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Isolation of Drug-Sensitive Eimeria Species from Wild Turkey Feces and Development of a Model Bioshuttle Program for Eimeria meleagritidis for Domestic Turkeys

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Isolation of Drug-Sensitive *Eimeria* Species from Wild Turkey Feces and Development of a Model Bioshuttle Program for *Eimeria meleagridis* for Domestic Turkeys

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Poultry Science

by

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University of Javeriana
Bachelor of Science in Industrial Microbiology, 2019

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Abstract

The objective of this thesis was to determine the anticoccidial profile of *Eimeria* spp. derived from wild turkeys. These drug-sensitive *Eimeria* spp. have potential to be used as vaccine candidates to control coccidiosis in commercial turkeys. Chapter two consists of a brief literature review focused on coccidiosis in turkeys. Chapter three addresses the methodologies utilized to isolate, recover, and speciate *Eimeria* recovered from wild turkey feces in the eastern two-thirds of the United States. From this, we successfully obtained single oocyst-derived stocks for *E. meleagrititis*, *E. dispersa*, *E. meleagridis*, *E. gallopavonis*, and *E. adenoides*. Chapter four describes the experiment conducted to assess the protective efficacy of an *E. meleagrititis* vaccine candidate isolated from wild turkeys with and without intermittent amprolium administration. Additionally, the impact of vaccination and/or challenge and a candidate bioshuttle program on gut permeability and the microbiome was evaluated. The *E. meleagrititis* vaccine candidate induced mild disease without affecting performance. Future research must be conducted to elucidate the impact of amprolium with and without single or multi-species live coccidiosis vaccination on the microbiome and gut barrier function in turkey poults.

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Dedication

This thesis is dedicated to my parents, John Jairo Trujillo and Sandra R. Peralta, and my brother, John Jairo Trujillo Peralta. Your unconditionally support, love, hard work, kindness, patience, and intellect during the path for this achievement.

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Chapter I. Introduction

In commercial poultry operations, intestinal coccidiosis caused by the Apicomplexan protozoa of the genus *Eimeria* results in significant economic losses on a global scale (Chapman, 2008; Chapman & Jeffers, 2014). In-feed anticoccidial drugs (ionophores and chemicals) have been used for decades to prevent coccidiosis outbreaks in commercial poultry flocks (Chapman & Jeffers, 2014). Unfortunately, *Eimeria* spp. exhibit partial or complete resistance to anticoccidial drugs if overused or if the drugs are not rotated properly. Coccidiosis control programs such as rotational, shuttle, and bioshuttle programs have been implemented in commercial settings to prevent the development of anticoccidial resistance and extend the life of the available drugs on the market (Chapman et al., 2005; Price, 2012; Chapman & Jeffers, 2014; Albanese et al., 2018). There is a need for alternatives drug-based anticoccidials to control coccidiosis due to limited number of available anticoccidial drugs on the market and evidence of some level of resistance to all commercially available anticoccidial drugs (Chapman, 2008; Chapman & Jeffers, 2014).

Introduction of drug-sensitive *Eimeria* spp. may exclude the drug-resistant wild-type *Eimeria* spp. in the environment. Live anticoccidial vaccines have been used to control coccidiosis, which prevent the overuse of anticoccidial drugs and promotes the development of immunity to *Eimeria* spp. included in the vaccine (Chapman et al., 2002). Additionally, bioshuttle programs consist of the combination of drugs and live vaccines that contain strains of *Eimeria* spp. obtained before most anticoccidial drugs were introduced or are drug-sensitive by nature (Chapman & Jeffers, 2014; Mathis et al., 2014; Kimminau & Duong, 2019). It has previously been proven that shifting the anticoccidial resistance profile or restoring sensitivity necessitates seeding the barn environment with anticoccidial-sensitive *Eimeria* spp. oocysts

(Chapman & Jeffers, 2014). According to Chapman & Jeffers (2014), this can be achieved by administering a live coccidiosis vaccine at hatch. During cycling, the sensitive isolates compete or interbreed with pre-existing wild-type parasites to restore anticoccidial sensitivity. Two or more subsequent flocks can be vaccinated to fully displace the pre-existing anticoccidial-resistant parasites (Chapman & Jeffers, 2014).

Although drug-sensitive live coccidiosis vaccines have been successfully used to control coccidiosis and renew drug sensitivity in commercial broiler and layer operations, there are limited species coverage in vaccines available for commercial turkey production, and insufficiency of research in turkey coccidiosis has resulted in a lack of knowledge for controlling this disease for commercial turkey production. Unfortunately, an alternative to the previously used drugs with live vaccination in turkey presents a lack of research in *in vitro* and *in vivo* studies. There is a need to evaluate the use of the live anticoccidial vaccine or a bioshuttle program to control coccidiosis in turkeys. Previously, we obtained wild turkey fecal samples and isolated the six major *Eimeria* spp. relevant to commercial turkey operations. Preliminary studies were conducted with *Eimeria* spp., recovered from wild turkeys to confirm monensin, zoalene, and amprolium sensitivity. The overall purpose of the project was to determine if a drug-sensitive *E. meleagritidis* strain recovered from wild turkey feces would induce protective immunity with and without amprolium intervention.

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Chapter II. Literature Review

Coccidiosis Control in Commercial Turkeys and the Search for Alternatives

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This literature review is in preparation to be submitted as a review article.

Abstract

Intestinal coccidiosis caused by Apicomplexan protozoans of the genus *Eimeria* has been historically controlled in commercial turkey operations by the dietary inclusion of anticoccidial drugs in the diet, such as ionophores or chemicals coccidiostats. There is a limited number of anticoccidial drugs available and the extended use in the field has led to the development of drug resistance. Commercial chicken producers have used drug-sensitive live coccidiosis vaccines to induce protective immunity to multiple *Eimeria* spp. and to renew sensitivity to available drugs for coccidiosis control. In contrast, the turkey industry is restricted to a single commercially available vaccine that has limited species coverage. This literature review highlights turkey coccidiosis research, methods to control coccidiosis in the field, and gaps in knowledge.

Introduction

Coccidiosis is caused by Apicomplexan protozoans of the genus *Eimeria* resulting in significant economic losses for the poultry industry on a global scale (Chapman, 2008; Chapman & Jeffers, 2014; Blake et al., 2020). Anticoccidial drugs have been used to control coccidiosis in commercial poultry flocks for decades (Chapman, 2009). Shuttle and rotational programs have been implemented to cycle different ionophores and chemicals to slow the development of drug resistance. Although the mode of action of ionophores and chemicals differs, multi-class drug resistance can occur. The inclusion of anticoccidial drugs in the diet is costly for producers, especially if the *Eimeria* spp. population has developed resistance. Furthermore, alternative strategies to control coccidiosis are necessary due to consumer pressure for antibiotic-free (ABF) or no antibiotics ever (NAE) poultry production. In the United States (US), ionophores have been categorized as antibiotics and cannot be used in ABF or NAE production systems. Although conventional production (ability to utilize antibiotics) is still widely implemented for commercial turkey rearing in the US, there is increased pressure to restrict the use of antibiotics. Moreover, there is a need for research focused on turkey coccidiosis due to the development of anticoccidial resistance, the limited availability of efficacious and approved drugs on the market, and the overall lack of research pertaining to turkey-specific *Eimeria* species. This literature review highlights turkey coccidiosis research, methods to control coccidiosis in the field, and gaps in knowledge associated with the impact of coccidiosis on intestinal permeability and the microbiome of turkeys.

***Eimeria* life cycle**

Eimeria are monoxenous parasites (one-host) that infect the gastrointestinal tract of

vertebrate animals and are primarily transmitted via the fecal-oral route (Barta, 2001; López-Osorio et al., 2020). Thus, *Eimeria* spp. that infect chickens will not cause overt disease in turkeys since these protozoa have evolved to inhabit the gastrointestinal tract of a specific host species (Vrba & Pakandl, 2015). The life cycle of *Eimeria* includes asexual and sexual replication stages with both exogenous and endogenous developmental phases (Barta, 2001; Chapman, 2008). Exogenous development, or sporogony, is an oxygen-demanding process that occurs outside the host. Unsporulated oocysts are non-infective and are excreted in the feces into the environment (Barta, 2001). Sporulation efficiency is affected by temperature, humidity, and oxygen availability. Oocysts tend to sporulate at ~25-30°C with ~40-70% humidity or greater, but optimal conditions vary by species (Reyna et al., 1983). A sporulated oocyst has four sporocysts, each containing two infectious sporozoites (Fayer, 1980; Barta, 2001). These infective oocysts can persist in the litter or feed for months to years due to the resilient oocyst wall (Barta, 2001). Coprophagy (i.e., consuming feces) is a natural behavior for chickens and turkeys which promotes ingestion of sporulated oocysts in the densely populated barn environment. Once ingested, the mechanical grinding action of the gizzard cracks the oocyst wall releasing the sporocyst. Excystation, or the release of the sporozoites into the intestinal lumen, occurs when sporocysts are exposed to proteolytic enzymes and bile salts (Chapman, 1978). The sporozoites penetrate and invade the host intestinal epithelial cells and develop in a parasitophorous vacuole where the trophozoites form into a schizont/meront that contains many merozoites. This process is referred to as merogony and is the asexual reproductive stage of the parasite that occurs within intestinal epithelial cells (Fayer, 1980; Barta, 2001). The number of merogonic cycles appears to be genetically fixed (Ahmad et al., 2016). Merozoite release lyses intestinal cells and the process continues up to 4x depending on the species and strain of

Eimeria. After several cycles of asexual replication, sexual replication or gametogony begins which is the development of two gametes female gamete (macrogametes) and then male gamete (microgametes) and subsequent fertilization (Barta, 2001). From this, a zygote will develop and an unsporulated oocyst will be expelled in the feces (Fayer, 1980; Barta, 2001). The durability of the oocyst wall protects against mechanical or chemical degradation in the environment (Quiroz-Castañeda & Dantán-González, 2015). The pre-patent period is the time between infection with the protozoa and excretion of unsporulated oocysts in the feces which varies by several factors including *Eimeria* spp. and strain (Vrba & Pakandl, 2014). When an immunologically naïve turkey consumes sporulated *Eimeria* oocysts, it becomes infected. Disease severity will depend on the *Eimeria* spp., the total number of oocysts ingested, and age and immune status of the host (Barta, 2001; Albanese et al., 2018). The pathological changes in the intestine are associated with the destruction of enterocytes and the host's immunological response to destroy infected cells.

Coccidiosis in turkeys

Although there has been a considerable amount of research focused on identifying the distribution and diversity of chicken *Eimeria* spp. in various geographic regions (Schwarz et al., 2009; Clark et al., 2016), there have been limited studies investigating the prevalence and diversity of *Eimeria* spp. in commercial and wild turkey populations in North America. Recent publications include the prevalence, distribution, and diversity of *Eimeria* in commercial turkey flocks in Canada (Imai and Barta, 2019), wild turkey populations in Ontario (MacDonald et al., 2019), and in commercial turkey flocks in the Midwestern United States (Duff et al., 2022). At present, there are seven documented *Eimeria* spp. (*E. meleagritidis*, *E. meleagridis*, *E. gallopavonis*, *E. dispersa*, *E. adenoeides*, *E. innocua*, and *E. subrotunda*) that infect wild and

domestic turkeys (*Meleagris gallopavo* [var. *domesticus*]) (Chapman, 2008). Another species, *E. edgari* (formerly *E. meleagrimitis* 2), has been recovered from commercial turkey flocks in the US and from wild turkeys in the Delmarva area (see Fitz-Coy et al., 2008). However, *E. meleagrimitis*, *E. adenoides*, *E. gallopavonis*, and *E. dispersa* are more notably pathogenic to commercial turkeys (Lund & Farr, 1965). However, a significant reduction in body weight gain has been reported after subclinical challenge with *E. dispersa* or *E. innocua* with minimal lesion development (Imai, 2018). This suggests that although certain species may not cause severe lesions in the gastrointestinal tract of turkeys, the subclinical infection can negatively impact performance which can be highly costly for producers.

E. meleagrimitis oocysts are small with dimensions ranging from 18-20 μ m by 15-17 μ m (Tyzzer, 1929; Clarkson, 1959; El-Sherry et al., 2015). Asexual development of *E. meleagrimitis* occurs in the small intestine, specifically in the duodenum and jejunum, and sexual development extends to the rectum in severe cases (El-Sherry et al., 2014b). As one of the more pathogenic species that infect turkeys, *E. meleagrimitis* hinders performance, causes occasional mortality, and necrosis of the gastrointestinal tract (El-Sherry et al., 2014b; Vrba & Pakandl, 2014). Macroscopic lesions occur predominantly in the duodenum and may extend into the jejunum (Gadde et al., 2020).

E. dispersa oocysts are ~26 μ m by 21 μ m in size and infect the upper small intestine, but lesions may be observed throughout the entire intestine (El-Sherry et al., 2014b; Vrba & Pakandl, 2014; El-Sherry et al., 2017). *E. innocua* is phylogenetically similar to *E. dispersa* (Vrba & Pakandl, 2014). Both species infect quail and turkeys, develop in the upper intestine, and have similar oocyst dimensions (Moore & Brown, 1952; Moore et al., 1954; El-Sherry et al., 2014a; El-Sherry et al., 2014b; Vrba & Pakandl, 2014). However, *E. innocua* is less frequently

detected in turkeys compared to the other species of *Eimeria*. *E. subrotunda* was originally described by Moore, Brown, and Carter (1954) and development primarily occurred in the duodenum (Moore et al., 1954). However, other investigators have not been able to detect *E. subrotunda* in turkey fecal samples suggesting that early speciation based on morphometrics was complicated by mixed species samples (Chapman, 2008; Ogedengbe et al., 2014).

E. adenoides, *E. meleagridis*, and *E. gallopavonis* are three species that infect the lower gastrointestinal tract of turkeys (Mastler & Chapman, 2006; El-Sherry et al., 2014a; Vrba & Pakandl, 2014; El-Sheryl et al., 2019). *E. gallopavonis* oocysts are one of the largest (~24-31µm by 16-21µm) of the *Eimeria* spp. that infect turkeys (El-Sherry et al., 2019) and have the longest pre-patent period at 144h (Hawkins, 1952). Development occurs primarily in the lower ileum, cecum, and rectum causing caseous plugs to form in the cecal neck in severe cases (Vrba & Pakandl, 2014; Gadde et al., 2020). *E. gallopavonis* can be confused with *E. meleagridis*, a less pathogenic *Eimeria* spp. with large oocysts (~18µm by 24 µm) and its pathogenicity is caseous material and plugs in feces and within the cecal pouch (Mastler & Chapman, 2006; El-Sherry et al., 2014a; Vrba & Pakandl, 2014; El-Sheryl et al., 2019). *E. adenoides* is characterized as the most pathogenic *Eimeria* spp. that infects the lower gastrointestinal tract of turkeys due to hemorrhages in the ceca and the formation of cecal cores (El-Sherry et al., 2014a).

Detection and speciation of turkey *Eimeria* spp.

