and CS diets (CS1, d7-21, and CS2, d21-38). The diets were similar to those in the first two experiments, but in different batches. All poults were fed the turkey starter diet during the first seven days. The two previously mentioned isolates of H. meleagridis were tested: Buford or PHL. Day-of-hatch poults were randomly distributed to one of eight groups: 1) NC, fed a LOW diet, CS-based (NC-CS, n= 45); 2) NC, LOW diet, WM based (NC-WM, n= 50); 3) positive control (PC), all turkeys directly inoculated with the Buford isolate, fed a LOW diet, WM based (PC-Buford-WM, n= 50); 4) PC, all turkeys directly inoculated with the PHL isolate, fed a LOW diet, WM based (PC-PHL-WM, n= 50); 5) HT with the Buford isolate, fed a LOW diet, WM based (HT-Buford-WM, n= 45); 6) HT with the Buford isolate, fed a LOW diet, CS-based (HT-Buford-CS, n= 45); 7) HT with the PHL isolate, fed a LOW diet, WM based (HT-PHL-WM, n=45); or 8) HT with the PHL isolate, fed a LOW diet, CS-based (HT-PHL-CS, n= 45). Due to a space limitation, we did not include positive controls with the CS diet. On day 10, 14 of 45 poults in the HT groups were directly inoculated with 105 histomonads/turkey and all turkeys (n= 50/group) in the PC groups. Mortality was recorded daily in both experiments. The PC-Buford-WM was terminated on day 24, 14 days post infection (d.p.i.), the PC-PHL-WM on day 36 (26 d.p.i.), and the remaining groups on day 38; mortality was monitored daily, and hepatic and cecal lesions were evaluated on a scale of 0-3. Bodyweight gain (BWG) from day 7 to 38 was measured only on the NC groups because the PC-Buford-WM

group had to be terminated on day 24, and the PC-PHL-WM was terminated on day 36. For those groups, BWG was measured from day 7 to day 21.

In Experiment 4, turkeys were raised in battery cages. The cage floor was covered with heavy paper from day 10 to day 25. The paper was changed daily. Three diets were tested: in addition to the two previously LOW diets (WM and CS), the diet surpassing the nutritional needs of young turkeys (turkey starter, TS) was used throughout the experiment for two groups. The TS diet was administered to all groups for the first seven days, then the WM diet was introduced on day 7 until termination (d30) (groups 2 and 5), or the CS diet was divided into two phases (CS1 and CS2), or the TS the whole period (groups 1 and 4). The CS1 was administered from day 7 to day 21 and CS2 from day 21 to termination (d30) (groups 3 and 6). One-hundred ninety-two poults were randomly divided into the following groups (n= 8 poults/cage, 4 replicates): 1) NC, fed the TS diet (NC-TS); 2) NC, fed a LOW diet, WM based (NC-WM); 3) NC, fed a LOW diet, CS-based (NC-CS); 4) HT group, fed the TS diet (HT-TS); 5) HT group, fed a LOW diet, WM based (HT-WM); or 6) HT group, fed a LOW diet, CS-based (HT-CS). Only the PHL isolate was used; on day 9, 2 of 8 poults were directly inoculated (seeders) with 10^5 histomonads/turkey. Body weight was recorded on day 7 and at termination (day 30) to calculate BWG. Mortality was recorded daily with an evaluation of lesions.

In Experiment 5, turkeys were again raised in battery cages, following the abovementioned practices. For this trial, only two diets were used: the LOW diet, WM-based with the addition of 3% of celite as a filler, and the corn-soy diet surpassing the nutritional needs of young turkeys (TS), mashed. The TS diet was administered to all groups for the first seven days, then the WM diet was introduced on day 7 until termination (d29) (groups 2 and 4) or the TS diet for the whole period (groups 1 and 3). Two hundred forty poults were randomly

allocated into 4 groups (n= 10/cage, 6 replicates): 1) NC, fed the TS diet (NC-TS); 2) NC, fed a LOW diet, WM based (NC-WM); 3) HT group, fed the TS diet (HT-TS); or 4) HT group, fed a LOW diet, WM based (HT-WM). On day 7, only 8 poults were kept in each cage. On day 9, 2 of 8 poults were directly inoculated (seeders) with 105 histomonads/turkey from the PHL isolate. Body weight was recorded on day 7 and at termination (day 27) to calculate BWG, and feed consumption was recorded. Mortality was recorded daily with an evaluation of lesions.

Statistical analysis

Mortality and frequency of lesions were compared with all possible combinations using the chi-square test of independence to determine significance in experiment 3, 4 and 5 ($P < 0.05$). Bodyweight gain data were subjected to multi-way analysis of variance for the randomized design using SAS's General Linear Models procedure. Means were separated with the Duncan test and considered significant at $P < 0.05$. Data were reported as mean ± SE. In experiment 3, for BWG each bird was the experimental unit. In experiments 4 and 5, the average of each cage was considered the experimental unit for performance data and individual birds for lesions scores. For all experiments, turkeys succumbing to infection or which were euthanized were subjected to lesion scoring.

