Ascaridia galli: Diagnosis, alternative treatments and influence on cytokine expression, microbiota, egg quality and digestibility

by

Maria Tereza Terra-Long

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Approved by

Ruediger Hauck, Chair, Assistant Professor of Pathobiology and Poultry Science
Bridget A. McCrea, Extension Specialist of Alabama Cooperative Extension System
Dianna V. Bourassa, Associate Professor and Extension Specialist of Poultry Science
Wilmer J. Pacheco, Chair, Associate Professor and Extension Specialist of Poultry Science
Xu Wang, Assistant Professor of Pathobiology

ABSTRACT

Because of the trend of cage-free egg production, infections with the nematode *Ascaridia galli* are receiving increased attention. The aim of the first study was to evaluate and quantify the parasitological challenge in pastured poultry production. Fecal samples from turkeys, broilers and layers were collected in two-week intervals to determine counts of coccidia oocysts and worm eggs. Seasonal differences suggested that higher temperatures might increase worm egg survival in the environment. Counts of coccidia were lower than published numbers in conventionally reared poultry, indicating that the rotation system effectively reduced the infection pressure. However, this was not true for worm eggs. Next-generation sequencing showed the presence of most described *Eimeria* spp. in these birds. The second study established a timeline for the influence of *A. galli* on the expression of key cytokines and on the composition of the jejunal microbiota. Birds were challenged with 500 embryonated *A. galli* eggs for 3 consecutive days starting at 24 days of age. Starting on day 31, three birds of each group were euthanized weekly until 8 weeks post infection (WPI). The number of larvae isolated from the intestinal wall decreased over time. At 5 WPI, there was a numerical upregulation of all cytokines in the infected group compared with the uninfected control and significant differences in the intestinal microbiota between groups. The upregulation of all cytokines evaluated might be the reason for resolution of the infection. A high infection dose and a more nutritionally dense feed might have contributed to the birds' immune system clearing the infection before the worms were able to reach maturity. The third study tested if *Artemisia absinthium* (0.02% in the feed) and pumpkin seed (1% in the feed) can alleviate potential negative effects of an *A. galli* infection on egg production and quality,

nutrient digestibility, cytokine expression and intestinal microbiota. Sixteen-week-old laying pullets were divided into three groups: untreated control, treated with artemisia or pumpkin seed. At 25 weeks, half of the birds were challenged with 250 embryonated *A. galli* eggs. At 35 weeks, birds were euthanized, intestines were checked for worms and samples were collected. For all nutrients, digestibility was lower in challenged birds. Differences in cytokine expression were minor. Jejunal microbiota of treated birds showed an increase on Lactobacillales. None of the treatments were effective against *A. galli*, but had minor effects on alleviating the calcium digestibility reduction. This research provides evidence that nematodes have high prevalence on alternative production systems and tools for controlling the diseases are still needed.

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I. INTRODUCTION

Alternative systems, such as cage-free are expanding with an increasing market demand for products with the least use of drugs. These requirements have the potential of resulting in greater challenges by endoparasites such as *Eimeria* and *Ascaridia galli*. Pasture farms rotate their use of pastures, which can help on the control of these parasites. However, *Eimeria* oocysts and nematodes eggs are very resilient in the environment and can last for years which makes their control more challenging.

A. galli affect the intestine of chickens and can impair their performance resulting in reduction of egg production and quality. It also has the potential to interact with the gut microbiota which may increase the bird's susceptibility to other pathogens. Parasites are known to stimulate the immune system mainly by a Th2-type response. However, in the case of roundworms in chickens, there is no information regarding how this immune response occurs during the development of the larvae.

In the chicken backyard flock community, the use of a variety of plant products against infections of chickens with roundworms is common. This information is anecdotal but might be accurate. Popular treatments include *Artemisia absinthium* and pumpkin seed, popularly known as wormwood. Testing these products scientifically will give backyard owners the information they need to make fact-based decisions on their use.

The research objectives of this work were:

1. To systematically investigate and characterize the parasitological profile of broilers, layers, and turkeys in an alternative management of system.