Microscopic identification of oocysts from fecal or litter samples and gross pathological changes are common metrics that have been used to diagnose coccidiosis in commercial poultry flocks. A simple fecal flotation technique using a saturated salt solution can be used to determine the presence or absence of *Eimeria* oocysts (Barta, 2001). Microscopic examination of mucosal

scrapings from affected tissues can be used to detect oocysts and histological preparation of tissues can be used to assess specific stages of endogenous development (Barta, 2001). However, the most common method of detection and for diagnosing coccidiosis in a flock is by gross evaluation of macroscopic lesions post-mortem (Conway & McKenzie, 2007; Chapman, 2008). A scoring system that ranges from 0-4 has been used to assess the severity of damage caused by species of *Eimeria* that infect chickens (Johnson and Reid, 1970). More recently, lesion scoring systems specific to turkey *Eimeria* spp. have been described (El-Sherry et al., 2014a; El-Sherry et al., 2014b; El-Sherry et al., 2019; Gadde et al., 2020). Mixed infections complicate species identification when using lesion scores as the sole metric. Supplementing with microscopic evaluation can prove to be useful, but further characterization using molecular methods is recommended when differentiating between species of *Eimeria* that infect turkeys. Compared to chicken *Eimeria* spp., there is more considerable overlap in oocyst morphology and size and location of endogenous development for *Eimeria* spp. that infect turkeys (Long et al. 1977; Chapman 2008; El-Sherry et al. 2015). Chapman (2014) emphasized the importance of combining molecular biology with phenotypic characteristics for precise identification of *Eimeria* spp. that infect turkeys since speciation based on strictly oocyst morphology and size has proved to be an inaccurate method for speciation. For turkey *Eimeria* spp., the partial sequences of the mitochondrial cytochrome c oxidase subunit I (mt COI) proved to be a more acceptable target for genotyping compared to 18S rDNA for PCR detection (Ogedengbe et al., 2014; Hafeez et al., 2015; El-Sherryl et al., 2013; Imai & Barta, 2019).

Coccidiosis control

Anticoccidial drugs

Anticoccidial drugs, either ionophores or chemicals, have been used to control coccidiosis in commercial poultry operations (Chapman, 1997; Allen & Fetterer, 2002). The mode of action is different for ionophores and synthetic anticoccidial drugs. Ionophores target sporozoites in the gut lumen before they invade a host cell, whereas chemical coccidiostats eliminate intracellular stages in the intestine (Chapman, 2007). Ionophores have been used to control coccidiosis for decades since resistance develops slowly and they do not fully inhibit parasite development, which promotes the development of immunity after subsequent exposures to *Eimeria* spp. in the field (Chapman 1999; Chapman, 2008; Chapman et al. 2010; Noack et al., 2019). Ionophores are compounds produced by the fermentation of *Streptomyces* spp. or *Actinomadura* spp. that target the membrane function of *Eimeria* spp. by altering ion transport and disrupting osmotic balance (Peek and Landman, 2011; Noack et al., 2019). Three classes of ionophores that disrupt the cell membrane of the parasite: 1) monovalent ionophores (ex: monensin (Augustine et al., 1992; Chapman & Rathinam, 2007; Rathinam & Chapman, 2009), narasin and salinomycin (Augustine et al., 1987) that are toxic for turkeys (Potter et al., 1986; Markiewicz et al., 2014)), 2) monovalent glycosidic ionophores (ex: maduramicin (McDougald et al., 1990; Milbradt et al., 2014) and semduramicin (Smith et al., 1998)), and 3) divalent ionophores (ex: lasalocid; Augustine et al., 1987; Chapman et al., 2010).

Chemicals, or synthetically derived anticoccidial drugs, may affect the protozoa's metabolism inhibiting any further development (Peek & Landman, 2011; Noack et al., 2019). The mode of action of synthetic anticoccidials may include: 1) inhibition of parasite mitochondrial respiration (ex: decoquinate (Joyner & Norton, 1973; Mathis et al., 2021), clopidol

(Joyner & Norton, 1973; Rathinam & Chapman, 2009; Mathis et al., 2021), toltrazuril (Greuel et al., 1991)), 2) inhibition of the folic acid pathway (ex: sulfonamides (Marsden & Martin, 1955; Greuel et al., 1991), sulfaquinoxaline (Reid, 1972; Joyner, 1973)), and 3) competitive inhibition of thiamine uptake (amprolium (Joyner, 1973; Cabel et al., 1991; Rathinam & Chapman, 2009)). Alternatively, the exact mechanism of action may be unknown for synthetic anticoccidials, such as zoalene (Hymas & Stevenson, 1962; Cabel et al., 1991; Mathis et al., 2021), diclazuril (Vanparijs et al., 1989; Rathinam & Chapman, 2009), halofuginone (Edgar & Flanagan, 1979; McDougald et al., 1986), nicarbazin (Ott et al., 1956; Cuckler et al., 1956; Mathis et al., 2021), and robenidine (Joyner & Norton, 1972). Coccidiosis control programs that have been adopted by the commercial turkey industry will be discussed below.

Vaccination

Live coccidiosis vaccination in commercial turkeys began in the 1950s, but the commercial vaccine was not introduced until 1984 (Williams, 2002; Chapman, 2008). Live coccidiosis vaccines may be attenuated or non-attenuated. Attenuated strains are generated from wild-type stocks by selecting for precociousness, or loss of one merogonic cycle, by passaging the very first oocysts shed in the feces (Shirley et al., 2005). The fecundity of non-attenuated and attenuated vaccine strains is critically important because there must be sufficient replication in the host to induce mild disease that promotes immune development with little to no impact on performance (Chapman et al., 2005; Chapman, 2008; Price, 2012). Using a live attenuated coccidiosis vaccine may be advantageous in the field since the replicative potential is decreased while remaining immunogenic. However, this can substantially increase vaccine production costs. Currently, only one live coccidiosis vaccine (Immunocox-T) for turkeys is available for

commercial producers that contain only two species, *E. meleagridis* and *E. adenoides* (Williams, 2002; Chapman et al., 2005; Chapman, 2008). Effective vaccination using live coccidiosis vaccines requires the administration of viable oocysts to initiate infection that results in oocysts being excreted in the feces and shed into the environment. There must be enough oocysts shed in the feces that efficiently sporulate to initiate a uniform secondary round of infection (Chapman, 2008; Chapman & Jeffers, 2014). Thus, protective immunity against *Eimeria* spp. included in live coccidiosis vaccines is stimulated by subsequent infection following vaccination. Since both live attenuated and live non-attenuated vaccines may lack relevant species, the generation of protective immunity is based on re-infection from the cycling of oocysts caused by the inoculation dose (Chapman, 2008; Price, 2012). Another important consideration regarding live coccidiosis vaccination is the method of vaccine application.

Concerted efforts to optimize live coccidiosis vaccination at the hatchery to promote improved uptake and success of the vaccine are essential. Since commercial turkey producers are limited to a single commercially available vaccine, research evaluating the optimal method to vaccinate turkey poults at hatch is lacking. However, for day-of-hatch chicks, several investigators have evaluated different administration methods for live coccidiosis vaccine application including drinking water (Jenkins et al., 2022), liquid or gel spray (Jenkins et al., 2013; Albanese et al., 2018), oral gavage (Imai & Barta, 2019; Price, 2012), eye-spray (Chapman, 1996; Chapman & Cherry, 1997b), and edible gel (Chapman & Cherry, 1997a; Danforth et al., 1997). Accurate vaccination application and proper uptake are critical to inducing a robust and protective immune response. Compared to the other application methods, there is less variability with oral gavage, but this is presently not feasible for use in commercial hatcheries.

Susceptibility to *Eimeria* spp. infection increases as the bird ages further highlighting the importance of proper coccidiosis control in commercial poultry operations. Vaccination administration is critical because turkeys who do not consume oocysts on the day-of-hatch may later be exposed to large numbers of oocysts in the litter (Chapman, 2008). Naïve poult exposed to massive quantities of virulent wild-type or vaccinal oocysts before developing immunity can have significant effects on performance or lead to clinical coccidiosis (ex. Clarkson, 1958; El-Sherry et al., 2014b). Compared to drug-based coccidiosis control programs, protective immunity develops earlier since poults are exposed to a controlled number of oocysts at hatch versus being exposed to the wild-type *Eimeria* spp. present on the farm. However, this absolutely requires uniform vaccine application and uptake at hatch and vaccination with all relevant species to a complex.

Coccidiosis control programs and restoration of drug sensitivity

The commercial poultry industry has adopted strategies to prevent the development of resistance and extend the life of the available anticoccidial drugs, such as rotational, shuttle, and bioshuttle programs (Chapman et al., 2005; Peek & Landman, 2011; Price, 2012). In a rotation program, various anticoccidial drugs are rotated between flocks, such as a synthetic anticoccidial and a divalent ionophore (Chapman et al., 1998; Clark, 2019; Agunos et al., 2019). A shuttle program uses different drugs with different modes of action in each diet phase to limit the development of anticoccidial resistance (Peek & Landman, 2011; Price, 2012; Clark, 2019; Agunos et al., 2019). Although rotation and shuttle programs have been used, alternative methods to extend the life of the currently available anticoccidial drugs are needed especially since ionophores cannot be used in ABF or NAE programs in the US. A bioshuttle program is a

strategy for preventing drug resistance that combines the administration of a live coccidiosis vaccine with intermittent drug prophylaxis in the feed or drinking water to control oocyst shedding during the second and third cycle in a flock (Reid et al., 1978; Chapman & Jeffers, 2014; Agunos et al., 2019).

Anticoccidial sensitivity testing is a method used to evaluate fecal samples from various farms within a production complex for sensitivity to various anticoccidial drugs under controlled conditions with the goal of predicting their efficacy in the field (Peek & Landman, 2011). *In vivo* challenge models have been used to determine if anticoccidial sensitivity has shifted or restored post-vaccination with drug-sensitive *Eimeria* spp. strains in the field (Joyner & Norton, 1972; Joyner & Norton, 1973; Peek & Landman, 2011). Drug-resistant wild-type *Eimeria* spp. may be displaced by drug-sensitive vaccinal strains by seeding barns with a significant number of anticoccidial sensitive *Eimeria* spp. (Jeffers, 1976; Peek & Landman, 2011; Chapman & Jeffers, 2014). Sensitive isolates compete or interbreed with pre-existing wild-type parasites and displacement of anticoccidial-resistant parasites has been observed after two or more subsequent flocks (Chapman & Jeffers, 2014). Anticoccidial sensitivity testing has been recognized as a useful tool for monitoring rotation and shuttle programs to ensure that coccidiosis control programs implemented in a commercial setting remain effective (Peek & Landman, 2011). Although drug-sensitive live coccidiosis vaccines have been successfully used in commercial broiler and layer production systems to control disease and renew sensitivity to available drugs (Chapman, 1994; Peek & Landman, 2006; Mathis & Broussard, 2006), no such vaccines are available for commercial turkey production. Understanding the sensitivity of field isolates to routinely used anticoccidials is essential for designing coccidiosis control programs for commercial turkey producers.

Since *Eimeria* spp. relevant to commercial turkeys also circulate within wild turkey populations (Ruff et al., 1988; MacDonald et al., 2019), these wild turkey-derived *Eimeria* spp. could be utilized to enhance coccidiosis control in commercial turkey operations and displace drug-resistant phenotypes in barns. To our knowledge, there have been no published studies on the restoration of anticoccidial sensitivity using drug-sensitive *Eimeria* spp. in commercial turkeys reared in the United States. Evidence suggests that wild-type, drug-resistant *Eimeria* spp. can be displaced by drug-sensitive vaccinal strains in commercial broiler flocks (Chapman & Jeffers, 2014; Snyder et al., 2021). Thus, similar results would be expected for commercial turkey flocks.

Impact of coccidiosis on gut health in turkeys

Although live coccidiosis vaccines have been shown to prevent coccidiosis in commercial turkeys, the effect of vaccination, with and without a bioshuttle program, on intestinal permeability and the gut microbiome has been largely understudied. Intestinal permeability in poultry indicates that there has been damage to the gut barrier (Bortoluzzi et al., 2019; Latorre et al., 2018). Fluorescein isothiocyanate dextran (FITC-d) has been used as a serum biomarker to evaluate gastrointestinal permeability associated with coccidiosis and necrotic enteritis challenge in chickens (Bortoluzzi et al., 2019; Latorre et al., 2018; Coles et al., 2021). There are many studies on the effects of coccidiosis on intestinal barrier function in broiler chickens (Pham et al., 2021; Teng et al., 2020). However, there is a lack of research on the effect of *Eimeria* spp. (vaccination and/or challenge) on intestinal barrier function and integrity in commercial turkeys. To our knowledge, the research is limited to one study focused on the effect of live vaccination with *E. meleagrimitis*, *E. gallopavonis*, *E. meleagridis*, and *E.*

adenoeides on performance and intestinal morphometrics compared to an ionophore (Milbradt et al., 2014) and another study regarding *Eimeria meleagridis* and *Clostridium perfringens* challenge to induce necrotic enteritis in commercial turkeys (Hardy et al., 2020). Dysbiosis caused by *Eimeria* spp. alters the intestinal mucosal and luminal environments which promotes proliferation of opportunistic pathogens such as *Clostridium perfringens* (Madlada et al., 2021). There are no reports related to the effects of *Eimeria* spp. on the intestinal microbiome composition of commercial turkeys. The relationship between *Eimeria* spp. and coccidiosis control programs on the intestinal microbiome in commercial turkeys are unknown.

Conclusion

Currently, only one commercial vaccine is available, and the number of licensed and available chemical anticoccidials for turkeys in the market is restricted. The absence of effective alternatives makes coccidiosis control difficult, especially when anticoccidial resistance develops. A recent review of the literature indicates that little research has been conducted on the relationship between coccidiosis vaccination, anticoccidial medication, and the diversity of gastrointestinal microorganisms in turkey poults.

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Chapter III. Isolation, speciation, and anticoccidial sensitivity of *Eimeria* spp. recovered from wild turkey feces in the United States

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Abstract

From 2018-2020, more than 100 wild turkey fecal samples from across the eastern two thirds of the United States were collected from areas where commercial turkey production is not common. We hypothesized that wild turkey fecal samples would contain *Eimeria* spp. possessing sensitivity to anticoccidial drugs. Samples containing *Eimeria* spp. oocysts were amplified *in vivo*. If propagation was successful, the samples were PCR-specified and subjected to turkey anticoccidial sensitivity (TACS) testing for major representatives of both the ionophore and chemical categories of anticoccidial drugs. The purpose of this study was to obtain the major *Eimeria* spp. important for commercial turkey production that had sensitivity to monensin, zoalene, and amprolium. Single oocyst-derived stocks would be used in future studies to evaluate the efficacy of wild turkey *Eimeria* spp. as vaccine candidates to control coccidiosis in commercial turkey flocks.

Key words: Coccidiosis, turkey, *Eimeria*, anticoccidial sensitivity testing, speciation

Introduction

Coccidiosis is caused by an Apicomplexan protozoan of the genus *Eimeria*, which causes significant economic losses for the commercial poultry industry (Chapman, 2008; Chapman & Jeffers, 2014). Historically, anticoccidial drugs, either ionophores or chemicals, have been used to control coccidiosis in commercial poultry operations. Coccidial resistance to ionophores develops slowly, and rotating programs with different ionophores, as well as a restricted number of effective chemical coccidiostats, has slowed the formation of multi-drug resistant coccidian species. However, alternative strategies to control coccidiosis are needed due to consumer-driven markets, increased regulatory environments, and evidence of multi-drug resistance (Chapman, 2008; Chapman & Jeffers, 2014). Drug-sensitive live coccidiosis vaccines have been used for commercial broiler and layer production, both to control disease and to renew sensitivity to available drugs for control, but the turkey industry is limited to commercially available vaccines with limited species coverage (Chapman et al., 2005; Chapman, 2008; Chapman & Jeffers, 2014).

Anticoccidial sensitivity testing is a method to evaluate field samples (usually litter or droppings) from various farms within a production complex for sensitivity to various anticoccidial drugs under controlled conditions with the goal of predicting their efficacy in the field (Peek & Landman, 2011). *In vivo* challenge models have been used to determine if anticoccidial sensitivity has been shifted or restored post-vaccination with drug-sensitive *Eimeria* spp. strains in the field (Peek & Landman, 2011). By seeding the barn with a significant number of anticoccidial-sensitive *Eimeria* species, the drug-resistant wild-type *Eimeria* spp. may be displaced by the drug-sensitive vaccinal strains (Chapman & Jeffers, 2014). The sensitive isolates compete or interbreed with pre-existing wild-type parasites to restore anticoccidial

sensitivity to the parasite population at a facility. Two or more subsequent flocks must be vaccinated to significantly displace the pre-existing anticoccidial-resistant parasites (Chapman & Jeffers, 2014). The scientific community recognizes anticoccidial sensitivity testing as a helpful technique for monitoring rotation and shuttle programs to ensure the proper use of anticoccidial drugs and vaccination strategies to control coccidiosis in a commercial setting (Peek & Landman, 2011). Although drug-sensitive live coccidiosis vaccines have been successfully used in commercial broiler and layer production systems to control disease and renew sensitivity to available drugs, there are no such broad vaccines available for commercial turkey production. Understanding how sensitive field isolates are to routinely used anticoccidials is essential for designing coccidiosis control programs.