RESULTS

Table 2 shows a summary of the results of the five horizontal transmission experiments in turkeys fed with different feed compositions and challenged with two different isolates of *Histomonas meleagridis*.

In pilot experiment 1, no horizontal transmission was observed (Table 2). In pilot experiment 2, the horizontal transmission was achieved, with 30.4% (7 out of 23) of the contacts presenting lesions in the ceca and/or liver, with only one mortality. Mortality of the seeder turkeys started on day 39 (21 d.p.i.), reaching 40% on day 44 (26 d.p.i.) when all seeders were humanely euthanized (Table 2). The trial was terminated on day 52 (34 d.p.i.).

In Experiment 3, no horizontal transmission was observed in turkeys fed with WM and challenged with either PHL or Buford strain of *H. meleagridis*. Interestingly, turkeys fed with CS and challenged with the PHL isolate showed 57% of horizontal transmission in the contacts (Table 2). Moreover, this group had 23.3% (7 out of 30) mortality in the contact turkeys (Table 2). The positive control groups PC-Buford-WM and PC-PHL-WM had mortality rates of 63.3% and 33.3%, respectively, with mortality of turkeys on the PC-Buford-WM group starting at 10 d.p.i. and having a sharp increase until 14 d.p.i., while mortality on the group PC-PHL-WM started 15 d.p.i., prolonging until 26 d.p.i. (Figure 1). Mortality in the seeders of the HT-Buford-WM group began at 11 d.p.i., 15 d.p.i. in the PC-PHL-WM group, 16 and 21 d.p.i. in the groups HT-PHL-WM and HT-PHL-CS, respectively. Mortality of the seeder turkeys in the HT groups was 42.9% in the HT-PHL-CS and HT-PHL-WM groups, 57.1% in HT-Buford-CS, and 64.3% in HT-Buford-WM (Table 2, $P > 0.05$). From day 7 to 21, the groups NC-WM and NC-CS had a similar BWG, differing from the group PC-Buford-WM and PC-PHL-WM (Table 3). From day 7 to 38, the group NC-WM had a higher BWG than the group NC-CS (Table 3).

In Experiment 4, the horizontal transmission was observed in all groups fed with different diet compositions (HT-TS, HT-WM, HT-CS). However, the group fed the turkey starter diet (HT-TS) had a lower percentage of contacts ($P > 0.05$) with lesions compared to both low-nutrient density diets (Table 2). In one of the four replicate cages of the group HT-TS, no

contacts had cecal nor hepatic lesions, while both seeders presented cecal lesions. No hepatic lesions were observed in both seeders and contacts from all groups. Only one seeder from the HT-WM group died (Table 2). Mortalities were not observed in the other groups. From day 7 to 30, the group NC-WM had the highest BWG, followed by the NC-TS, HT-WM, HT-TS, NC-CS, and HT-CS (Table 3).

In Experiment 5, the horizontal transmission was observed in both groups (HT-TS and HT-WM). Agreeing with the previous experiment, a lower transmission level was observed in the group fed the turkey starter diet (HT-TS; Table 2). Four of six replicate cages of the HT-TS group had no contacts with lesions in the ceca or liver, although both seeders developed severe lesions, except one in one cage where only one seeder had lesions. The contacts of two cages of the HT-WM did not develop lesions in the ceca and liver. Two seeders died in the HT-TS group and five seeders and one contact died in the HT-WM group. The BWG from day 7 to 27, the group NC-TS had the highest BWG, followed by HT-TS, NC-WM, and HT-WM. Feed intake followed a similar pattern, being higher in the NC-TS and HT-TS groups, followed by NC-WM, and HT-WM (Table 3).

DISCUSSION

In the present study, variation was observed between experiments conducted under similar conditions. In Experiment 3, we could not achieve horizontal transmission using the same isolate (PHL) and diet (low-nutrient density wheat middlings diet) used in the pilot experiment 2. In addition to that, variability was observed within treatments in experiments 4 and 5 conducted in battery cages, with the horizontal transmission not being observed in some cages, although

having the same conditions, agreeing with the variability of horizontal transmission observed in other studies (Armstrong and McDougald 2011).