2. To better understand the immune response (gene expression of key cytokines) and microbiota changes in early and late stages of *A. galli* infections.

3. To determine if raw pumpkins seed and wormwood reduce the worm burden of *Ascaridia galli* in chickens and prevent a reduction in egg production.

4. To investigate if raw pumpkin seed and artemisia prevent possible negative changes in the intestinal microbiota after infection with *A. galli*.

II. LITERATURE REVIEW

INTRODUCTION

Over the last few decades, there have been changes in the market. Consumers are more concerned about leaving a healthy lifestyle and have started to demand more natural products. Tools widely used in agribusiness such as pesticides, growth promoters, anticoccidials and antimicrobials are heavily criticized.

Another concern of consumers is in regard to animal welfare. Inside cages, especially if the density is high, birds can rub against cage walls and each other which can result in damage of their plumage and affect thermoregulation (1). Additionally, depending on the material of the cage's floor, it is common to see overgrown claws that can result in higher risks of birds being trapped and getting hurt (2). Conversely, cage-free birds can express natural behaviors which may give the consumer the perspective of having better living conditions.

Traditional caged layer egg production is being replaced by cage-free production and other alternative system models and one of the reasons for this chance is to satisfy consumer demand (3). In fact, the United States Department of Agriculture's Agricultural Marketing Service predicts that by 2026, 71% of U.S. hens will be housed in these types of systems (4). In the year 2021, there were already 9 states that banned cages for egg-laying hens in the United States. These states are Nevada, Utah, Colorado, Michigan, Washington, Oregon, California, Massachusetts and Rhode Island (5).

CAGE FREE EGG PRODUCTION SYSTEMS

Examples of alternative systems are cage-free, free-range, organic, and pastured productions. Cage-free birds are confined in barns, while free-range animals are raised in barns

with outside access of varying of sizes. The minimum space area is 2 square feet per hen. Organic productions only feed animals with certified organic feed that does not contain animal by-products, antibiotics, or genetically modified grains. On pastured farms, animals are raised on pastures with a rotation system for the majority of their lives (6).

Alternative systems claim to be better for the environment. For example, these systems claim to be more sustainable, and to provide better animal welfare since they allow the birds to exercise natural behavior, such as scratching and foraging for feed on the ground (7,8). The introduction of environmental enrichments, such as elevated resting-places and perches, are growing in the poultry production especially in European countries (9). Additionally, a study was able to show that eggs from pastured-raised layers had twice as much vitamin E and long-chain omega-3 fats compared to conventionally raised eggs (10).

Another common practice seen in alternative productions, as well as the entire poultry industry is the use of nutritional alternatives such as prebiotics, probiotics, organic acids, essential oils, and natural phytochemical extracts. They can potentially improve gut health by changing the microbiota, improving the intestinal barrier, and modulating the immune system especially when used in combination with each other (11,12).

CHALLENGES IN ALTERNATIVE PRODUCTION SYSTEMS

In free-range systems, the mortality rates can come close to 20% during production resulting in considerable economical losses (13). Animals raised in pastures are exposed to predators including coyotes, undomesticated cats, foxes, and eagles (14). Chickens are also exposed to environmental challenges including heat and cold stress that can impact the bird's health by depressing the immune system response and increasing chances of disease (15). This

will not only affect the welfare of the birds but also reduce feed consumption and, consequently, reduce egg quality parameters such as egg weight and eggshell thickness (16).

Likewise, one of the greatest differences between all of the alterative systems and conventionally raised layers is that these birds can come in contact with their feces which increases their chances of infection with endoparasites (17).

ASCARIDIA GALLI

a. Prevalence in poultry systems

Helminthic parasites, such as *Ascaridia galli* and *Heterakis gallinarum*, are commonly found in alternative chicken production systems in the production of chickens (18,19). Chickens are the main host, but turkeys and wild birds can also be infected (20).

The risk of infection can be influenced by different factors. Temperature and humidity are some examples. A study done with organic free-range layers in Germany showed that due to higher temperatures and humidity during the summer season, the risk of infection with nematodes was 50% higher when compared to the risk during the winter (21). Another study evaluating the environmental tolerance of worm eggs also suggested that indoor areas might favor the embryonation of eggs which can potentially increase the risks of birds being infected (22). However, a study comparing barn system (cage-free) with conventional free-range and organic systems did not see significant differences in nematode prevalence between these different housing systems (23).