Eimeria spp. oocysts have different morphologies that can be observed microscopically and have evolved to infect and cause macroscopic lesions in different locations of the gastrointestinal tract (El-Sherry et al., 2013; Chapman, 2014; El-Sherry et al., 2015; Imai & Barta, 2019). Although the species identification based on strictly oocyst morphology and size has been demonstrated to be an inaccurate method for turkey *Eimeria* species, Chapman (2014) emphasized the importance of combining molecular biology with phenotypic characteristics for precise identification of *Eimeria* spp. that infect turkeys. Previously, species confirmation of turkey *Eimeria* spp. using molecular techniques, such as genotyping the mitochondrial oxidase 1 (mt COI) gene, has proven effective for species-specific identification of turkey *Eimeria* turkey by PCR (El-Sherry et al., 2015; Imai & Barta, 2019; Duff et al., 2022).

Since *Eimeria* spp. relevant to commercial turkeys also circulate within wild turkey populations (Ruff et al., 1988; MacDonald et al., 2019), we hypothesized that wild turkey fecal samples collected from geographical regions absent of commercial turkey operations would

contain drug-sensitive *Eimeria* species. Data obtained in the current study provides a better understanding of the prevalence and distribution of drug-sensitive *Eimeria* spp. who are circulating in wild turkey populations in the United States. Perhaps these wild turkey-derived *Eimeria* spp. could be utilized to enhance coccidiosis control in commercial turkey operations and displace drug-resistant phenotypes in the barn. To our knowledge, there are no published studies on the restoration of anticoccidial sensitivity using drug-sensitive *Eimeria* spp. in commercial turkeys that are reared in the United States. However, evidence suggests that wild-type, drug-resistant *Eimeria* spp. can be displaced by drug-sensitive vaccinal strains in commercial broiler flocks (Chapman & Jeffers, 2014; Snyder et al., 2021). Live vaccination with drug-sensitive *Eimeria* spp. and rotating available anticoccidial drugs appropriately limit the risk of selecting drug-resistant phenotypes in the field. It is essential for antibiotic-free production systems in the United States that cannot utilize ionophores. The present study aimed to isolate drug-sensitive strains of *E. adenoides*, *E. gallopavonis*, *E. meleagritidis*, *E. meleagridis*, and *E. dispersa* from wild turkey feces. The drug-sensitive *Eimeria* spp. could be used to generate a multi-species live vaccine as an alternative vaccination program for the commercial turkey industry.

Materials and Methods

A schematic representation of isolation, speciation, and anticoccidial sensitivity test of *Eimeria* spp. recovered from wild turkey feces is presented in Figure 1.

Recovery of wild turkey Eimeria spp.

Fecal samples were collected from wild turkeys by individuals and state agencies across the eastern two-thirds of the United States. Samples were placed on wet ice packs and shipped to

the UADA Poultry Health Laboratory for further processing. Light microscopy was used for the initial detection of *Eimeria* spp. oocysts. A subset of the fecal sample was diluted with saturated salt solution and evaluated using a McMaster counting chamber. The chamber was examined for the presence/absence of oocysts and recorded as oocyst-positive or oocyst-negative. Oocyst-positive samples were partially processed and oocysts were sporulated as previously described by Reid & Long (1979) and El-Sherry et al. (2013). No further processing was done for oocyst-negative samples. Due to the low number of oocysts present in the majority of fecal samples obtained, *in vivo* amplification of oocysts and *in vitro* sporulation was conducted to obtain a fresh oocyst stock to use for TACS testing.

Post-amplification, oocyst-positive fecal samples were partially purified following previously described methods (Imai & Barta, 2019). Fresh fecal samples were suspended in a sterile saturated salt solution, blended on high speed for 30-60 seconds, and the homogenate was sieved to remove coarse debris. Then the filtrate was centrifuged at 1,250xg for 10 minutes to float oocysts away from fecal debris. Post-centrifugation, the supernatant containing the oocysts was diluted in sterile distilled water (10x) and centrifuged at 1,250xg for 10 minutes. The supernatant was decanted, then the pellet of partially purified oocysts was resuspended in 2.0 % or 2.5% potassium dichromate (w/v, aqueous) and transferred to an Erlenmeyer flask capped with a sterile gauze plug. The volume was mixed and adjusted to approximately ¼ of its total volume to permit adequate aeration and then placed on a rotary platform shaker operating at ~100 rpm at 26°C for 3-4 days or until sporulation was confirmed by microscopy. Following sporulation, the oocysts were resuspended in fresh 2.5% potassium dichromate and held at 4°C.

Molecular Characterization and Speciation

DNA Extraction

DNA was isolated from sporulated *Eimeria* spp. samples using methods previously described by El-Sherry et al. (2013) with some slight modifications. Sporulated oocysts were transferred to a 1.5 mL microcentrifuge tube and pelleted by centrifugation $1,200 \times g$ for 2 minutes. The pelleted oocysts were resuspended in 100 μL DNAzol reagent (Invitrogen® Life Technologies Inc.), and sterile 0.5 mm glass beads were added until there was a layer of dry beads observed above the liquid surface of the sample. The contents were then vortexed for approximately 60 seconds. Oocyst breakage was confirmed microscopically and additional rounds of disruption were used until most of the oocysts had been lysed. Once sufficient oocyst breakage was confirmed microscopically, an additional 900 μL of DNAzol was added to the sample. The tubes were placed on a rocker at room temperature for a minimum of 18 hours. Post-incubation, the sample was centrifuged at $13,000 \times g$ for 15 minutes at 4°C . The supernatant was then transferred to a new 1.5 mL microcentrifuge tube to remove insoluble debris and glass beads. The pelleted DNA was added to 500 μL of 100% ethanol (Life Technologies) and mixed by inversion, then sat at room temperature for 5 minutes, followed by centrifugation at $14,000 \times g$ for 8 minutes at 4°C . The pelleted DNA was washed twice with 500 μL of 70% cold ethanol at 4°C , then mixed by centrifugation at $10,000 \times g$ for 5 minutes. The DNA pellet was air-dried by inversion and was then resuspended in 40 μL of EB Buffer (Qiagen / 10mM Tris-Cl). A BioTek spectrophotometer (BioTek, Winooski, VT) was used to determine the purity and quantity of DNA. After the spectrophotometric-generated concentration (A260/A280 ratio should be 1.7-1.9) and molecular weight from 20-100Kb, an aliquot of DNA was analyzed by agarose gel electrophoresis to confirm. DNA samples were stored at -20°C . If

the DNA concentration was estimated to be between 2 - 999ng/μL, a 1:10 dilution of the DNA was made using nuclease-free water. If the DNA concentration was estimated to be >999ng/μL, a 1:100 dilution was made using nuclease-free water.

Polymerase Chain Reaction (PCR) Identification

Primary PCR

Purified gDNA extracted from sporulated *Eimeria* recovered from wild turkey feces was used for primary PCR to amplify *Eimeria* species-specific mitochondrial oxidase 1 (mt COI) gene, a 1,272-base pair (bp) fragment, as described by El-Sherry et al. (2013), Hafeez et al. (2015), and Imai & Barta (2019). The primer sequences and PCR parameters are listed in Table 1. Applied Biosystems SimpliAmp Thermal Cycler (ThermoFisher Cat. No. A24811) was used for PCR. LongAmp Taq PCR kit (#E5200S New England Biolabs, Ipswich, MA) was used for PCR per the manufacturer's instructions. Magnesium sulfate was not included in the reaction. Primers and nuclease-free water was obtained from Integrated DNA Technologies, Coralville, Iowa. In the present study, each primary PCR tube consisted of 2 μL purified genomic DNA (50ng), 5X PCR Buffer, 10mM dNTPs, 2,500units/mL LongAmp® Taq DNA Polymerase, 0.4 μM forward primer, 0.4 μM reverse primer, and nuclease-free water to make up a final volume of 50 μL. The primary PCR samples were run PCR reaction at 94°C for 30 seconds, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds and extension length at 65°C for 60 seconds, followed by a final extension at 65°C for 5 minutes to complete the PCR process. The product size was estimated by electrophoresis using 1% agarose gel containing 5μL/100mL agarose of ethidium bromide in 1X Tris-Acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) at 80V and 150mA for ~60 minutes. Primary PCR products estimated to be ~1,272kb in size were purified using Pure Link PCR purification Kit

(Life Technologies Inc.), and electrophoresis was repeated to confirm the size of the purified primary PCR product as described above. Then, purified primary PCR products were diluted at 1:10 in nuclease-free water with a final volume of 20 μ L. Consequently, a 2.0 μ L aliquot of the appropriately diluted primary reaction solution was used as the template for each subsequent species-specific PCR reaction.

Nested PCR

A species-specific PCR-based assay using diluted primary PCR products as templates was conducted. Briefly, 2.0 μ L aliquots of each diluted (1:10) primary PCR product were used as templates for secondary PCR amplification using species-specific primers for *E. adenoides*, *E. gallopavonis*, *E. dispersa*, *E. innocua*, *E. meleagridis*, or *E. meleagrimitis* based on Hafeez et al. (2015) and Imai & Barta (2019) with minor modifications in annealing temperatures (Table 1). The resulting PCR products were separated using a 1% or 2% agarose gel as described above based on the expected size of the product. All positive-nested PCR products were sent to Eton Bioscience (Eton Bioscience, Inc., Raleigh, NC, USA) for Sanger sequencing as secondary confirmation. Sequences were evaluated by mapping sample sequences to reference sequences obtained from the NCBI database using Geneious bioinformatics software version 2021.2.

Turkey Anticoccidial Sensitivity Test (TACS)

Anticoccidial sensitivity was calculated based on the reduction of oocyst shedding in the medicated, challenged group compared to the non-medicated, challenged control group (CC). For TACS, there were 6 turkeys utilized per sample/evaluation (n=3 for CC, n=3 for medicated). Anticoccidial medication, either monensin (75g/ton), zoalene (454g/ton), or amprolium (0.024%/drinking water), began 2 days prior to the challenge. At 9 or 10 days of age, ~100 sporulated oocysts (aged <6 months) per mL were orally administered to respective CC and

medicated group. Feces were collected from day 5-9 days post-challenge. Based on total oocyst output between CC and medicated groups, samples were deemed resistant (<30% reduction), partially sensitive (31-79% reduction), or sensitive (>79% reduction) to monensin, zoalene, or amprolium.

Single oocyst-derived stocks

To obtain pure stocks of individual drug-sensitive *Eimeria* spp., one sporulated oocyst was isolated from a mixed species sample and was administered orally to one turkey between 12-14 days-of-age. The process was conducted as described by El-Sherry et al. (2015). To summarize, sporulated oocysts (aged no more than 3 months) were diluted to a concentration of approximately 500 or 1,000 sporulated oocysts/mL. A micropipette was used to apply 1µL of the stock onto the surface of sterile agar (pour plates). The 1µL drop was examined microscopically to verify that the drop contained a single sporulated oocyst. Then, a gelatin capsule (XPRS Nutra Size 3 Empty Capsules, Amazon) was opened and used to remove the agar piece containing the 1µL drop. The gelatin capsule was closed and orally administered to a single turkey poult, followed by 3 mL of sterile saline. Feces were collected from day 4-10 post-administration of a single oocyst and were partially processed, concentrated as appropriate, and sporulated as previously described above by salt flotation and were suspended in 2.5% potassium dichromate at 4°C (Reid & Long, 1979; El-Sherry et al., 2013).

Statistical Analysis

Purified gDNA obtained from each sample was used for PCR (n=1/sample). For sequence confirmation, species-specific PCR-positive products were sequenced and subjected to analysis (n=1/PCR product/sample). When necessary, data were presented as a number out of the total to generate percentage values.

Results

Recovery and amplification of *Eimeria* spp. from wild turkey fecal samples

A total of 106 fecal samples were collected from wild turkeys across Arkansas (AR), Louisiana (LA), Texas (TX), Delaware (DE), Pennsylvania (PA), and Maine (ME). *Eimeria* spp. oocysts were detected in 78.3% (83/106) of the samples, including 100% of the samples recovered from DE (36/36), PA (38/38), or ME (9/9) (Table 2). Although fecal samples from wild turkeys located in regions of AR, TX, and LA were received, the quality of the samples was poor, and the samples could not be appropriately evaluated. Due to the low concentration of *Eimeria* spp. oocysts in the majority of the samples obtained from DE, PA, and ME, an amplification step (passage through *Eimeria*-free turkey poults) was required to obtain a fresh stock of oocysts for TACS testing and speciation. Viable sporulated oocysts were recovered after the amplification and sporulation steps for 49.4% (41/83) of the samples. Of the 41 samples, there were 22 DE, 11 PA, and 8 ME samples subjected to monensin TACS testing (Table 3).

Turkey Anti-Coccidial Sensitivity (TACS) testing, speciation, and generation of single-oocyst-derived stocks

There were 11/41 (26.8%) sensitive, 22/41 (53.7%) had reduced sensitivity, and 8/41 (19.5%) were resistant to monensin (Table 3). There were 20/41 (48.8%) samples with a sensitivity threshold >70% for monensin that were subjected to zoalene TACS testing. Of the 20 samples, 9/20 (45%) were resistant, 8/20 (40%) had reduced sensitivity, and 3/20 (15%) were sensitive to zoalene (Table 3). Speciation results for 35/41 samples subjected to monensin and/or zoalene TACS are shown in Table 4 and Figure 1. More than one *Eimeria* spp. was detected in 25/35 (71.4%) of the samples, whereas 10/35 (28.6%) samples contained only *E. meleagrimitis*.

The prevalence of *E. meleagritidis*, *E. dispersa*, and *E. adenoeides* in wild turkey fecal samples collected in the present study was 85.7%, 54.3%, and 42.8%, respectively. Top candidates were selected based on sensitivity to monensin and zoalene and used to generate single oocyst-derived stocks. An amplification step was required for candidate stocks to obtain enough oocysts to confirm the recovery of a single *Eimeria* spp. (PCR and sequence confirmation) and to re-confirm sensitivity to monensin, zoamix, and amprolium. Seven stocks were generated, and 7/7 (100%) were confirmed to be sensitive to amprolium (Table 3).

Discussion

There has been limited research investigating the prevalence and diversity of *Eimeria* spp. in wild turkey populations within North America (Kozicky, 1948; Ruff et al., 1988; MacDonald et al., 2019). Early studies specifically used microscopic evaluation of oocyst morphology and size to differentiate species of turkey *Eimeria*. It cannot be the only method used to speciate *Eimeria* that infect turkeys because there is substantial overlap in oocyst size and morphology across commercially relevant *Eimeria* species (El-Sherry et al., 2013; Chapman, 2014; El-Sherry et al., 2015). At a minimum, PCR-based speciation is necessary to speciate turkey *Eimeria*. There is also considerable sequence homology between some of the turkey *Eimeria* spp., which requires sequencing of positive PCR products to capture false positives (El-Sherry et al., 2013; Hafeez et al., 2015; Imai, 2018). The use of molecular detection methods to assess the prevalence and diversity of *Eimeria* spp. in turkeys has been predominantly limited to commercial turkeys (Rathinam et al., 2015; Imai & Barta, 2019; Duff et al., 2022). However, MacDonald et al. (2019) used molecular techniques to speciate *Eimeria* spp. circulating in wild turkey populations in Ontario, Canada.