Armstrong and McDougald (2011) investigated the rate of transmission of histomonosis between turkeys exposed to directly inoculated turkeys (referred to as seeders) or to contaminated cages, where directly inoculated turkeys were previously present. The authors also compared the transmission rate between turkeys raised on bare-wire cage floors, paper-covered cage floors, or on floor pens with pine shavings. Differences were not detected in the rate of transmission between groups of turkeys placed on cages covered with paper or on floor pens with pine shavings. A higher rate of transmission was observed with turkeys that had directly contacted seeders, while turkeys exposed to contaminated cages had a lower infection rate. Interestingly, in one of three experiments conducted in similar conditions, having two seeder birds and six contact turkeys in battery cages with the floor covered, contact turkeys were not infected (Armstrong and McDougald 2011). Still, the seeders developed severe lesions of histomonosis, similarly to what was observed in some cages of experiments 4 and 5 of the present study. The variation in infection rate was not explained by the authors. McDougald and Fuller (2005) compared the horizontal transmission rate of histomonosis in turkeys on battery cages. Each cage had a total of eight poults, with two, three, or four of them being directly inoculated with *H. meleagridis*, and each treatment had three replicates. The horizontal transmission was achieved in all groups, with 72.2%, 80.0% and 75.0% of the contacts positive for histomonosis in the groups with two, three, and four seeder turkeys, respectively. The authors also investigated the impact of the length of exposure on contamination. Poults were exposed to seeders birds (two seeders and six contacts) for one, two, three, or four days. Each treatment had two replicates. Contact turkeys presented lesions at a rate of 16.7%, 100%, 87.5% and 100%

when exposed to seeder turkeys for 1, 2, 3, or 4 days, respectively. The authors presented the results as the average of the replicates, not stating if variation was observed between replicates. Nevertheless, no information about the diets was provided (McDougald and Fuller 2005). In a study by Landman *et al.* (2015), ten turkeys were directly inoculated with *H. meleagridis,* and 30 turkeys were exposed to them for 14 days. Seeders and contact turkeys were 14 days old. All contact turkeys were positive for histomonosis (mortality or lesions) at the end of the experiment. Only one study was reported without replication. Turkeys were fed a standard turkey feed containing 2292 cal/gram and 25.8% crude protein, but once again, no dietary ingredients were reported (Landman *et al.* 2015).

Variability in the incidence and outcome of histomonosis in outbreaks in turkey farms is also commonly reported, with only one house within a farm with multiple houses being affected; only one sex in mixed flocks, or only one section within a house (Callait-Cardinal *et al.* 2010, Sulejmanović *et al.* 2019). Sulejmanović *et al.* (2019) reported three outbreaks of histomonosis in turkey houses where toms and hens were raised together but in different compartments separated by a wire mesh. In the three outbreaks, only toms were severely affected by histomonosis. At the same time, female turkeys were infected, detected by the presence of antibodies evaluated by ELISA but did not manifest clinical signs. The presence of histomonads was also confirmed in high numbers in dust samples by PCR. The authors hypothesized that the gut microbiota and a variation in the immune response between males and females could be responsible for the difference in the disease outcome (Sulejmanović *et al.* 2019). Unfortunately, the stocking density was not reported in the two compartments of the house, nor the composition of the diets nor if the diets were different among males and females (Sulejmanović *et al.* 2019).

It is still not clear if histomonosis can be transmitted by contacting fresh feces in the litter or only by direct cloacal contact between turkeys and which factors would affect the survival of histomonads in the litter. Lotfi, Abdelwhab, and Hafez (2012) showed that histomonads could survive 9 hours in turkey feces and non-chlorinated water, raising the possibility that the protozoa can be transmitted by contact with the litter and other environmental sources. In the present study, both pilot studies (Experiments 1 and 2) and Experiment 3 were conducted in floor pens with wood shavings as bedding material. Although it was not quantified, in pilot Experiment 2, litter moisture was apparently higher than in Experiment 3 in the floor pen of the group HT-WM. Moreover, in Experiment 3, a difference was noticed in the quality of the feces between groups fed the LOW diets, the corn-soy based, or the wheat-middlings based, with turkeys from the HT-PHL-CS group presenting watery feces. Interestingly, that was the only group that experienced horizontal transmission. It is noteworthy that although the same diet formulation for the wheat middling diet was used in experiments 2, 3, 4, and 5, the nutritional composition of the diets varied and that in experiment 5, the WM diet had the addition of 3% of celite as a filler (Table 4). Another hypothesis, although not evaluated, is that the diets used in the different experiments had ingredients with different levels of mycotoxins. It is known that mycotoxins interact with the intestinal microbiota, possibly leading to dysbiosis (Guerre 2020), which could potentially favor the development of histomonosis. Only one study investigated horizontal transmission of histomonosis in turkeys fed a diet containing ingredients contaminated with aflatoxins (Fudge *et al.* 2022). The influence of other mycotoxins in histomonosis is unknown and requires further investigation.