Additionally, it is intuitive to assume that birds that are kept in cages have lower prevalence of nematodes since they do not come in direct contact with feces. In battery cages, prevalence rates vary from 2.4 to 4.3% (24) while the prevalence of roundworms in cage-free production can range from 69.5% infected (22) to nearly 90% (25).

b. Life cycle of *Ascaridia galli*

Adult forms of *A. galli* are yellowish-white and there is sexual dimorphism in which females can measure between 60 and 116 mm in length while males can measure between 50 and 76 mm. The eggs are 71 to 92 micrometers by 45 to 57 micrometers. The shell of the worm eggs is smooth and thick. *A galli* worm eggs can survive in the external environment for periods longer than one year (26).

The birds are infected by the ingestion of embryonated eggs present in feces containing L2 larvae. After ingestion, the eggs release the larvae L2 that will migrate to the mucosa of the jejunum (27). There, they will settle and develop into larvae stage L3. Between 18 to 30 days later, these larvae molt becoming adult worms. The histotrophic phase can last up to 7 weeks and adults will migrate to the lumen and start laying eggs (28,29).

Adults are free in the lumen and can be found from 4 weeks post-infection (P.I.). Egg shedding in chicken feces is expected to start 5 weeks P.I. These eggs contain immature larvae that need 14 to 30 days in the environment to become infective (30). The embryonation is affected by oxygen availability, humidity, and temperature conditions. For sufficient development to occur, it is necessary humidity levels above 70% and aerobic conditions (22). Moreover, environmental temperatures around 28 to 30° Celsius will result in shorter incubation periods than temperatures at 15°C (31).

Other studies have described the possibility of an erratic migration of adults where adults move along the intestinal tract until they reach the cloaca where they ascend the oviduct until the shell gland. If the egg is decalcified when this migration occurs, the worm can settle in it and stay alive or be calcified. Such migration brings not only an unpleasant surprise to the consumer, but these worms can mechanically carry fecal microorganisms with zoonotic potential (32–35).

An atypical case was also described of roundworms present in the proventriculus and gizzard causing proventriculitis in a poultry bird. The lesions seen in histopathology showed denuded epithelium of coating, subepithelial hemorrhage associated with fibrosis and inflammatory infiltrate of monocytes (36).

c. Clinical signs, Lesions and Histopathological findings

Ascariasis infections are usually asymptomatic. In severe cases, it is possible to notice clinical signs such as cachexia, reduction in growth rates and increase of mortality depending on the severity of the disease (37). At necropsy, adult worms can be found in the intestine, can obstruct partially or totally the lumen, and mixed infections with different parasites are common (38). Histopathology shows a mild to moderate inflammatory infiltrate of eosinophils (acute phase) or mast cells (chronic phase) in the lamina propria. The mucosa can be thickened (edema) and may contain inserted larvae (29).

Regarding egg production, studies have shown contradictory results. While Tarbiat at al., (2020) have shown that birds infected with *A. galli*, left untreated or with a non-strategic use of anthelmintics, had reduced egg production per bird, egg mass and feed conversion (39), other authors did not see significant differences between control and infected groups (26,42).

Since larvae and adults are found in the jejunum, *A. galli* can potentially affect nutrient digestibility and absorption. A study evaluating the pathogenicity of ascaridiosis in turkeys suggested that roundworms may produce toxins that are capable of reducing the bird's capacity to absorb nutrients (43). In fact, a reduction of 4% of dietary metabolizable energy has been reported in chickens infected with *A. galli* (44).

d. Immunology

Throughout their life cycle, roundworms are immunogenic and affect both cellular and humoral pathways stimulating and inhibiting an immune response (45–47). Infections with *A. galli* have been associated with an increase of T cell populations in the lamina propria as well as an accumulation of CD4+ lymphocytes in the epithelium (47)**.**