In the present study, we evaluated the prevalence of *Eimeria* spp. recovered from wild turkey feces collected in regions within the eastern two-thirds of the United States. According to our results, 78.3% (83/106) of the samples obtained contained oocysts upon microscopic evaluation. It should be noted that the samples obtained from wild turkeys located in regions of Arkansas (AR), Texas (TX), and Louisiana (LA) were of poor quality, and the presence or absence of oocysts could not be determined. However, oocysts were detected in 100% of the samples collected from Delaware (DE), Pennsylvania (PA), and Maine (ME). These data suggest that *Eimeria* cycling is ongoing in wild turkey populations in these regions. Furthermore, the detection of oocysts in the present study was similar to the results published by MacDonald et al. (2019), where 77.1% of the samples obtained from hunter-harvested wild turkeys contained oocysts in Ontario, Canada. Of the 83 oocyst-positive samples obtained in the present study, oocysts were successfully recovered from 41/83 (49.4%) samples post-amplification and subjected to anticoccidial sensitivity testing for major representatives of the ionophore and chemical categories of anticoccidial drugs, including monensin, zoalene, and amprolium.

Monensin has been the most utilized ionophore for controlling coccidiosis in commercial turkey flocks (Chapman, 2008; Chapman et al., 2010). However, there are few investigations regarding monensin sensitivity for wild-type *Eimeria* spp. recovered from commercial turkey operations (Jeffers & Bentley, 1980; Chapman & Rathinam, 2007; Chapman, 2008; Chapman et al., 2010). In the present study, only 20/41 (48.8%) of the wild turkey *Eimeria* spp. were considered to be sensitive to monensin (>70% reduction in total oocyst output) and were subjected to zoalene TACS testing. There have been limited studies regarding in-feed zoalene to control coccidiosis in commercial turkeys (Hymas & Stevenson, 1962; Mitrovic et al., 1971; Cabel et al., 1991). To our knowledge, there are no reports of zoalene sensitivity for *Eimeria* spp.

recovered from wild turkey populations. Although only three *Eimeria* spp. samples recovered from Maine exhibited true sensitivity to zoalene, the “leakiness” caused by the increased excretion of oocysts after drug treatment may have skewed the total oocyst output results in the present study. Samples with reduced or complete sensitivity to monensin and zoalene were selected as potential vaccine candidates. From this, single oocyst-derived stocks were generated from stocks with sensitivity to monensin and reduced or complete sensitivity to zoalene. These candidates moved forward to the speciation (PCR and DNA sequencing) and cloning process. The cloning process is tedious and required the administration of a single *Eimeria* spp. oocyst isolated from a mixed species sample. The inherent viability of the single *Eimeria* spp. oocyst at the time of administration markedly influenced the recovery rate of oocysts from feces (19% recovery rate). For candidates recovered from the cloning process, an amplification step was required to obtain enough oocysts to confirm recovery of a single *Eimeria* spp. (confirmation with PCR and DNA sequencing) and further in vivo testing. Seven stocks were generated for amprolium TACS testing. Amprolium, a synthetic anticoccidial, is a thiamine uptake inhibitor that can be added to the diet or drinking water to prevent or treat coccidiosis (Chapman, 2008). Rathinam & Chapman (2009) assessed the monensin and amprolium sensitivity profile of *Eimeria* spp. isolated from commercial turkey flocks. Of those evaluated, 4/33 (12.1%) isolates of *Eimeria* were sensitive to monensin, but none were sensitive to amprolium. In the present study, monensin sensitivity was higher for wild turkey-derived *Eimeria* spp. than has been previously reported for commercial turkeys (Rathinam & Chapman, 2009). This highlights the difference in the frequency of exposure to anticoccidial drugs between wild and commercial turkeys and the probable impact on the development of anticoccidial-resistant *Eimeria* spp. phenotypes in commercial settings.

Based on our speciation results of wild turkey *Eimeria* sp. recovered from DE, PA, and MA, *E. meleagritidis*, *E. meleagridis*, *E. dispersa*, *E. gallopavonis*, and *E. adenoides* are prevalent in wild turkey populations in the eastern two-thirds of the US. These findings were similar to Imai & Barta (2019) who detected the same six species in commercial turkey flocks in Canada. However, only four of the six *Eimeria* spp. were prevalent in commercial turkeys in the midwestern US (Duff et al., 2022). It is important to note that the PCR-positive products were not sequenced to confirm PCR results (Duff et al., 2022). In contrast, PCR-positive species-specific PCR products were sequenced in the current study and the study published by Imai & Barta (2019).

The most prevalent *Eimeria* species from wild turkey fecal samples collected in the current study was *E. meleagritidis*. This aligns with others reporting a high prevalence of *E. meleagritidis* detected by PCR in commercial or wild turkey populations in North America (Rathinam et al., 2015; Imai & Barta, 2019; Duff et al., 2022). The prevalence of *E. meleagritidis* and *E. adenoides* in the wild turkey fecal samples was similar to those published by Imai & Barta (2019) and Duff et al. (2022). The high prevalence of *E. meleagritidis* and *E. adenoides* in commercial turkey flocks is likely associated with live coccidiosis vaccination at hatch (Immuxo-T®) which only contains those two species. However, there was no information about anticoccidial or vaccination programs for either study. Previous investigations noted that the most common *Eimeria* spp. recovered from pen-raised wild turkeys was *E. meleagritidis* based on oocyst morphology (Ruff et al., 1988). However, speciating turkey *Eimeria* spp. solely based on morphology proved to be inaccurate (El-Sherry et al., 2015). *E. dispersa* prevalence was detected at low levels in commercial turkey flocks in Canada (Imai & Barta, 2019) or absent in commercial turkey flocks in the midwestern US (Duff et al., 2022), but detected frequently in

the wild turkey samples collected in the current study. The low prevalence of *E. meleagridis* in the present study was similar to a report by Duff et al. (2022) that evaluated the prevalence of *Eimeria* spp. in commercial turkey operations in the midwestern US. In another study, only 4/6 species (*E. adenoeides*, *E. meleagrimitis*, *E. gallopavonis*, and *E. meleagridis*) were detected in the fecal samples obtained from wild turkey populations in Ontario (MacDonald et al., 2019). Co-infection with more than one *Eimeria* spp. in the present study suggests that wild turkeys may be effective reservoirs that could impact commercial turkey operations. Interestingly, this is the first report using molecular methods to speciate and assess the prevalence of *Eimeria* spp. circulating in wild turkey populations in the US.

Due to consumer-driven markets and increased regulatory environments, there are no major pharmaceutical investments for developing new drugs. Although drug-sensitive live coccidiosis vaccines have been successfully used for commercial broiler and layer production for years, both to control the disease and to renew sensitivity of field-resident coccidial populations to available drugs for control, there are no such broad vaccines available for commercial turkey production. As there are no available libraries of these turkey *Eimeria* species retained from pre-anticoccidial drug usage, there are no available drug-sensitive strains upon which to develop vaccine-based control programs. We have collected turkey *Eimeria* samples with help from cooperators (hunters, state wildlife departments, and wildlife refuges) from wild turkeys across the eastern two thirds of the United States that had never been directly treated with anticoccidial drugs. The drug-sensitive single-oocyst derived stocks generated from wild turkey fecal samples collected in the eastern US may have the potential to be used as potential vaccine candidates.

Conclusion

Six of the seven described turkey *Eimeria* spp. were detected in wild turkey fecal samples collected in the eastern US suggesting that these protozoa are readily circulating in wild turkey populations. Those wild turkey populations geographically disparate from commercial turkey operations are likely reservoirs for pan sensitive *Eimeria*. Isolates of *E. meleagritidis*, *E. meleagridis*, *E. dispersa*, *E. adenoeides*, and *E. gallopavonis* possessing sensitivity to monensin, zoalene, and amprolium recovered in the present study have the potential to be used as vaccine candidates in commercial turkey operations. Immunogenicity studies have been conducted or are underway.

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Tables

Table 1. Primers and PCR parameters for turkey *Eimeria* spp. identification.

Species	Primer name	Sequence (5' to 3') *	Annealing temperature	Extension Length	Cycles	Size (bp)
mtCOI	COI_UNI_199F	5'-ATGATYTTCTTTGTAGTTATGCC-3'	54°C	60s	35	1,272
	mtRNA20_UNI_R	5'-GTATGGATTTACACGGTCAA-3'				
<i>E. adenoeides</i>	E.ad.CO1_427F	5'-CCAACCTCAGTAGATCTAATTGTA-3'	52°C	60s	30	713
	E.ad.CO1_1186R	5'-GTGAAGTGAGCAATGACA-3'				
<i>E. gallopavonis</i>	E.gal.CO1_292F	5'-AGAGTGAATTGTGTATCACTATTAT-3'	52°C	60s	30	861
	E.gal.CO1_1153R	5'GAGATAATACGAAATGGAAGTGG-3'				
<i>E. meleagrimitis</i>	E.mel.CO1_474F	5'-CTCAAGTTTCCTATCCTCAG-3'	50°C	35s	30	554
	E.mel.CO11_1028R	5'-GCGTACCAGATATCTAAGGAG-3'				
<i>E. dispersa</i>	E.dis.CO1_577F	5'-ACAGCTATTATGTTAATTGGT-3'	52°C	35s	30	451
	E.dis.CO1_1028R	5'-GCATACCAAGTATCTATTGAA-3'				
<i>E. meleagridis</i>	E.md.CO1_431F	5'-CCTCAGTAGATTTAATTGTC-3'	48°C	60s	35	1,012
	E.md.CO1_1443R	5'-TTAGAAGATTAGGGAATATAA-3'				
<i>E. innocua</i>	E.inn.CO1.396F	5'-TCCATTAAGTACATCCCTG-3'	50°C	25s	30	209
	E.inn.CO1.604R	5'-GAAGTGTACCAATTAACATAATG-3'				

* *Eimeria* species-specific PCR primers and parameters from Hafeez et al (2015) and Imai & Barta (2019) and with a minor modification in the annealing temperature and extension length.

Table 2. Wild turkey fecal samples received by geographical location

State	Samples Received	Oocyst-Positive	Positive/Total (%)
AR	9	0	0/9 (0)
DE	36	36	36/36 (100)
LA	1	0	0/1 (0)
PA	38	38	38/38 (100)
ME	9	9	9/9 (100)
TX	13	0	0/13 (0)
Total	106	83	83/106 (78.3)

Table 3. TACS (monensin, zoalene, and amprolium) testing for select wild turkey *Eimeria* spp. recovered from Delaware (DE),

Location	Monensin ¹			Zoalene			Amprolium ²		
	R	RS	S	R	RS	S	R	RS	S
DE	6/22 (27.2)	11/22 (50.0)	5/22 (22.7)	6/10 (60.0)	4/10 (40.0)	0/10 (0.0)	0/3 (0.0)	0/3 (0.0)	3/3 (100)
PA	1/11 (9.0)	7/11 (63.6)	3/11 (27.2)	2/4 (50.0)	2/4 (50.0)	0/4 (0.0)	-	-	-
ME	1/8 (12.5)	4/8 (50.0)	3/8 (37.5)	1/6 (16.7)	2/6 (33.3)	3/6 (50.0)	0/4 (0.0)	0/4 (0.0)	4/4 (100)
Total	8/41 (19.5)	22/41 (53.7)	11/41 (26.8)	9/20 (45.0)	8/20 (40.0)	3/20 (15.0)	0/7 (0.0)	0/7 (0.0)	7/7 (100)

Pennsylvania (PA), and Maine (ME).

¹ Samples with >70% sensitivity to monensin were evaluated for zoalene sensitivity (n=20)

² Single oocyst-derived stocks (n=7) were generated from select mixed species samples and evaluated for amprolium sensitivity

Classification: <30% reduction, R = resistant; 31-79% reduction, RS = reduced sensitivity; >79% reduction, S=sensitive

Table 4. Speciation of wild turkey *Eimeria* spp. propagated from fecal samples collected in Delaware (DE), Pennsylvania (PA), and Maine (ME).

Location	<i>E. adenoides</i>	<i>E. dispersa</i>	<i>E. gallopavonis</i>	<i>E. innocua</i>	<i>E. meleagridis</i>	<i>E. meleagritidis</i>
DE	10/17 (58.8)	10/17 (58.8)	2/17 (11.8)	3/17 (17.6)	2/17 (11.8)	15/17 (88.2)
PA	4/10 (40.0)	7/10 (70.0)	1/10 (1.0)	5/10 (50.0)	1/10 (1.0)	8/10 (80.0)
ME	1/8 (12.5)	2/8 (25.0)	0/8 (0.0)	0/8 (0.0)	1/8 (12.5)	6/8 (75.0)
Total	15/35 (42.8)	19/35 (54.3)	3/35 (8.5)	8/35 (22.9)	4/35 (11.4)	30/35 (85.7)

Note: Positive species-specific PCR products were subjected to sequencing to confirm results

Figures

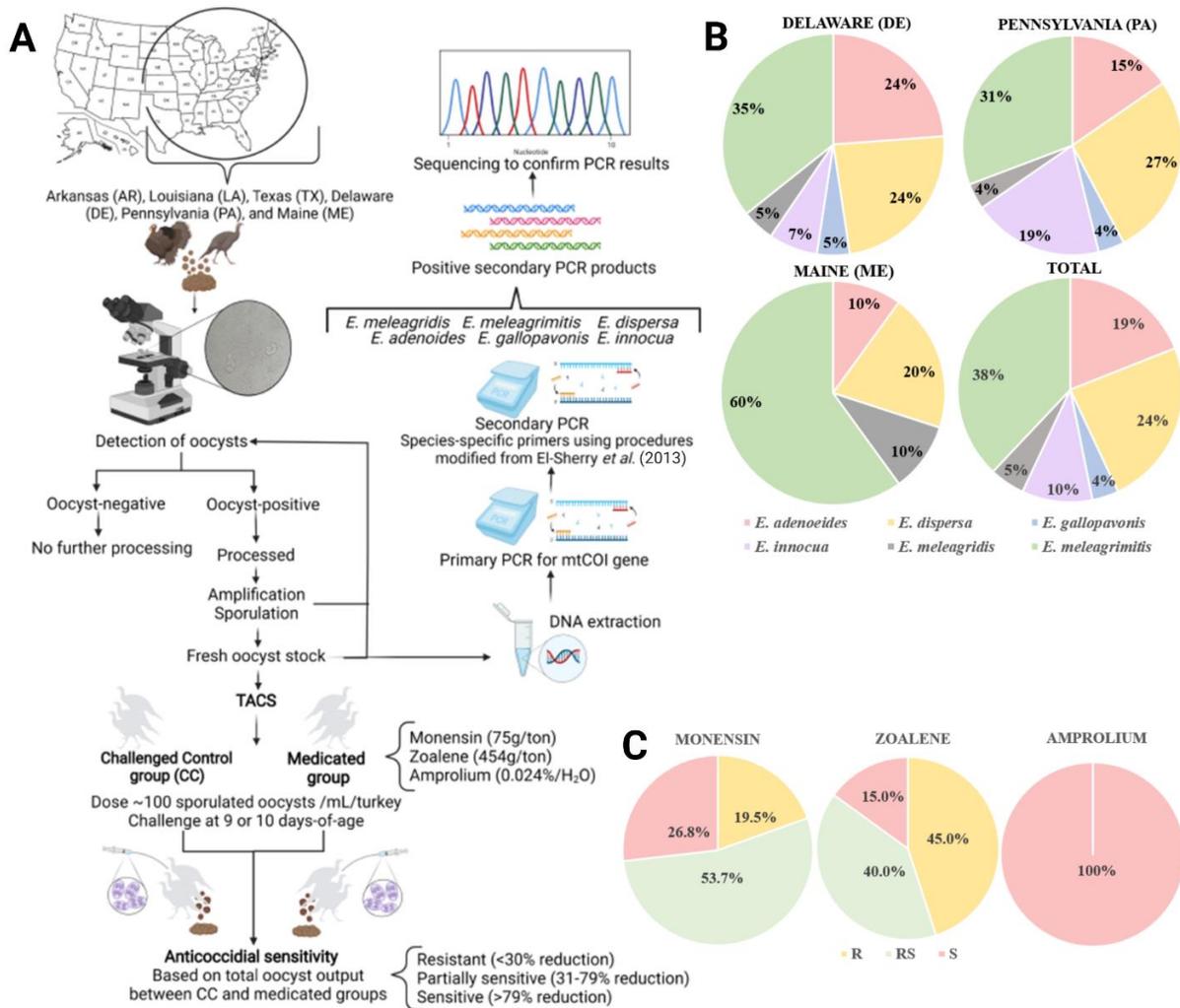


Figure 1. A. Schematic representation of isolation, speciation, and anticoccidial sensitivity testing of *Eimeria* spp. recovered from wild turkey feces. Recovery and detection of *Eimeria* spp. oocysts from wild turkey feces was followed by *in vivo* amplification to obtain a fresh stock of oocysts for TACS testing and speciation (PCR and sequence confirmation). B. Prevalence of *Eimeria* spp. (*E. adenoides*, *E. dispersa*, *E. gallopavonis*, *E. innocua*, *E. meleagridis*, and *E. meleagritimitis*) recovered from wild turkey fecal samples collected in DE, PA, and ME. C. TACS (monensin, zoalene, and amprolium) testing for select wild turkey *Eimeria* spp. recovered DE, PA, and ME. Samples with >70% sensitivity to monensin were evaluated for zoalene sensitivity. Single oocyst-derived stocks (n=7) were generated from select mixed species samples and evaluated for amprolium sensitivity. Classification: <30% reduction, R = resistant; 31-79% reduction, RS = reduced sensitivity; >79% reduction, S = sensitive. Created with BioRender by Carolina Trujillo Peralta.