Histomonas meleagridis has a particular relationship with bacteria (Ganas *et al.* 2012, Mazumdar *et al.* 2017, Bilic and Hess 2020). Some studies suggest that bacteria can serve as a

food source or provide specific compounds necessary for *H. meleagridis* survival (Dumas *et al.* 2011, Pan and Yu 2014, Adhikari *et al.* 2020). In other intestinal protozoa affecting human beings, such as *Entamoeba histolytica* and *Trichomonas vaginalis*, the bacteria can affect the virulence and adhesion of the protozoa (Burgess *et al.* 2017, Hinderfeld and Simoes-Barbosa 2020). In the case of *H. meleagridis*, the protozoa are probably influenced by the intestinal microbiota and the litter microbiota (Dumas *et al.* 2011, Pan and Yu 2014, Adhikari *et al.* 2020, Bindari *et al.* 2021). There is a direct correlation between gut and litter microbiota, and both are the reflection of the interaction between feed, ventilation, air quality, water quality, gender, among others (Dumas *et al.* 2011, Pan and Yu 2014, Adhikari *et al.* 2020, Bindari *et al.* 2021). One hypothesis is that depending on the bacteria and consequently by-products of bacterial fermentation present in the litter and ceca, the ability of *H. meleagridis* to adhere and invade host cells can be impaired. *Histomonas meleagridis* is a pleomorphic microorganism, assuming a rounded, flagellated form on the cecal lumen, transitioning to amoeboid during the invasion of tissues (Munsch *et al.* 2009, Gruber, Ganas, and Hess, 2017). Under challenging conditions, the shape and behavior of histomonads can change to a cyst-like stage (Munsch *et al.* 2009, Gruber, Ganas, and Hess, 2017, Zaragatzki *et al*. 2010). The role of this cyst-like stage in the infectivity and transmission of histomonosis is unknown. One hypothesis is that the ability to invade tissue, causing infection, is reduced in these stages. Callait-Cardinal *et al.* (2010) evaluated factors impacting the incidence and severity of histomonosis in free-range turkey flocks in France and the authors observed an interaction between hygiene and litter quality to the presence and severity of histomonosis. The authors hypothesized that higher levels of moisture in the building, caused by diarrhea, poor hygiene, and wet litter, could increase the contact between turkeys and their excreta.

To remove the variability of the litter, experiments 4 and 5 were conducted in battery cages with heavy paper covering the floor, allowing contact of turkeys with excreta. Nevertheless, variability was observed within treatments, which are puzzling findings. It could be hypothesized that the fecal moisture was different within treatments, impacting the survival or activation of histomonads; however, the position of each replicate cage in the room was randomized, therefore, a ventilation effect is not probable. Water consumption was not measured, but comparing the feed consumption of experiment 5, there was a low variation within treatments, suggesting that it is unlikely the possibility of some cages having a lower water consumption.

Interestingly, the BWG of turkeys eating the LOW, WM diet was higher than the BWG of turkeys eating the turkey starter in the experiment. The same was not observed in Experiment 5. In experiment 5, poor poult quality was observed, with 4.2% seven-day mortality. All groups were fed the turkey starter diet during the first seven days and poor poult quality reflected on the overall performance, as can be observed comparing the BWG of experiments and 5. The effect on BWG does not seem to be associated with transmission of histomonosis since, in experiment 4, turkeys in the HT-TS group had no difference in BWG compared to the HT-WM group; however, the HT-TS had a lower number of contacts presenting lesions. Poor poult quality has been linked with the severity of histomonosis (Chadwick and Beckstead 2020).

Regarding the two isolates of *H. meleagridis* used in the present study, we were not able to achieve horizontal transmission with the Buford isolate, only with the PHL isolate. The Buford isolate was recovered from layer pullets around 20 years ago and supplied to us by Dr. Lorraine Fuller, University of GA, Athens. It is possible that although the isolate is still able to cause clinical disease and that initially, it led to horizontal transmission in experiments

conducted by other research groups (personal communication); the isolate lost its ability to infect other turkeys by direct transmission during sequential *in vitro* passages during the years. The PHL isolate is contemporary, and it was isolated from turkeys. The Buford isolate is more virulent than the PHL isolate, causing the formation of cecal cores and inflammation of the ceca, together with severe hepatic lesions, and having a shorter incubation period compared with the PHL isolate. The PHL isolate causes severe typhlitis, much more severe than the one caused by the Buford isolate, sometimes leading to perforation of the cecal wall and peritonitis. Although the present study did not evaluate the genetic variation between the isolates Buford and PHL, based on the mortality rate, clinical signs, and lesions, it can be assumed that the isolates belong to different genetic clusters. In outbreaks in commercial flocks, usually, a combination of isolates of *H. meleagridis* can be involved in an infection, potentially explaining the variability in disease manifestation (Lund, Wehr, and Ellis 1966).