Helminth infections are known to induce Th2 helper cell-based cytokine responses with upregulation of IL-13 and IL-4 locally (48). This response is triggered mainly by the attachment of larvae in the mucosa in the first weeks and the migration of young adults to the jejunal lumen. A study in Denmark investigated the systemic immune response at different time points after an induced *A. galli* infection confirmed this stimulation of a Th2 response and showed an up regulation of DEF β-1 (antimicrobial peptide) caused by initial larval migration. Additionally, in this study, the authors found that, at later stages of the infection (6 weeks post infection), there was first an upregulation of pro-inflammatory cytokines followed by a downregulation of Th1 response related cytokines such as INF-γ at 9 weeks post infection (49).

Interestingly, the downregulation of Th1 response is something commonly seen in helminth infections in both mammals as well as avian species. This is known as Th1/Th2 polarization of the immune system (45,50,51). This inhibition of Th1 response caused by helminths has been investigated in other species such as *Ascaris suum* and studies suggested that proteins from these parasites could potentially have therapeutic applications in autoimmune diseases (52). Moreover, some studies already demonstrated an association between chronic infection with nematodes and reduced vaccine response, which shows again the downregulation of Th1 response caused by worms (53,54).

Worm infections can also influence the expression of other cytokines related to immune modulation. The upregulation of IL-10 and TGF-β normally occurs locally in the early stages of the infection in order to reduce the immune response and preventing excessive damage caused by the inflammation process (45,48,55,56).

Regarding to humoral response, Marcos-Atxutegi et al. (2009) showed that *A. galli* (larvae and adults) can stimulate a local and intense inflammation process as well as a systemic humoral immune response (IgG antibodies). These antibodies were detected in the blood and egg-yolk (46). Another study evaluating possible maternal protection against *A. galli* also found antibodies in the yolk. However, there was no significant differences in worm burden between the offspring from an infected and an uninfected hen (57). Systemic IgY antibodies were also detected in infected birds which illustrates a humoral response. However, the correlation with the number of worms detected was weak which suggested that this humoral response does not protect birds against infection (47).

e. Diagnosis

There are direct and indirect tools to diagnose an infection with roundworms. Anorexia and depression can be suggestive, and, in cases of severe infection, adult worms can be seen in feces. Another non-invasive diagnostic tool is the parasitological evaluation of feces under the microscope for identification of worm eggs using a McMaster chamber (58). The eggs of *A. galli* and *Heterakis* are very similar to each other (59) and differentiation between them under the microscopic can be hard for a untrained individuals. Postmortem examination of the jejunum (60) and the identification of adult worms is also a simple test to be done, and histopathology can be used for identification of larva in the intestinal wall (29).

An ELISA can also be used as an indirect tool to identify hens infected with roundworms as well as to quantify levels of antibodies (46). The ELISA can be done with blood samples or egg yolk and is a specific and non-invasive technique that can give information on which roundworm is infecting the animal (61).

Additionally, molecular tools such as PCR and sequencing of DNA extracted from worm eggs, larvae or adults are options as diagnostic tools that are highly specific. They can be used for differentiation of worm species as well as for population studies of parasite's dynamics (62). Another molecular technique that was recently validated as a tool of diagnostic, quantification, and differentiation between *A. galli* and *H. gallinarum* is the Droplet Digital™ PCR (ddPCR) which is a technology for DNA quantification that does not need standard curves. This characteristic makes it possible for direct comparisons of infections to be measured in different laboratories possible (63).

f. Treatment

While several classes of antiparasitic drugs are effective against *A. galli* such as levamisole (64) and ivermectin (65), in the United States, the only pharmacological base, with a withdrawal time for eggs of zero-days, registered by the Food and Drug Agency and approved to be used in commercial poultry is fenbendazole. The restriction of only being able to use benzimidazoles-pharmaceuticals against helminthics is also true in the European Union where cage production systems were completely banned, and where diverse studies demonstrated high prevalence of roundworms (21,25,37). Benzimidazoles are known to bind to β-tubulin inhibiting microtubules formation. As a result of that, it will cause instability of the muscular skeleton and affecting mobility as well as disruption of any microtubule-based process such as mitosis and spermatogenesis in adult worms (66).