Chapter IV. Impact of *Eimeria meleagrimitis* and intermittent amprolium treatment on performance and the gut microbiome of turkey poults

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Abstract

Although drug-sensitive live coccidiosis vaccines have been successfully used to control coccidiosis and renew drug sensitivity in commercial broiler/layer operations, limited species coverage vaccines are available for commercial turkey production. This study aimed to assess the protective efficacy of an *E. meleagritidis* vaccine candidate against homologous challenge. Experimental groups included: 1) Non-vaccinated, non-challenged control (NC); 2) Non-vaccinated, challenged control (PC); 3) candidate vaccine (VX) + amprolium; or 4) VX. For VX with and without amprol, 50% of the poults (directs) were orally vaccinated at DOH with 50 sporulated *E. meleagritidis* oocysts and were comingled with non-vaccinated (contact) poults for the duration of the study. Group 3 was treated with amprolium (0.024%) in the drinking water from d10-14. At d23, all groups (except NC) were orally challenged with 95K *E. meleagritidis* oocysts/mL/poult. The following parameters were evaluated: BW/BWG, intestinal lesion scores (LS) and FITC-d 6 days post-challenge, and daily fecal and litter oocysts per gram (OPG). At d29, ileal and cecal contents were collected for 16S rRNA gene-based microbiome analysis. VX did not impact performance during the pre-challenge period. During the post-challenge period (d23-29), BWG for the VX groups was significantly ($P < 0.05$) higher than the PC group. LS was significantly ($P < 0.05$) reduced for both contacts and directs of VX groups compared to PC. As expected, amprolium administration from d10-14 markedly reduced fecal and litter OPG compared to the group that did not receive amprolium. The ileal and cecal content results showed that the PC group had different bacterial diversity and structure, including alpha and beta diversity, compared to NC. Linear discriminant analysis Effect Size (LEfSe) identified that *Lactobacillus salivarius* (ASV2) was enriched in PC's ileal and cecal content. Compared to controls (NC and PC), the experimental groups showed no distinct clusters, but there are

similitudes in the ileal and cecal community based on Bray-Curtis and Jaccard distances. In conclusion, these data suggest that vaccination with this strain of *E. meleagridis*, with or without amprolium intervention, caused a very mild infection that induced protective immunity and challenged markedly affected both the ileal and cecal microbiome.

Key words: *Eimeria*, coccidiosis, vaccination, turkey, microbiome, amprolium.

Introduction

Coccidiosis is caused by the genus *Eimeria*. These obligate intracellular protozoa invade and destroy host intestinal epithelial cells. Host health and performance can be significantly affected depending on the *Eimeria* spp. and the number of ingested sporulated oocysts.

Chemoprophylaxis has been used for over a century to control coccidiosis in commercial poultry (Chapman, 2014). However, *Eimeria* spp. has been shown to develop resistance to anticoccidial drugs (Chapman, 1997). Due to the emergence of multidrug-resistant *Eimeria* spp., the turkey industry has limited options for coccidiosis prevention in commercial turkey flocks.

Anticoccidial rotational and shuttle programs have extended to the use of some drugs as well as live vaccination with drug-susceptible *Eimeria* spp. may displace drug-resistant wild-type *Eimeria* strains in the barn environment (Chapman & Jeffers, 2014). A bioshuttle program (i.e. application of live coccidiosis vaccine followed by delayed anticoccidial intervention in the feed or drinking water) permits the development of immunity, and it improves performance compared to ionophore treatment alone (Montoya & Quiroz, 2013).

At present, there are seven documented *Eimeria* spp. that infect domestic turkeys (*Meleagris gallopavo* [var. *domesticus*]) (Chapman, 2008). Only four of the seven species (*E. meleagrimitis*, *E. adenoides*, *E. gallopavonis*, and *E. dispersa*) are more notably pathogenic in commercial turkeys (Lund & Farr, 1965). However, infection with multiple species makes it difficult to truly estimate the effects of a single species in the field. For vaccination, turkey integrators have been limited to commercially available live *Eimeria* spp. Vaccines that currently do not contain all relevant species. There is evidence of wild turkey fecal samples harboring *Eimeria* spp. that are frequently detected in commercial turkey populations (Ruff et al., 1988). Although drug-sensitive live coccidiosis vaccines have been successfully used to control

coccidiosis and renew drug sensitivity in commercial broiler and layer operations, the only commercially available coccidiosis vaccine for turkeys contains two species, *E. meleagrimitis* and *E. adenoides*. There is no evident cross-protection between *Eimeria* species infecting turkeys (Imai, 2018). As a result, an optimal vaccine formulation to displace the drug-resistant wild-type *Eimeria* spp. would consist of those currently affecting the farm. To properly shift the population of *Eimeria* oocysts from pan-resistant to pan-sensitive in a flock, vaccination with drug-sensitive strains would be the most cost-effective option available. Tailoring a vaccine to contain only the species relevant to a particular house would be ideal to avoid introducing non-relevant strains. Since *Eimeria* spp. are also prevalent in wild turkey populations (Ruff et al., 1988) and the probability of exposure to anticoccidials is low, *Eimeria* spp. recovered from wild turkeys should be evaluated as potential vaccine candidates. Live vaccination can negatively impact performance and amprolium has been briefly applied to the drinking water to reduce vaccine-related effects without disrupting immune development (Chapman, 1997). However, it appears that the timing of application post-vaccination should be selected based on oocyst cycling to not impede immunity (Pohl, 2012).

At present, there are no reports on the impact of live coccidiosis vaccination and/or intermittent amprolium intervention on the intestinal microbiome or gut integrity of turkeys. The complex interactions between the host and gut microbiome affect digestion and overall health of the host (Salzman, 2011). Consequently, the replication of *Eimeria* spp. within the host may shift the composition of the microbiome and increase intestinal permeability. These effects may be directly or indirectly related. A serum biomarker, fluorescein isothiocyanate-dextran, also known as FITC-d, has been used to assess gastrointestinal permeability in necrotic enteritis and coccidiosis models in chickens (Latorre et al., 2018; Bortoluzzi et al., 2019). However, the

relationship between live coccidiosis vaccination with and without amprolium intervention and effect on intestinal integrity and the gut microbiome post-challenge with *E. meleagritidis* has not been evaluated.

Previously, we obtained wild turkey fecal samples and generated single oocyst-derived stocks for five of the major *Eimeria* spp. relevant to commercial turkeys: *E. meleagritidis*, *E. dispersa*, *E. meleagridis*, *E. gallopavonis*, and *E. adenoides*. We found that *Eimeria meleagritidis*, one of the more pathogenic species that infect turkeys (Imai & Barta, 2019), was the most dominant species in wild turkey fecal samples. *E. meleagritidis* was also the most prevalent species detected in commercial turkey flocks in the midwestern United States (Duff et al., 2022). However, this study included flocks vaccinated at hatch with the commercially available vaccine containing only two species: *E. meleagritidis* and *E. adenoides*. Although not all turkey *Eimeria* spp. induces clinical disease, flock performance can be severely impacted (Imai & Barta, 2019). The flock performance must be considered when vaccinating commercial turkey flocks, especially if additional *Eimeria* spp. have been detected in previous flocks. The present study aimed to assess the protective efficacy of a wild turkey-derived, anticoccidial-sensitive (monensin, zoalene, and amprolium) *E. meleagritidis* vaccine candidate derived from wild turkeys against homologous challenge. Furthermore, the effect of vaccination and/or challenge with and without amprolium on the gut microbiome and intestinal permeability was assessed.

Materials and Methods

Eimeria meleagridis

The strain of *E. meleagridis* used to vaccinate and challenge in the present study was isolated from a wild turkey fecal sample collected in Maine, USA. The sample was submitted to the University of Arkansas Division of Agriculture Poultry Health Laboratory in 2019. Sensitivity to monensin, zoalene, and amprolium was confirmed. A single oocyst-derived stock was generated, identity confirmed (PCR and sequencing), and used for vaccination and challenge. Oocysts were kept in potassium dichromate suspension at 4°C until the inoculum was prepared within 24 hours (h) of use.

Preparation of vaccine and challenge stocks

Freshly sporulated *E. meleagridis* oocysts were centrifuged at 1300×g for 10m. The supernatant was discarded, and the pelleted oocysts were resuspended in 0.9% sterile saline. A McMaster chamber was used to determine the concentration of the stock solution or sporulated oocysts/mL (Conway & McKenzie, 2007). For vaccination, oocysts were prepared to achieve a final concentration of ~50 sporulated *E. meleagridis* oocysts/0.25mL/poult. The same procedure was followed to prepare the inoculum for the challenge at a concentration of ~ 95,000 sporulated *E. meleagridis* oocysts/1mL/turkey.

Amprolium

Amprolium (Amprol® 9.6% Oral Solution: Huvepharma), a synthetic anticoccidial, was administered in the drinking water at a concentration of 0.024% per manufacturer's guidelines. Only the VX – Amprol group received amprolium daily in the drinking water from d10-d14.

Experimental Design

A total of 350 day-of-hatch (DOH) female turkey poultts were obtained from a local commercial hatchery. Poultts were neck-tagged individually and randomly allocated into the following treatment groups: 1) non-vaccinated, non-challenged control (NC), 2) non-vaccinated, challenged control (PC), 3) *E. meleagrimitis* candidate vaccine + Amprol (VX + Amprol), or 4) *E. meleagrimitis* candidate vaccine (VX). The VX + Amprol treatment group received amprolium (0.024%) in the drinking water from d10-d14 at the University of Arkansas Poultry Health Laboratory. There were two vaccination levels for the vaccinated groups: directly vaccinated (directs) or indirectly vaccinated (contacts). For the vaccinated groups, 50% of the poultts assigned to those groups received ~50 sporulated *E. meleagrimitis* (VX) oocysts/0.25mL/poult immediately prior to placement by oral gavage. The NC, PC, and contacts did not receive any treatment prior to placement. At d23, turkeys in all groups, excluding the NC group, were orally challenged with ~95,000 sporulated *E. meleagrimitis* oocysts/1mL/turkey by oral gavage. Individual body weights (BW) were recorded at DOH, d8, d23, and d29 (termination) to determine average body weight gain (BWG).

Each treatment group was housed in a single 7x7ft floor pen with fresh pine shavings (n=60-70 poultts/pen). From DOH-d10, poultts for each treatment group were housed to simulate commercial brooding density (0.475 sq. ft./poult). From d10-d29 (termination), density was 0.817 sq. ft./poult. All turkey poultts were provided feed and water *ad libitum* throughout all experiments. The lighting program followed management guidelines for commercial turkey hens (Aviagen, 2018). All animal handling procedures complied with the Institutional Animal Care and Use Committee (IACUC protocol #21117) of the University of Arkansas.

Fecal and litter OPG

Oocyst shedding during the duration of the study was monitored by collecting fecal and litter samples from d5-d28 post-vaccination. Individual fecal samples were collected from a subset of the directs and contacts in the vaccinated groups (n=10 samples from d5-d6; n=1 pooled sample at d7; n=5 samples at d8; and n=3 from d9-d28). The difference in the number of individual fecal samples collected throughout the duration of the experiment was due to the sheer amount of time it took to collect the samples. Initially, the goal was to collect n=10 individual samples per group and vaccination level. However, n=3 individual samples were a more feasible number to collect. Pooled fecal samples were collected for the NC and PC group (n=3) from d5-d29. Pooled litter samples were collected for each treatment group from random sections of the pen (n=3). To determine fecal and litter oocysts per gram of feces (OPG), all fecal and litter samples were collected in 5mL microcentrifuge tubes or 50mL polypropylene centrifuge tubes, respectively, and then weighed and suspended in 2.5% PDC (Sigma-Aldric, Co) at a final concentration of 1:2 (w/v). Fecal or litter samples were processed to determine OPG counts using a McMaster counting chamber using a standard formula that includes the initial weight of the sample, volume of saturated NaCl solution, and any additional dilutions that were required (Hodgson, 1970; Long & Rowell, 1975; Conway & McKenzie, 2007). For example, 100 μ L of fecal homogenate was mixed with 900 μ L saturated NaCl and immediately loaded into a McMaster counting chamber. The chamber was left undisturbed for 5 minutes to allow the oocysts to float. The oocysts within the McMaster chamber grid were enumerated. If less than 20 oocysts were counted in the first lane of the grid, all six lanes were counted. If more than 20 oocysts were counted in the first lane, the sample was washed from the slide, and a further

dilution of the original sample was made with saturated NaCl before the McMaster chamber was reloaded.

Lesion Scores

At d29, or 6d post-homologous challenge, macroscopic intestinal lesion scores (duodenum to lower intestine) were evaluated and recorded for each group and vaccination level (n=18-20) in order to evaluate macroscopic lesions using methods similar to El-Sherry et al. (2014) and Gadde et al. (2020). A lesion score of “0” represents a healthy organ whereas a score of “4” represents severe coccidiosis.

Serum FITC-d

Serum levels of FITC-d (ng/mL) were used as a biomarker to evaluate intestinal permeability, as described by Baxter et al. (2017). At the end of the trial, turkey poults (n=18-20 for NC and PC; n=13-15 for vaccinated groups) were orally gavaged with 8.32 mg/kg of body weight of fluorescein isothiocyanate-dextran (FITC-d, MW 3–5 KDa; Sigma-Aldrich Co). One hour after FITC-d administration, turkeys were euthanized by CO₂ inhalation. Blood samples were collected from the femoral vein and centrifuged (1000× g for 30m at 4°C) to separate the serum.