To conclude, we were able to reproduce horizontal transmission of histomonosis in four out of five experiments, more consistently on battery cages with the floors covered with paper and with diets with low-nutrient density. Transmission of histomonosis is multifactorial and not fully understood. Further studies are needed to investigate the role of litter moisture, diets, and morphological stages of *H. meleagridis* on the transmissibility of histomonosis.

Table 1. Composition of the experimental diets.

Ingredient (%)	Low-nutrient density diet corn soy based (LOW-CS)		Diet surpassing the nutritional needs of young turkeys (TS)	Low-nutrient density diet with wheat middlings (LOW-WM)
	Corn-soy 1 (CS1)	Corn-soy 2 (CS2)		
Corn	57.90	75.64	43.33	61.75
Soybean meal	30.23	19.09	42.24	13.20
Wheat middlings	-	-	-	20.50
Animal protein concentrate[§]	5.00	0	7.50	-
Poultry fat	3.58	1.00	3.40	-
Limestone	1.10	1.59	0.66	-
Calcium	-	-	-	1.52
Monocalcium phosphate	-	-	-	2.13
Dicalcium phosphate	1.10	1.57	1.52	-
Salt	0.40	0.41	0.24	0.25
Bicarbonate	-	-	-	0.20
Methionine	0.20[1]	0.20[1]	0.38[1]	0.16[3]
Lysine	-	-	0.42[2]	0.02[4]
L-threonine	-	-	0.11	-
Vitamin/mineral premix	0.20/0.10[†]	0.20/0.10[†]	0.15[‡]	0.23[€]
Choline chloride (60%)	0.20	0.20	0.05	0.02[£]
Enzymes[¶]	-	-	-	0.02
Calculated composition (%)				
Crude protein	22	15	28	14
AME (kcal/kg)	3,098	3,082	3,020	2,800
Total Ca	1.27	1.07	1.49	1.15
Available phosphorus	0.56	0.43	0.76	0.58
Dig TSAA	0.82	0.67	1.06	0.55
Dig Lys	1.02	0.69	1.64	0.62
Dig Thr	0.68	0.49	0.96	0.43
Dig Ile	0.79	0.56	1.01	0.49
Dig Val	0.91	0.66	1.12	0.59
Dig Trp	0.22	0.15	0.28	0.14
Dig Arg	1.34	0.88	1.75	0.80

[§]Composition: crude protein, 57%; crude fat, 8.5%; calcium, 7.94%; phosphorus, 3.59%; sodium, 0.49%; potassium, 0.38%; chloride, 0.73%; cysteine, 1%; methionine, 0.71%; lysine, 3.13%; histidine, 0.91%; tryptophan, 0.34%; threonine, 1.97%; arginine, 3.78%; isoleucine, 1.88%; leucine, 3.71%; phenylalanine, 2.09%; valine, 2.77% (H.J. Baker's ProPlus 57%).
[†]Supplied per kg of feed by vitamin premix (0.2%): Vitamin A, 61,740 IU; vitamin D3, 44,100 ICU; vitamin E, 441 IU; vitamin B12, 0.1 mg; menadione, 12 mg; riboflavin, 52.9 mg; D-pantothenic acid, 79.4 mg; niacin, 308.6 mg; folic acid, 7.1 mg; pyridoxine, 22 mg; thiamine, 12.3 mg; biotin, 0.7 mg. Mineral premix (0.1%): calcium, 767 mg; total phosphorus, 0.8 mg; potassium, 1.2 mg; sodium, 1.2 mg; magnesium, 1.0 mg; sulfur, 1,228 mg; iron, 15 mg; zinc, 100 mg; manganese, 100 mg; copper, 15 mg; iodine, 1.2 mg; selenium, 0.3 mg.
[‡]Supplied per kg of feed by vitamin and mineral premix (0.15%): Vitamin A, 13,227 IU; vitamin D3, 6,613 ICU; vitamin E, 66 IU; calcium, 51 mg; manganese, 124.5 mg; zinc, 124.5 mg; copper, 7.5 mg; iodine, 2.1 mg; selenium, 0.3 mg.
[€]Supplied per kg of feed by Vitamin and mineral premix (0.225%) Vitamin A, 13,230 IU; vitamin D3, 66,100 ICU; vitamin E, 100 IU; vitamin B12, 0.0248 mg; vitamin E EQ, 100 mg; biotin, 0.33 mg; menidione, 4 mg; riboflavin, 15 mg; d-pantothenic acid, 24.26 mg; niacin, 88.2 mg; folic acid, 1.1 mg; pyridoxine, 6.9 mg; thiamine, 2.21 mg; manganese, 125.1 mg; chelated manganese, 40 mg; zinc, 125.1 mg; chelated zinc, 40 mg; iron, 2.1 mg; copper, 7.5 mg; iodine, 2.1 mg; selenium, 0.3 mg.
[£]Choline chloride (70%).
[¶]Rovabio® Advance Phy L: endo-1,4-β-xylanase ≥ 6,250 VU/ml, endo-1,3(4)-β-glucanase ≥ 4,300 VU/ml, arabinofuranosidase ≥ 23,000 VU/ml, 6-phytase ≥5,000 FTU/ml.
[1]DL-methionine.
[2]L-lysine HCl.
[3]Methionine hydroxy analog (88% methionine).
[4]BIOLYS-77® (60% lysine).