Since there is only one option of treatment available for animals in production, there are concerns regarding resistance against fenbendazoles (67). Even though a study done in 2017 did not show resistance to this drug by *A. galli* (68), other parasites in different species have experienced this. In sheep, genes of resistance against benzimidazole were identified in *H. contortus* (69). In turkeys, one random isolate of *A. dissimilis*, out of four tested, showed resistance against fenbendazole (70).

Finally, although resistance has not yet been reported, there are studies showing that the effect of deworming does not last, and it is not rare to see the necessity of multiple deworm treatments on the same flock (71). This is because these drugs are capable of eliminating the parasites in the birds, however, if this treatment is initiated late, the environment will already be contaminated with eggs that were shed which results in high contamination load and increases chances of re-infection soon after treatment (72).

g. Prevention

The control of worms can be complex and challenging. Due to the high tenacity of worm eggs in the environment, infections with roundworms are difficult to control with biosecurity and good sanitation alone. For these reasons, routine use of anthelmintics is still considered the most effective strategy against *A. galli* (73). However, this is not an option in organic flocks since this type of production does not allow the use of drugs. Moreover, in flocks that can use anthelmintics there are limitations on drug options. This can increase the selection pressure and, consequently, increases the chances of resistance against these anthelmintics. Another strategy that could be used to reduce environmental parasitic load over time is to frequently monitor worm eggs shedding through fecal exams and establish a threshold for starting treatment (74).

Additionally, there are no vaccines that have been shown to be efficient against nematodes. This is possibly explained due to the fact that the parasite has different life stages, and it stays in the lumen and mucosa regions of the jejunum which are places of limited access for effector cells (75). A study published in 2013 was able to induce a humoral and cell-mediated immune response in birds injected with an extract of the roundworm. However, this response was not able to protect birds against challenges (76).

Other strategies that can be used are the use of single natural compounds or combinations, known as nutritional alternatives to growth promoters. They have the potential to modulate the microbiota and the immune system (77,78). More specifically, plant-based compounds are being studied and can be utilized on production system that cannot use drugs such as organic.

CHICKEN INTESTINAL MICROBIOTA

a. Microbiota composition

The term microbiota refers to all microorganisms present in an environment and includes viruses, bacteria, fungi, and protozoa (79). The chicken gut microbiota is a very complex environment, and studies have shown that different sections of the gut have clear differences in the relative abundance and absolute numbers of bacteria (80,81).

The most common phyla in duodenum, jejunum, ileum, and colon are Firmicutes which includes the orders Lactobacillales, Bacillales and Clostridiales. The most common phyla in the cecum is Bacteroidetes, which includes the families *Bacteroidales, Flavobacteriales* and *Sphingobacteriales*. Cyanobacteria is also commonly found in jejunum samples, while they are rare in cecum (81,82).

b. Interaction between *A. galli* and the microbiota

Microorganisms interact with each other, and their compositions can be affected by changes in the environment. This can be caused by exogenous bacteria, parasites or viruses as well as diet changes and the immune system (83–85). These changes can result in an imbalance known as dysbiosis which gives the opportunity for pathogens to cause diseases.

More specifically, possible interactions between *Ascaridia galli* and bacterial microbiota where also investigated in a few studies. Research evaluating the interaction between *A. galli* and *Pasteurella multocida* concluded that birds had worse performance (lower weight gain and egg production) whenever they were infected with both pathogens compared to the birds infected with only one. Moreover, an infection with *A. galli* followed by *P. multocida* resulted in more severe pathological lesions then the opposite order of infection. These results are suggestive of a synergistic effect. Still, the mechanism behind these interactions have not been yet demonstrated. (86).