Microbiome

Ileal and cecal contents were collected from 29-day-old turkey poult hens (n=6/treatment). Samples were stored at -20°C in an RNA/DNA shield until DNA extraction was performed. Total genomic DNA of ileal and cecal content samples was extracted using the DNeasy Power Lyzer Power Soil Kit (Qiagen, Germantown, MD, USA) according to the manufacturer’s protocol. The concentration of DNA was measured using a NanoDrop One

(Thermo Fisher Scientific, Madison, WI, USA) and was diluted to 10 ng/μL with Dnase- or RNase-free water. The V4 region of the 16S rRNA gene was amplified using primer sequences (forward: 5'-GTGCCAGCMGCCGCGGTAA-3' and reverse: 5'-GGACTACHVGGGTWTCTAAT-3') attached with gene-specific Illumina adapters for each sample (Caporaso et al., 2011). PCR amplification was performed by using a T100 thermal cycler (Bio-Rad, Hercules, CA, USA). All 16S PCRs conditions were followed by 30 s initial denaturation at 95°C; 30 cycles at 95°C for 10 s, annealing at 55°C for 30 s, at 72°C for 1 min, at a 72°C final extension for 10 min. The PCR products were determined on a 1% agarose gel and then normalized plates using a SequalPrep™ Normalization Plate Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendation. All purified PCR amplicons were pooled together to generate a sequencing library (Kozich et al., 2013). After the concentration and quality of the library were confirmed by KAPA Illumina Library Quantification Kits (Roche, Indianapolis, IN, USA) via a quantitative PCR (qPCR, Eppendorf, Westbury, NY, USA) assay and an Agilent 2100 Bioanalyzer System (Agilent, Santa Clara, CA, USA), respectively.

The library was sequenced on a MiSeq sequencer (MiSeq Reagent Kit v2, 500 cycles (Illumina, San Diego, CA, USA)). To prevent contamination from reagents, a mock community (ZymoBIOMICS™ Microbial Community Standard (Zymo, Irvine, CA, USA)) and a negative of DNA extraction and PCR amplification were included in sequencing as well. The sequencing files obtained from the Illumina sequencer were pre-processed, quality filtered ($Q > 30$), and analyzed using the QIIME2 (2021.4 release) software (Bolyen et al., 2019). Deblur algorithm was used for sequence trimming, de-noising, chimera removal, and features binning at the amplicon sequence variants (ASV) level (Amir et al., 2017). Naïve Bayes classifier was employed for the assignment of all sequences into bacterial taxonomy using the Greengenes

(v13_8 clustered at 99% identity) reference database. The raw data are available in the NCBI SRA database with the BioProject ID PRJNA.

Statistical Analysis

All data were subjected to analysis of variance (ANOVA) as a completely randomized design using JMP Pro 14 software. Significant differences among the means were determined by Tukey's multiple comparison test for BW, BWG, and serum FITC-d, where statistically significant differences between the means were set at a $p \leq 0.05$. The LS data were determined using Proc Mixed Analysis by SAS 9.4 at $p \leq 0.0001$. Oocysts per gram of feces (OPG) and litter data were expressed as mean using JMP Pro 14 software.

Alpha diversity, including the Shannon Index and the number of Observed ASVs, was compared using a two-tailed Wilcoxon signed-rank test between two groups. Beta diversity based on Bray-Curtis and Jaccard distances was tested using an analysis of similarity (ANOSIM). The outputs of diversity were visualized using the "ggplot2" package in R (v4.1.2). The linear discriminant analysis (LDA) effect size (LefSe), an analytical tool for discovering and interpreting biomarkers of high-dimensional data, was used to identify the signature bacteria associated with the growth stages and intestinal segments. LDA score > 2 was used as a criterion for judging the significant effect size (Segata et al., 2011). The signature bacteria were visualized in a heat map using the 'pheatmap' function in R.

Results

Performance

For average BW, there were no significant differences between all groups at DOH or d8 (Table 1). However, there were significant ($p \leq 0.05$) differences in BW at d23 only between positive control (PC) and VX + Amprol – contact, with the VX group having the lower BW at d23. The BW at d29 showed that VX – contact had a markedly higher value than PC with significant ($p \leq 0.05$) differences; in contrast, the other groups evaluated had no significant differences.

For average BWG from DOH-d8, there were no significant differences between all groups. The DOH-d23 BWG (pre-challenge BWG) was significantly ($p \leq 0.05$) higher in PC than contact in VX + Amprol. However, the post-challenge (d23-d29) BWG of the PC group was significantly ($p \leq 0.05$) affected due to the challenge with 95,000 sporulated *E. meleagrimitis* oocysts/mL/turkey at d23. Though, d23-d29 BWG was considerably ($p \leq 0.05$) improved for the direct and contact of the VX + Amprol and VX compared to the PC and NC. DOH-d29 average BWG was only significantly different between the PC and VX – contact groups.

Lesion scores and Serum FITC-d

At d23, all turkeys, except for the NC, were orally challenged with *E. meleagrimitis* (95,000 sporulated oocysts/mL). After 6d post-challenge, intestinal lesion scores were evaluated using methods similar to El-Sherry et al. (2014) and Gadde et al. (2020). Lesion scores were significantly ($p \leq 0.0001$) reduced in direct and contact of VX and VX + Amprol as compared to the NC and PC (Table 1, Figure 1). There were no significant differences in average lesion scores in the vaccinated level group. The distribution of lesion scores at d29 has been presented in Figure 1. The vaccinated and NC groups had less severe lesion scores than the PC group.

Serum FITC-d levels in the PC group were significantly ($p \leq 0.05$) higher than VX – contact (Table 1). However, serum FITC-d levels for the VX + Amprol – contact group was significantly ($p \leq 0.05$) higher than the VX – contact group or the contacts in the vaccinated group that did not receive amprolium from d10-d14.

Fecal and litter OPG

There was a sharp increase in fecal OPG at d6 for turkeys that received 50 *E. meleagritidis* oocysts at DOH compared to those that did not directly receive the vaccination (Figure 2A). Drinking water administration of amprolium from d10-14 reduced mean fecal OPG for direct and contact poult in VX + Amprol group (Figure 2A). However, turkeys that did not receive vaccination (VX – contact), but were commingled with turkeys that did receive 50 *E. meleagritidis* oocysts at DOH (VX – direct), presented a sharp initial peak and had a higher mean fecal OPG at d13. Furthermore, the naïve contact poult that did not receive any drug intervention to attenuate oocyst cycling had a sharp increase in fecal OPG from d11-d15 compared to all other groups. The importance of proper coccidiosis vaccination methods was represented by the difference in fecal OPG between contact and direct of the VX group and between contacts of the VX group and contacts of the VX + Amprol. The greatest peak in OPG for the PC was at d24, which was ~24h post-challenge with *E. meleagritidis* (95,000 sporulated oocysts/mL). There was a peak in fecal and litter OPG in the NC group at d25 and d24, respectively. This was unexpected and suggests that there was cross-contamination at the time of challenge although the poult in the NC were not handled after challenging the other groups at d23.

For litter OPG (figure 2B), there were differences in OPG peaks between vaccinated groups associated with or without the administration of amprolium. For instance, the group that

did not receive drug intervention to attenuate oocyst cycling (VX) had multiple peaks in litter OPG after d14 whereas the VX + Amprol group had more uniform litter OPGs with only a single sharp increase between d6 to d9 (Figure 2B).

Microbiome

Microbiome analysis of the bacterial communities in the ileal and cecal contents have been summarized in Figures 3-7.

Figure 3 shows the phylum and genus composition of the ileal and cecal contents by the group. At the phylum level, Firmicutes (89.3-95.9%) was the most dominant taxa, followed by Proteobacteria (2.7-10.0%) for both ileal and cecal contents for all groups assessed (Figure 3A). However, following Proteobacteria, Actinobacteria (0.4-2.2%) was enriched in ileal contents and Tenericutes (0.8-3.1%) was enriched in cecal contents for all groups.

Lactobacillus was the predominant genus in the ileum for all groups except for the VX – Amprol contact group which had a higher abundance of *Streptococcus* compared to all treatment groups (Figure 3A). The group with the highest abundance of *Lactobacillus* in the ileum was the PC group (54.2%). For the VX groups, the proportion of *Lactobacillus* was higher in the VX group (37.1% for directs and 26.6% for contacts) than in the VX + Amprol group (17.0% for directs and 9.5% for contacts) in ileal contents. Additionally, a higher abundance of *Clostridium* was in the VX group (15.4% for direct and 18.8% for contact) than in the VX + Amprol group (10% in both at the level group of VX). The VX + Amprol group (24.6% for directs and contacts) was dominated by *Turicibacter* in the ileum compared to all other groups.

At the genus level, the dominant genera in the cecal contents were *Faecalibacterium*, *X. Ruminococcus* and *Lactobacillus* (Figure 3B). The highest abundance of *Faecalibacterium* was in the VX + Amprol group (14.7% for directs and 15.8% for contacts)

followed by the NC (10.9%) and VX contacts (11.0%) in cecal contents. However, *X*, *Ruminococcus* abundance was elevated for VX contact group (11.5%) and VX + Amprol – contact group (10.2%) compared to the VX direct group (8.3%) and VX + Amprol – direct group (7.8%). *Clostridium* abundance in the cecal contents was highest in the VX – contact group (4.1%) compared to all treatment groups.

The results showed that ileal and cecal content in the PC group did not exhibit a different bacterial diversity and structure, including alpha and beta diversity, when compared to NC (figure 4 and figure 5). Alpha diversity was measured using the Shannon Index and the number of observed ASVs. There were no significant ($p>0.05$) differences for alpha diversity in ileal or cecal contents across all vaccinated groups (Figure 4). However, for ileal contents, the PC group had significantly ($p<0.10$) lower alpha diversity (Shannon Index) in the ileal contents compared to all vaccinated groups, except for the VX + Amprol contact group. No distinct clusters between cecum and ileum-based Bray-Curtis and Jaccard distances across all groups (figure 5).

Linear discriminant analysis Effect Size (LEfSe) was employed to identify bacterial biomarkers for each group. In the PC group, *Lactobacillus salivarius* (ASV2) was enriched in cecal and ileal content (Figures 6 and 7). *Faecalibacterium prausnitzii* (ASV7 in cecal and ASV15 in ileal) was enriched in the cecal and ileal community of the VX + Amprol – contact group, while *Turicibacter* (ASV4) was overrepresented in VX + Amprol – direct group (Figures 6 and 7). *Pepetostreptococcaceae* (ASV65) was the one only enriched in VX – contact group or ileal contents (Figure 7), while *Ruminococcaceae* (ASV25) and *Lachnospiraceae_Ruminococcus* (ASV23) were greater in VX – direct and VX – contact group cecal contents, respectively (Figure 6).

Discussion

The importance of proper coccidiosis vaccination uptake in turkey poult was demonstrated in the present study by comparing the difference in fecal OPG between contacts and direct of the VX group and the contacts of VX group and contacts of the VX + Amprol group. Only numerical differences in BW or BWG between the vaccinated groups were observed suggesting that this strain of *E. meleagritidis* is relatively non-pathogenic especially considering the “contact” poult were not directly vaccinated and the number of oocysts that were ingested was not controlled. Since susceptibility to *Eimeria* spp. infection increases with age and severity of disease is associated with the number of oocysts ingested and *Eimeria* spp., a negative impact on performance was anticipated for contact poult, especially those that did not receive intermittent amprolium treatment. Interestingly, the contact and directs in the VX group were numerically heavier than those that received amprolium in the drinking water from d0-14 and improved gut integrity compared to all other treatments. Even though there were no differences in lesion scores between VX groups, the serum FITC-d levels of the VX + Amprol group were similar to the PC groups which may suggest that the timing of amprolium administration (d10-14) was perhaps too early. In the field, amprolium is generally administered in the diet ~d16 to mitigate performance losses associated with live coccidiosis vaccination at hatch. We hypothesized that arresting *E. meleagritidis* development shortly after initiation of the second cycle by administering amprolium in the drinking water would have beneficial effects overall. A more comprehensive study is currently underway to validate these results.

This present study provides an initial evaluation of the effects of *E. meleagritidis* and amprolium on the gut microbiome, specifically the ileal and cecal microbiome, of turkeys. *Eimeria* spp. infection impedes digestion and absorption of nutrients by impairing the intestinal

barrier function, causing bacterial translocation, and disrupting gut homeostasis (Shang et al., 2018). The gut microbiota and microbiome influence host performance and resistance to enteric pathogens (Madlala et al., 2021) and the effects of the gut microbiome on overall performance and health of chickens have been described (e.g. Yeoman et al., 2012; Danzeisen et al., 2013; Wei et al., 2013). However, the microbiome of chickens and turkeys are only 16-19% similar at the genus level (Wei et al., 2013) indicating distinct variations between the two avian species. Several investigators have assessed the impact of coccidiosis on the gut microbiome of chickens (e.g., Orso et al., 2020; Wu et al., 2014). In contrast, research evaluating the effect of live coccidiosis vaccination and anticoccidial drugs on the turkey gut microbiome is lacking.

In the current study, there was increased heterogeneity in the microbiome composition of cecal contents compared the ileal contents. This aligned with previous findings described by D'Andrea et al. (2017) who assessed the gut microbiome of healthy vs. hemorrhagic enteritis (*Adenovirus*)-infected turkeys. Furthermore, the lack of significant differences for alpha and beta diversity across treatment groups is similar to a report published by Macdonald et al. (2017). The investigators demonstrated that live coccidiosis vaccination did not affect alpha diversity in ceca of broiler chickens. Although there were no significant differences in alpha or beta diversity associated with *E. meleagrimitis* vaccination and/or challenge in the present study, there was an apparent shift in the microbiome composition at both the phylum and genus level. For example, *E. meleagrimitis* challenge at d23 increased the abundance of *Lactobacillus salivarius* in the ileum of the PC group compared to the NC and the vaccinated groups which was unexpected. Interestingly, Latorre et al. (2018) observed the same phenomenon in necrotic enteritis-challenged chickens. Bacteria in the small intestine compete with the host to acquire and utilize amino acids whereas the bacteria in the ceca capitalize on the amino acids or nutrients that

bypass the small intestine (Apajalahti & Vienola, 2016). It has been estimated that Lactobacilli in the small intestine acquire up to 6% of the dietary protein (Apajalahti & Vienola, 2016). If nutrient absorption within the small intestine is depleted, the resident bacteria will use the nutrients for growth. Perhaps the increased abundance of *Lactobacillus* is associated with the over proliferation of lactobacilli due to the malabsorption of nutrients by the host associated with *E. meleagrimitis* challenge. In contrast, amprolium administration was associated with a reduction in *Lactobacillus* in the ileum, but an increase in *Turicibacter*, a known butyric acid producer associated with a normal/healthy gut in chickens (Latorre et al., 2018). Although synthetic anticoccidials do not have antimicrobial effects, these drugs may have an indirect effect on the host's gut microbiome since they affect parasite metabolism after intracellular invasion. The complexity of the host-microbiota-protozoa interaction and effects on host immune development and performance requires further investigation.

Based on the results from the present study, vaccination with a non-attenuated strain of *E. meleagrimitis* obtained from wild turkey feces induced a mild infection providing protective immunity with and without amprolium intervention which affected gut integrity and shifted the ileal and cecal luminal microbiome in turkey poults. The impact of a bioshuttle program on the intestinal microbiome requires further investigation.

Conclusion

Vaccination with *E. meleagrimitis* obtained from wild turkey feces induced a mild infection providing protective immunity based on lesion scores and performance. However, attenuated shedding but increased permeability in Amprol treated group suggests the timing of administration (d10-14) was too early. Since this was a pilot study, additional experiments with more replicate pens will be conducted to validate these results. Large-scale studies will be

conducted to evaluate combinations of drug-sensitive wild turkey *Eimeria* spp. as a candidate live coccidiosis vaccine, as a standalone, or implemented with a bioshuttle program. If successful, this will provide the turkey industry with a strategy to control coccidiosis that could be customized based on complex needs.

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Table

Table 1. Effect of *Eimeria meleagrimitis* vaccination, with and without amprolium intervention, and/or *E. meleagrimitis* challenge on average BW, BWG, LS, and serum FITC-d in turkey poult.