Table 2. Summary of the results of the horizontal transmission, evaluated as the % frequency of lesions in ceca and/or liver in contact turkeys for all five experiments evaluating different feed compositions and isolates of *Histomonas meleagridis*.

Experiment	Diet	Isolate	Number of turkey seeders/ Number of turkey contacts	% Horizontal transmission in contacts	% Mortality rate (seeders / total)	% Mortality rate (contacts / total)
Pilot experiment 1 (Floor pen)	CS[1]	Buford	7/18	0 %	100 % (7/7)	0 % (0/18)
Pilot experiment 2 (Floor pen)	WM[2]	PHL	10/23	30.4 % (7/33)	40 % (4/10)	4.3 % (1/23)
Experiment 3 (Floor pen)	WM CS	Buford or PHL	14/30	57 % (17/30) PHL-CS [a] 0 % (0/30) PHL-WM [b] 0 % (0/30) Buford-WM [b] 0 % (0/30) Buford-CS [b]	42.9 % (6/14) PHL-CS 42.9 % (6/14) PHL-WM 64.3 % (9/14) Buford-WM 57.1 % (8/14) Buford-CS	23.3 % (7/30) PHL-CS [a] 0 % (0/30) PHL-WM [b] 0 % (0/30) Buford-WM [b] 0 % (0/30) Buford-CS [b]
Experiment 4 (Battery cages)	WM CS TS[3]	PHL	2/6	100 % (23/23) WM [a] 83.3 % (20/24) CS [b] 45.8 % (11/24) TS [c]	12.5 % (1/8) WM [a] 0 % CS [b] 0 % TS [b]	0 % (0/6) WM 0 % (0/6) CS (0 %) (0/6) TS

Experiment 5	WM	PHL	2/6	61.1 % (22/36) WM [a]	41.6 % (5/12) WM	2 % (1/36) WM [a]
(Battery cages)	TS			16.7 % (6/36) TS [b]	4.2 % (2/12) TS	0 % (0/6) TS [b]

`[1]CS: low nutrient density diet, corn-soy based.
[2]WM: low nutrient density diet, wheat middlings based.
[3]TS: diet surpassing the nutritional requirements of young turkeys, turkey starter diet.
[a-c] Values within columns in each Experiment with different superscripts differ significantly ($P < 0.05$).

Table 3. Evaluation of body weight gain (BWG) or feed intake (FI) of turkeys inoculated with two different isolates of *Histomonas meleagridis* (Buford or PHL) and fed different experimental diets in validation experiments 3, 4 and 5.

Experiment 3	BWG (d7-21)	BWG (d7-38)
NC[1]-WM[2]	233.1 ± 2.65 [a]	870.9 ± 16.31 [a]
NC-CS[3]	233.6 ± 5.89 [a]	610 ± 11.97 [b]
PC[4]-Buford-WM	176.7 ± 11.86 [c]	ND
PC-PHL-WM	210.1 ± 6.95 [b]	ND

Experiment 4	BWG (d7-30)	
NC-TS	701.0 ± 10.44 [b]	ND
NC-WM	834.0 ± 20.86 [a]	ND
NC-CS	524.7 ± 16.03 [c]	ND
HT-TS	586.0 ± 22.63 [c]	ND
HT-WM	599.7 ± 29.02 [c]	ND
HT-CS	425.0 ± 15.91 [d]	ND

Experiment 5	BWG, g (d9-27)	FI, g (d9-27)
NC-TS	497.7 ± 7.48 [a]	751.2 ± 21.96 [a]
NC-WM	307.5 ± 8.07 [c]	608.3 ± 10.61 [b]
HT-TS	442.5 ± 20.09 [b]	761.3 ± 40.51 [a]
HT-WM	215.0 ± 22.22 [d]	561.7 ± 24.26 [b]

[1]NC: non-challenge control.
[2]WM: low nutrient density diet, wheat middlings based.
[3]CS: low nutrient density diet, corn-soy based.
[4]PC: positive control (all turkeys directly inoculated on day 10 with the Buford or PHL isolates, 10^5 histomonads/turkey, intracloacally).
ND: not determined.

Data express as Mean ± SE. [a-c]Values within columns with different superscripts differ significantly ($P < 0.05$).

Table 4. Analysis of the three different batches of wheat middling based low-nutrient diet used in experiments 2, 3, 4, and 5.

Items	Experiment 2[§]	Experiment 3[£]	Experiment 4[¥]	Experiment 5[€]
HT[1] achieved	Yes	No	Yes	Yes
DM, %	89.3	89.6	90.2	90.8
Crude protein, %	14	17.4	19.7	16.4
Gross energy, kcal/kg	3,858	3,934	3,960	3,805
Fiber NDF, %	17.4	12.3	12.3	15.9
Fiber ADF, %	6.36	4.05	3.7	5.83
Al, mg/kg	109	75	49.1	169
Ca, mg/kg	10,743	9,941	9,014	14,133
Cu, mg/kg	123	20.4	31.7	52.4
Fe, mg/kg	247	125	122	517
K, mg/kg	4,666	5,291	5,974	5,568
Mg, mg/kg	1,672	1,760	1,767	1,868
Mn, mg/kg	131	164	149	181
Na, mg/kg	1003	887	1051	1,242
P, mg/kg	7,857	7,994	7,619	9,715
S, mg/kg	1,810	2,237	2,504	2,090
Zn, mg/kg	532	172	136	376

HT: horizontal transmission of histomonosis.

[§]Experiment 2: inoculation of the PHL isolate on day 18 in 10 seeders (34 contacts). Poults fed the TS diet for the first 14 days and, on day 15, the WM diet was introduced until the end of the experiment (day 52).

[£]Experiment 3: inoculation of Buford or PHL on day 10 in 14 seeders (31 contacts) in a floor pen. All groups fed the TS diet the first seven days, then the WM diet from day 7 to 38 (groups 2, 3, 4, 5, 7), or the CS diet, divided into two phases (CS1 and CS2), from d7-21 (CS1) and d21-38 (CS2) (groups 1, 6, and 8).

[¥]Experiment 4: inoculation of PHL isolate on day 9 in 2 seeders/cage (6 contacts/cage, 4 cages). All groups fed the TS diet for the first seven days, then the WM diet was introduced on day 7 until termination (d30) (groups 2 and 5), or the CS diet was divided into two phases (CS1 and CS2), the CS1 from day 7 to day 21 and CS2 from day 21 to termination (d30) (groups 3 and 6), or the TS the whole period (groups 1 and 4).

[€]Experiment 5: inoculation of PHL isolate on day 9 in 2 seeders/cage (6 contacts/cage, 6 cages). All groups fed the TS diet for the first seven days, then the WM diet was introduced on day 7 until termination (d29) (groups 2 and 4), or the TS diet for the whole period (groups 1 and 3).