Treatment	NC	PC	VX + Amprol Direct	VX + Amprol Contact	VX Direct	VX Contact
BW (g) ¹						
DOH	57.96 ± 0.61	58.31 ± 0.57	57.63 ± 0.77	59.23 ± 0.79	57.00 ± 0.62	57.29 ± 0.68
d8	154.86 ± 2.41	158.28 ± 2.12	163.11 ± 3.66	157.50 ± 3.37	159.00 ± 2.54	155.31 ± 4.84
d23	535.08 ± 7.71 ^{ab}	559.81 ± 8.83 ^a	524.70 ± 11.62 ^{ab}	515.33 ± 12.23 ^b	533.43 ± 11.58 ^{ab}	536.40 ± 9.70 ^{ab}
d29	730.60 ± 11.92 ^{ab}	706.60 ± 11.90 ^b	750.83 ± 20.55 ^{ab}	736.47 ± 18.44 ^{ab}	764.73 ± 16.63 ^{ab}	783.73 ± 13.67 ^a
BWG (g) ¹						
DOH-d8	96.84 ± 2.27	99.97 ± 2.02	105.49 ± 3.47	98.68 ± 3.19	102.00 ± 2.38	98.03 ± 2.46
DOH-d23	477.00 ± 7.56 ^{ab}	501.17 ± 8.75 ^a	467.07 ± 11.44 ^{ab}	456.47 ± 12.05 ^b	476.33 ± 11.58 ^{ab}	478.80 ± 9.53 ^{ab}
DOH-d29	672.52 ± 11.74 ^{ab}	648.00 ± 11.78 ^b	693.20 ± 16.11 ^{ab}	677.60 ± 14.79 ^{ab}	707.63 ± 12.88 ^{ab}	726.13 ± 10.20 ^a
d23-d29	195.52 ± 5.40 ^b	145.05 ± 5.10 ^c	226.13 ± 9.85 ^a	221.13 ± 7.01 ^a	231.30 ± 566 ^a	247.33 ± 5.24 ^a
LS ²	0.05 ± 0.05 ^c	2.21 ± 0.16 ^a	1.39 ± 0.12 ^b	1.55 ± 0.14 ^b	1.30 ± 0.11 ^b	1.45 ± 0.14 ^b
FITC-d [ng/mL] ¹	141.37 ± 29.78 ^{abc}	269.74 ± 25.25 ^a	206.61 ± 11.69 ^{abc}	250.11 ± 32.87 ^{ab}	74.72 ± 29.89 ^{bc}	65.38 ± 58.77 ^c

Data are expressed as the mean ± standard error. At d23, turkeys were orally challenged with *E. meleagrimitis* (95,000 oocysts/mL) except for negative control (NC). At d29, a subset of the turkeys from each group orally received FITC-d orally and were lesion scored. ^{a,b,c} Different superscripts indicate significant differences between the treatments at $P \leq 0.05$. ¹ Statistical evaluation using ANOVA followed by post hoc Tukey's range test. ² Statistical differences between lesion scores detected using SAS Proc Mixed Analysis.

Figure

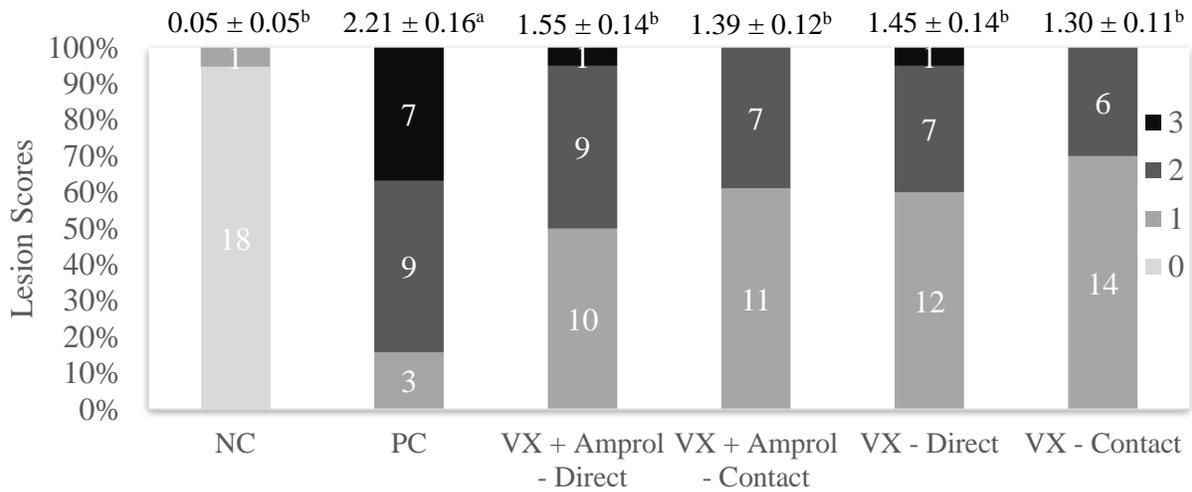


Figure 1. Cumulative lesion scores 6 days post-challenge. At day 23, all poulters, except for the NC, were orally challenged with *E. meleagridis* (95,000 sporulated oocysts/mL). Six days post-challenge (day 29), a subset of the poulters from each group and vaccination level (n=18-20/group) were lesion scored. A lesion score of “0” represents a healthy organ whereas a score of “4” represents severe coccidiosis. No lesion scores of 4 were observed. Numbers within columns indicate the number of poulters evaluated for each lesion score (0-3). Mean lesion score ± standard error presented above columns. Means further separated using Proc Mixed Analysis (SAS 9.4).^{a-c} Different superscripts between treatment groups indicate means differ significantly ($P \leq 0.05$).

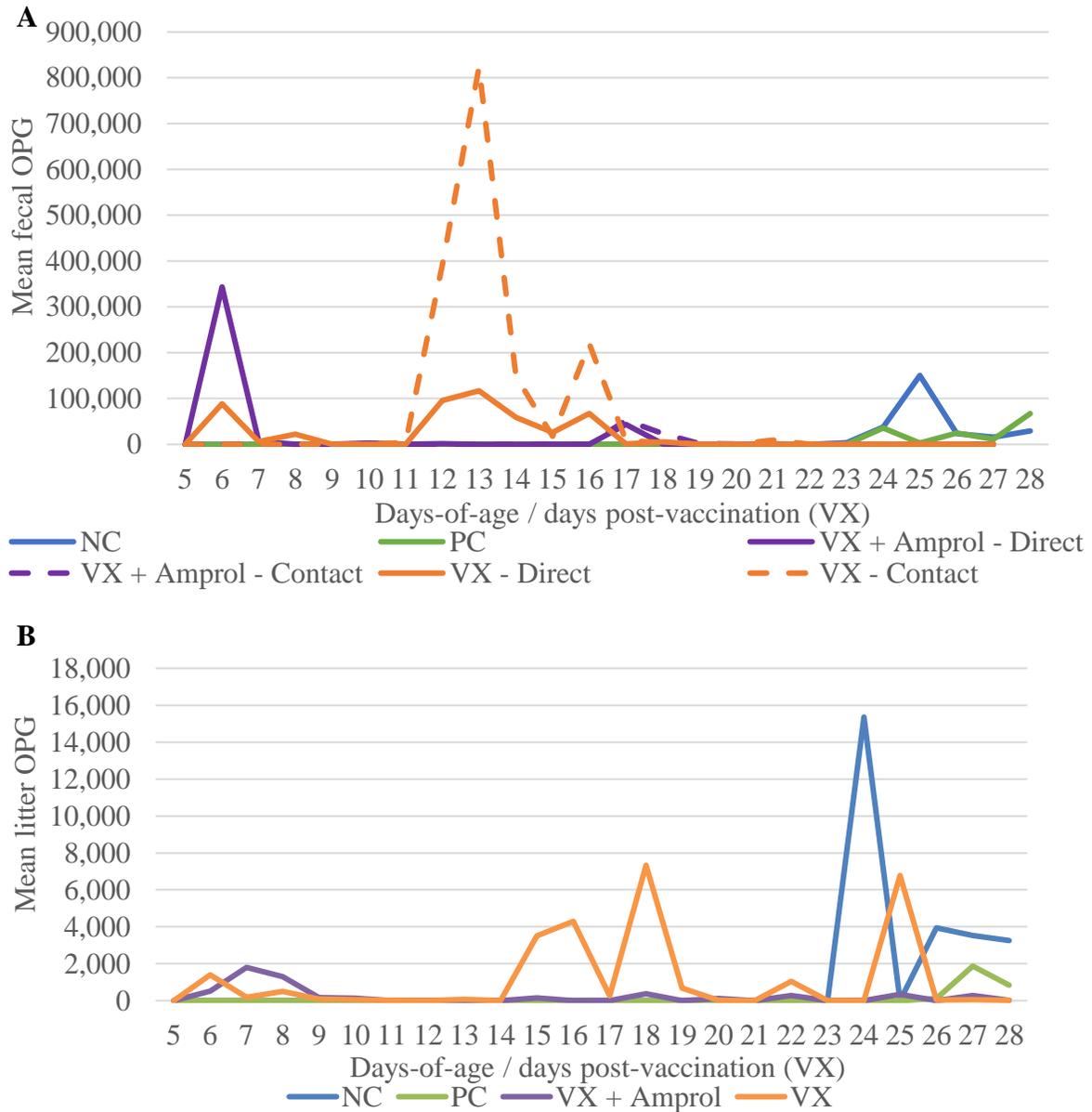


Figure 2. Effect of *E. meleagridis* vaccination and/or challenge, with and without amprolium intervention, on (A) mean fecal oocyst per gram (OPG) and (B) mean litter OPG. For fecal OPG, individual fecal samples were collected from the direct and contact poult (n=3-10 individual fecal samples/group/vaccination level/day) and pooled fecal samples were collected for NC and PC. At DOH, 50% of the poult in the vaccinated groups orally received 50 sporulated *E. meleagridis* (VX) oocysts immediately prior to placement. The NC, PC, and contacts did not receive any treatment prior to placement. VX + Amprol group received amprolium in the drinking water from d10 - d14 at 0.024%. At d23, turkeys were orally challenged with *E. meleagridis* (95,000 oocysts/mL) except for negative control (NC). Pooled litter samples were collected for each treatment group (n = 3).

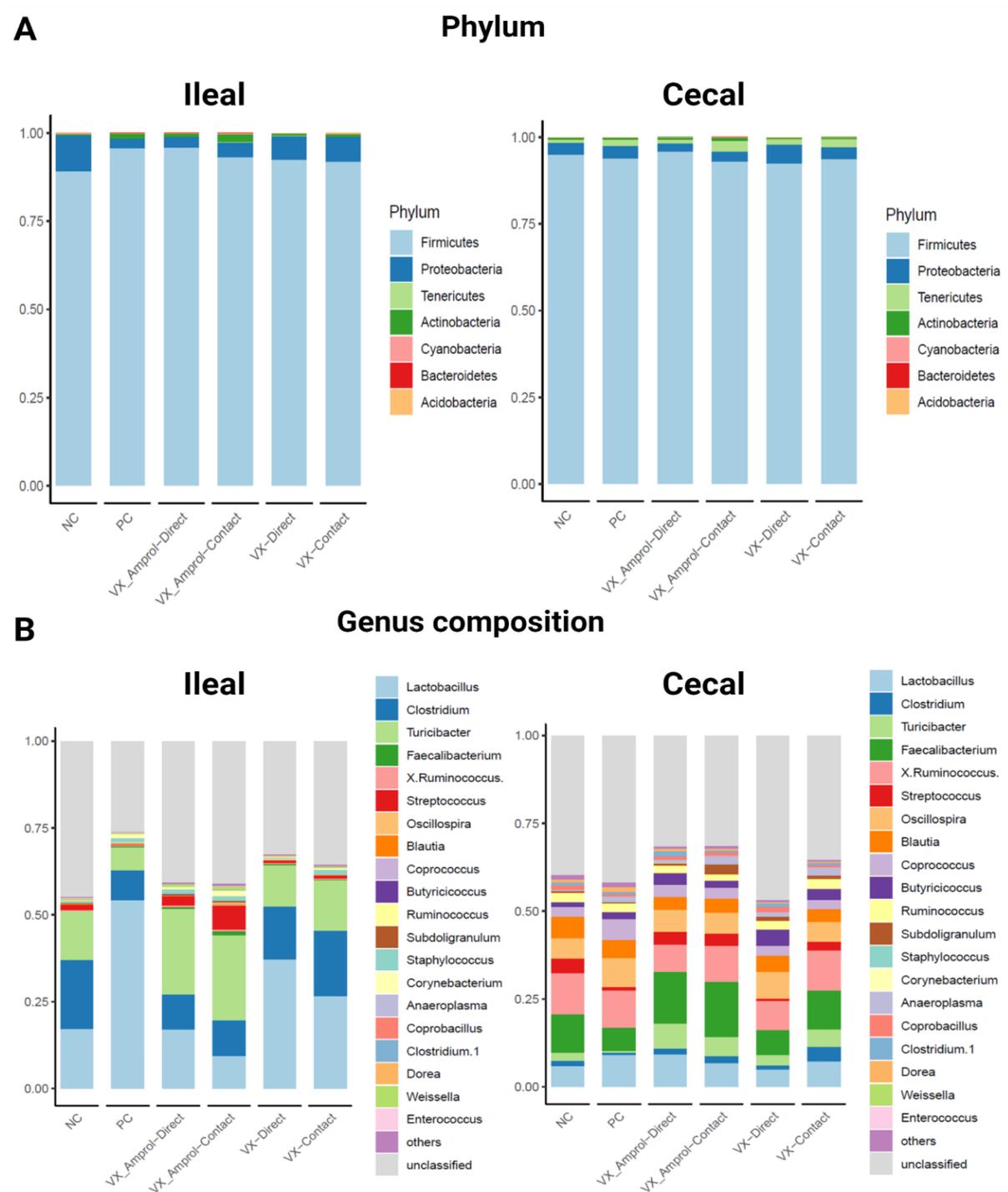


Figure 3. Effect post-challenge of *Eimeria meleagridis* vaccination, with and without amprolium intervention, on (A) phylum and (B) genus composition in ileum and cecal contents. All groups, except the NC group, were challenged with 95,000 sporulated *E. meleagridis* sporulated oocysts at d23. At d29, or 6d post-challenge, ileal and cecal contents were collected from 29-day-old turkey poult hens (n=6/treatment/vaccination level).

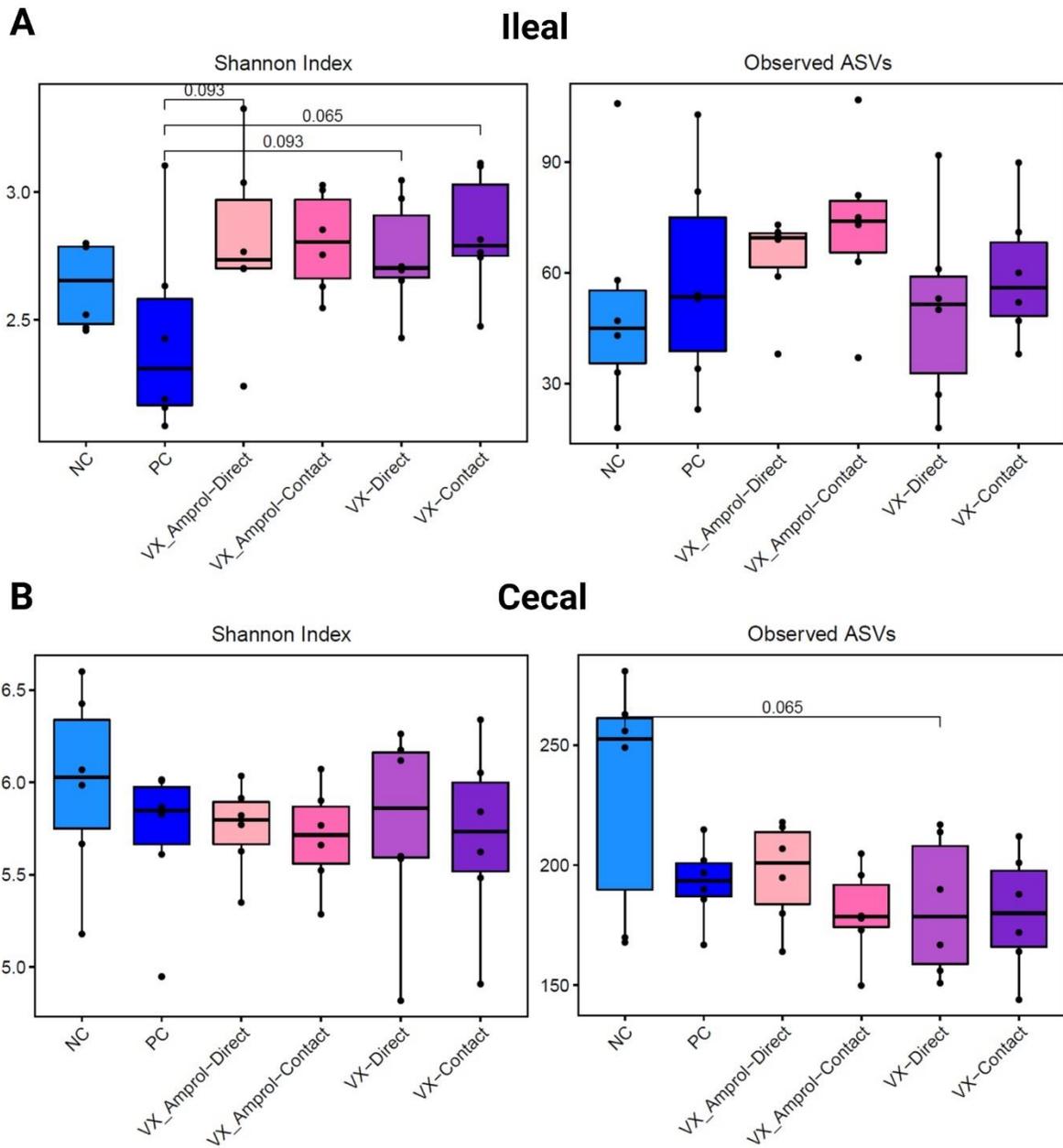


Figure 4. Alpha diversity of (A) ileal and (B) cecal contents collected at d29 (6d post-challenge). Alpha diversity was measured using Shannon Index (left) and number of Observed ASVs (right). Statistical comparison was made using the two-tailed Wilcoxon signed-rank test between two groups.

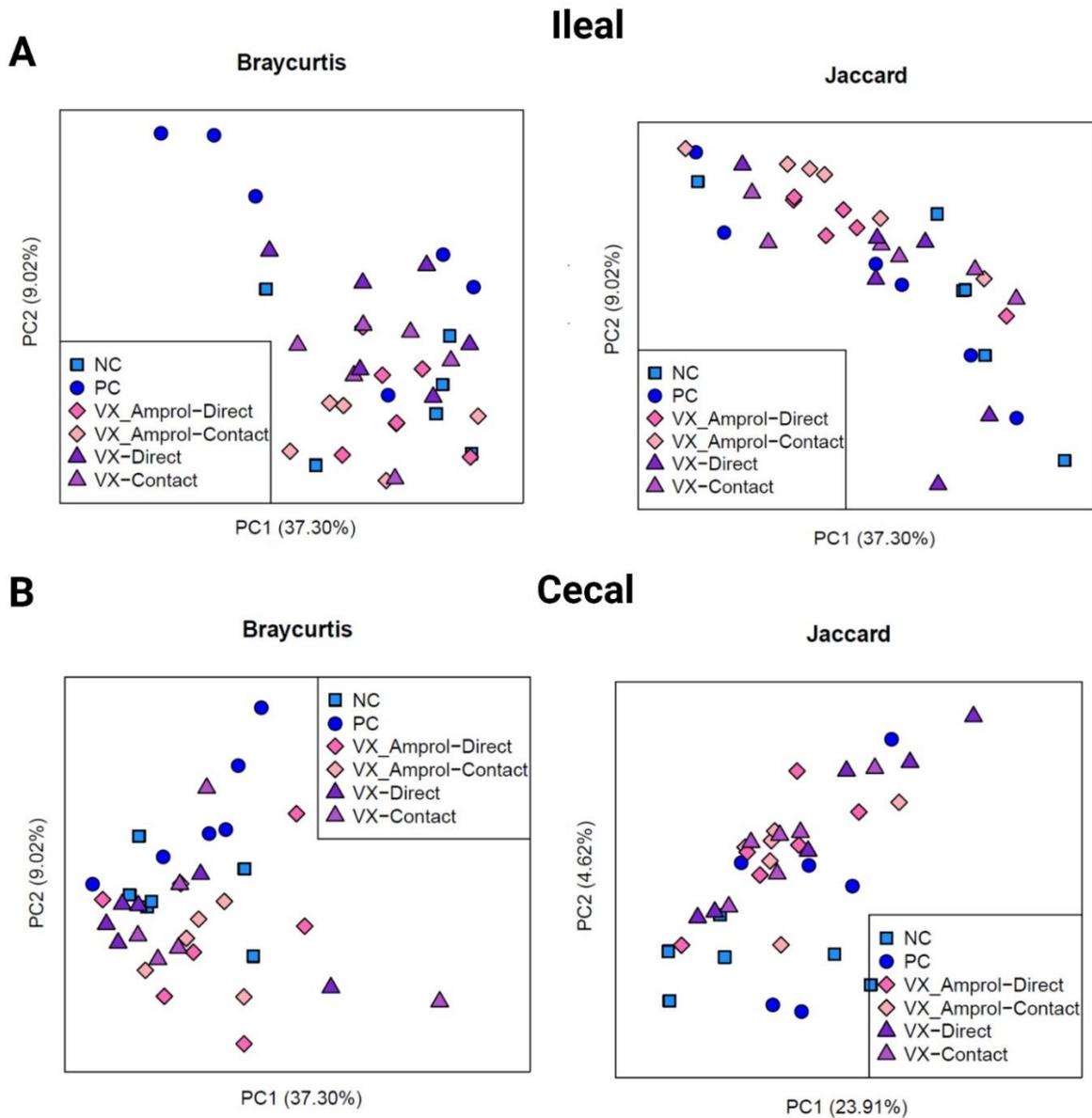


Figure 5. Beta diversity of (A) ileal and (B) cecal contents collected at d29 (6d post-challenge). Beta diversity was evaluated using Bray Curtis (left) and Jaccard (right) distances. The outputs of diversity were visualized using the “ggplot2” package in R (v4.1.2). Analysis was conducted using an analysis of similarity (ANOSIM).

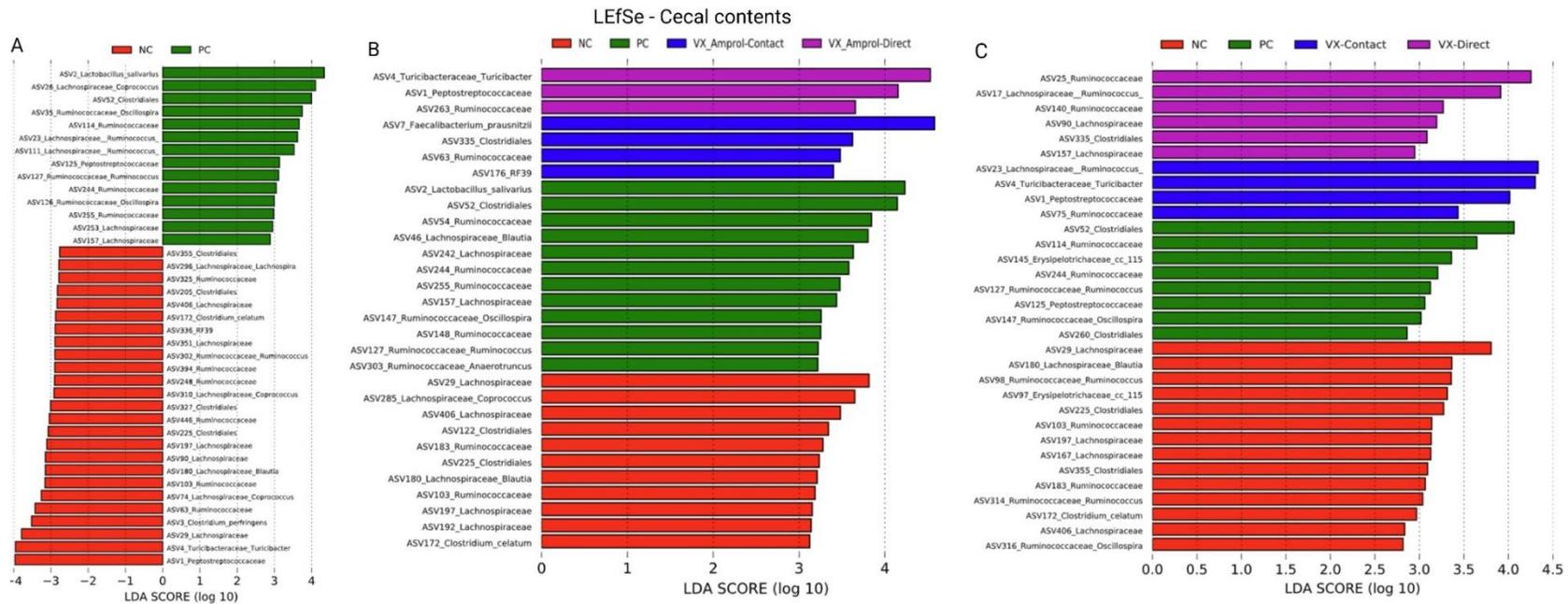


Figure 6. Linear discriminant analysis effect size (LEfSe) on effect post-challenge of *Eimeria meleagridis* vaccination, with and without Amprol intervention, in turkey cecal bacterial populations at the genus level for Negative control (NC) and positive control (PC) (A), PC, VX+Amprol-Contact, and VX+Amprol-Direct (B), and PC, VX-Contact and VX-Direct (C). LEfSe was used to identify the signature bacteria associated with the growth stages and intestinal segments. LDA score >2 was used as a criterion for judging the significant effect size.

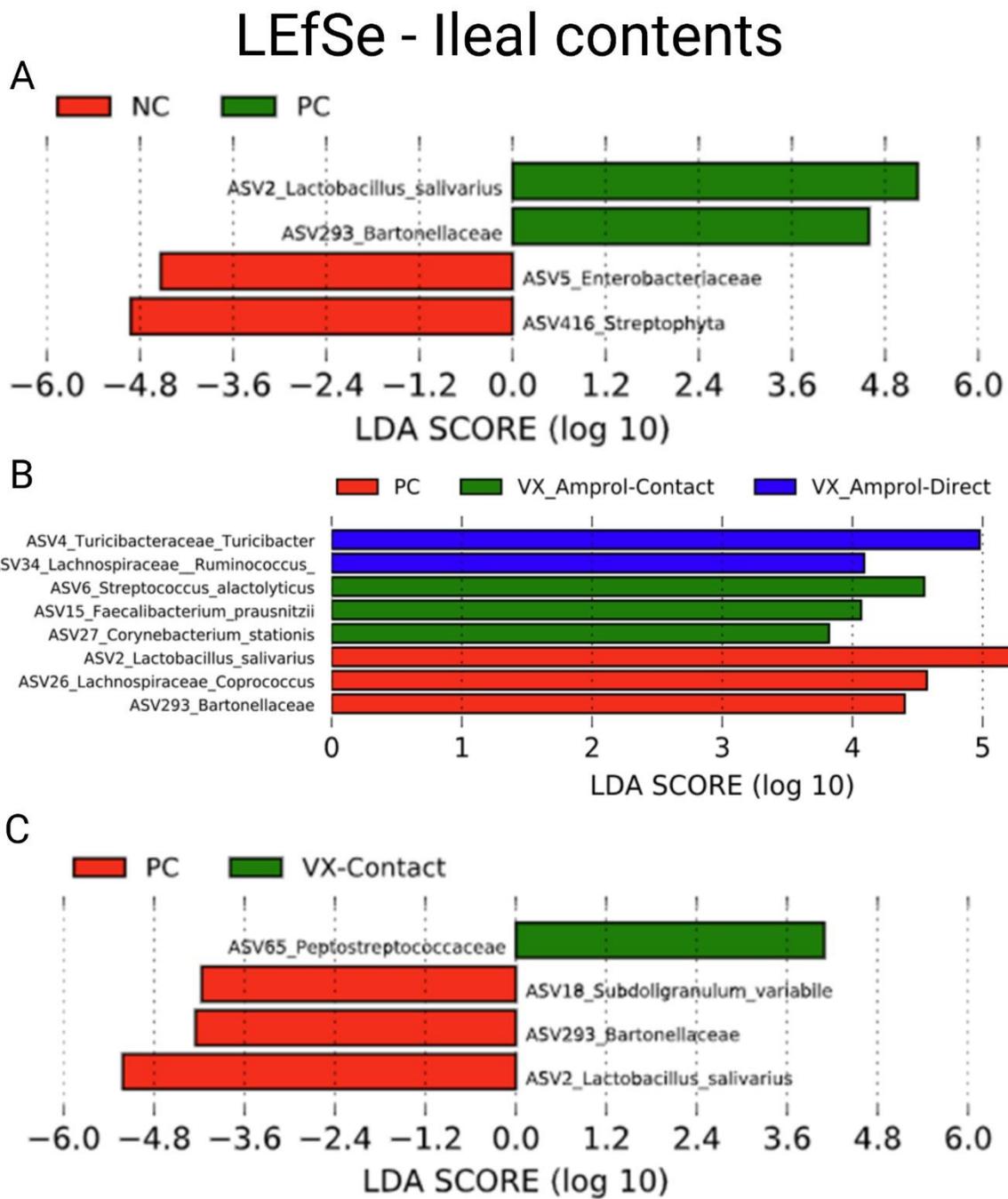


Figure 7. Linear discriminant analysis effect size (LEfSe) for Negative control (NC) and positive control (PC) (A), PC, VX+Amprol-Contact, and VX+Amprol-Direct (B), and PC and VX-Contact (C) in ileal contents at the genus level. LEfSe was used to identify the signature bacteria associated with the ileal contents. LDA score >2 was used as a criterion for judging the significant effect size.

Chapter V: Conclusion

There is currently only one commercially available live coccidiosis vaccine for turkeys and the number of anticoccidial drugs approved for coccidiosis control is limited. Coccidiosis mitigation is difficult due to a lack of effective alternatives, especially when anticoccidial resistance develops. *Eimeria* spp. derived from wild turkeys that are relevant to commercial turkeys could be used to improve coccidiosis control in commercial turkey operations and displace drug-resistant phenotypes in barns. Presently, six of the seven described turkey *Eimeria* spp. were found in fecal samples collected from wild turkeys in the eastern United States. These *Eimeria* spp. have potential to be vaccine candidates used in commercial turkey operations based on the initial research conducted with the *E. meleagritidis* vaccine candidate. More research focused on evaluating the effects of *Eimeria* spp. vaccination and/or challenge and bioshuttle programs on performance, gut integrity, and luminal and mucosal microbiome of turkeys is needed.

Appendix



DIVISION OF AGRICULTURE
RESEARCH & EXTENSION

University of Arkansas System

To: Billy Hargis
Fr: Beth Kegley - Ag-IACUC, co-Chair
Date: May 24th, 2021
Subject: IACUC Approval
Expiration Date: May 20th, 2024

The Division of Agriculture Institutional Animal Care and Use Committee (Ag-IACUC) has APPROVED your protocol # 21117 *Efficacy of drug-sensitive wild turkey Eimeria spp. samples as potential vaccine candidates to prevent coccidiosis in commercial turkeys.*

In granting its approval, the Ag-IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the Ag-IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond May 20th, 2024 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy, the Ag-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-Isaias, Danielle Graham, Christine Vuong, Callie McCreery Selby, Makenly Coles, Roberto Senas Cuesta, Aaron Forga, Lesleigh Beer, Thaina Barros, and Lucas Graham. Please submit personnel additions to this protocol via the modification form prior to their start of work.

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

BMH/tmp