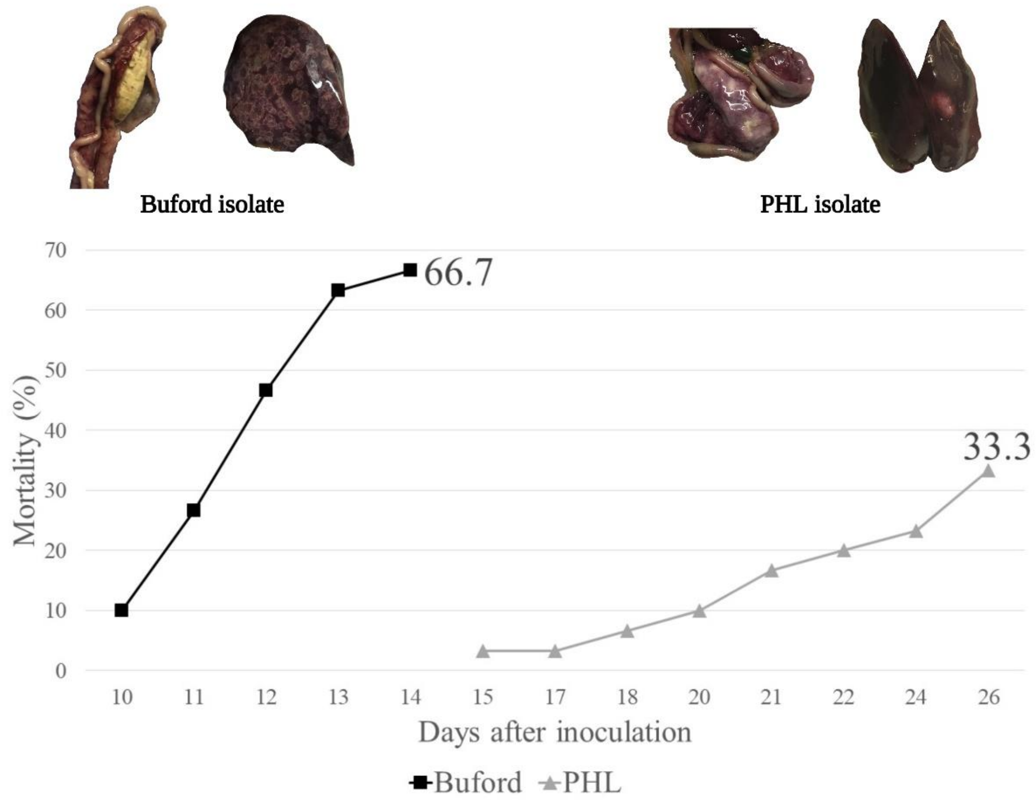

Figure 1. Mortality of turkeys directly inoculated (intra-cloacal) with two different isolates of *Histomonas meleagridis* (Buford or PHL) in experiment 3. Created with BioRender.com.

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APPENDIX

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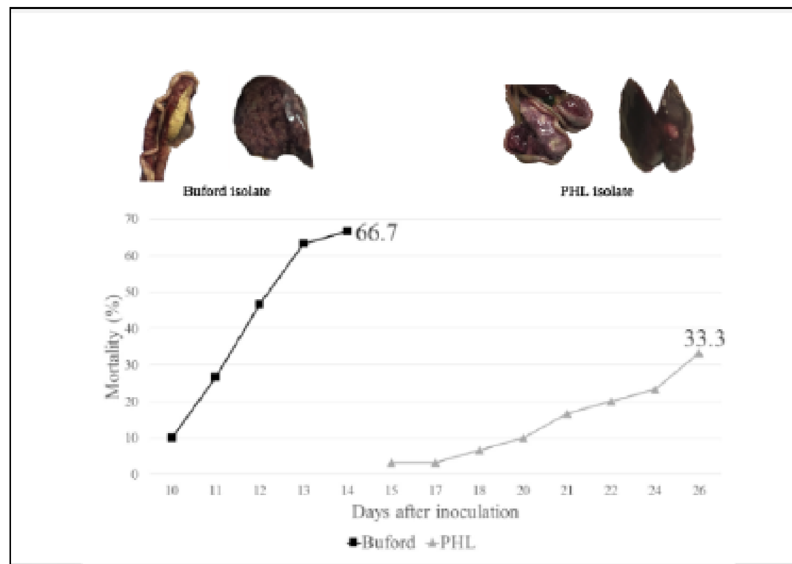

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VI. CONCLUSION

Histomonas meleagridis has characteristics and interactions that need to be understood to offer tools to control the disease. Based on the studies here presented, sodium chlorate and sodium nitrate were able to reduce the growth of histomonads *in vitro* but the same effect could not be observed *in vivo*. Impact of *Eimeria adenoeides* on histomonosis depends on the dose and pathogenicity of the oocysts. Horizontal transmission was affected by certain feed ingredients and nutritional density, and possibly by litter conditions. The variation observed within treatments and between experiments exemplifies the complexity of the disease and agrees with variation reported in outbreaks in turkey flocks and the inconsistency of some published studies. Further studies are needed to investigate the influence of litter conditions, microbiota, genetic variability, and the impact of the different morphological stages on infectivity and transmission of histomonosis.

VII. APPENDICES

UNIVERSITY OF ARKANSAS

Office of Research Compliance

To:	Billy Hargis
Fr:	Craig Coon
Date:	June 10th, 2019
Subject:	IACUC Approval
Expiration Date:	June 6th, 2022

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **19118:** *Evaluation of the effect of Eimeria adenoides or Eimeria tenella in the pathogenesis of Histomoniasis in turkeys and the prophylactic use of alternative compounds or probiotics*.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond June 6th, 2022 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Billy Hargis, Guillermo Tellez, Thaina Barros, Lesleigh Beer, Cheryl Lester, Christine Vuong, and Aaron Ashcraft. Please submit personnel additions to this protocol via the modification form prior to their start of work.

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

CNC/tmp

19118

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May 2nd, 2022

To Whom It Might Concern:

I confirm that Thaina Landim de Barros is the lead author and the main contributor of the literature review entitled "Factors influencing histomonosis in turkeys", available in the section II - Literature Review; the research paper entitled "Research Note: Evaluation of Dietary Administration of Sodium Chlorate and Sodium Nitrate for *Histomonas meleagridis* prophylaxis in turkeys", available in Chapter I (section III); the research paper entitled "Evaluation of *Eimeria adenoeides* pre-infection on the severity of histomoniasis in turkeys", available in Chapter II (section IV); and the research paper entitled "Feed composition and isolate of *Histomonas meleagridis* alter horizontal transmission of histomonosis in turkeys. Proof of concept", available in Chapter III (section V).

B.M. Hargis
Distinguished Professor and Director
JKS Poultry Health Laboratory
Tyson Endowed Chair for Sustainable Poultry Health
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