Another study showed that an infection with *A. galli* can also reduce the diversity and numerical abundance of intestinal microbiota in hens. These changes can be caused not only by the presence of adults, but also larvae as well (87). Finally, the Animal Health Laboratory's newsletter from the University of Guelph showed that a large number of *Ascaridia galli* larvae acted as a predisposing factor for the occurrence of necrotic enteritis in broiler breeder chickens (88).

c. Methods of investigating the chicken microbiota

The chicken gut microbiome is a highly diverse environment with more than 900 microbial species already identified (89). This is due to of the advancements and popularization of molecular tools. Initial microbiota studies relied on traditional culturing methods (90). This can bring

information about few microbes, but most of gut bacteria are not culturable or have extremally low abundance. Because of that, techniques such sequencing of the 16S gene of bacteria became popular given that there is an amplification step in its workflow.

The 16S rRNA is a highly conserved gene in all prokaryotic organisms. This makes it well suitable as a target for gene sequencing in samples from complex biological environments such as soil, water, and gut microbiome (91). Another important characteristic of the 16 S gene is that it has 8 highly conserved regions and 9 variables regions (92). The conserved regions of the gene are the target for primer design. Using these regions as primers also offers the possibility of using a single pair of primers to amplify a large number of diverse individuals that will be differentiated through the variable regions (93,94).

Taxonomic analysis is performed by comparing the results with other available databases such as SILVA, Greengenes and NCBI 16S microbial data bases. The diversity of communities can be measured within each individual sample (alpha diversity) or between the samples (beta diversity) (95). The use of molecular tools, at the same time, improved the knowledge we have regarding the diversity of the different intestinal sections, and have the potential to be a great tool to investigate how we can modulate the gut microbiome thus ensuring intestinal health.

ALTERNATIVE APPROACHES TO DRUGS

The banning of caged systems in Europe, as well as in some states in the United States can illustrate how consumer behavior can influence poultry production. One of most obvious consequence of these changes seen in the past 10 years is the increase in prevalence of helminths (24,58). Nutritional alternatives are being investigated and have been potential improvements on gut health by interfering on the microbiota population, improving the intestinal barrier, and

modulating the immune system. Some examples of these strategies are probiotics, prebiotics, organic acids, essential oils, and natural phytochemical extracts in poultry diets (96,97).

a. **Prebiotics and Probiotics**

Probiotics are microbes fed to animals in order to promote gut health. The most common probiotics are *Bacillus*, *Lactobacillus*, *Enterococcus* and *Bifidobacterium* (77,98). These bacteria can reduce the colonization of pathogenic bacteria by competitive exclusion, modulating gut-associated immune cells and macrophages or producing bacteriocins. The can also generate toxic microbial fermentation products that reduces harmful bacteria viability in the gut such as volatile fatty acids, ethanol, and organic acids (99–101).

Prebiotics can modulate the gut microbiota by stimulating the beneficial bacteria. Examples of prebiotics are inulin, fructo-oligosaccharides (FOS), mannan-oligosaccharides (MOS), and xylo-oligosaccharides (102–104). There are also products called symbiotics which are a combination of prebiotics and probiotics. The symbiotics are a combination of a prebiotic and a probiotic. They are known to bring more benefits to birds than the two products alone since the use of them together can increase the chances of the probiotic's survival and modulate the gut microbiota composition (105,106).

b. Essential oils

Essential oils such as oregano, carvacrol, thymol, and cinnamaldehyde are another alternative for antimicrobial drugs. They can stimulate immune cells and promote upregulation of cytokines such as INF-gamma and IL-6 which might help birds challenged with *Eimeria tenella*, for example (107). It is also common to find blends of organic acids such as benzoic and essential oils that have been proven to improve feed conversion ratio and carcass yield (108,109).

c. Plant extracts

Plant-based extracts are potential alternatives to drugs and have been used for years in human medicine for the control of not only parasites but also fever, liver disease and cancer (110–113). These plant extracts have a diversity of compounds with anti-inflammatory properties, but the mechanisms of action of most of them are still being characterized and studies that focus on host-microbe interaction are needed (114). More specifically in this work, two phytogenics commonly used by backyard producers with the potential to be used against *Ascaridia galli* were tested: *Artemisia absinthium* and Pumpkin seeds.

i. *Artemisia absinthium*

Artemisia absinthium is an herb that has cut-out gray leaves and a remarkably bitter taste. There are reports of its use as a medicinal plant, more specifically as an antihelminth, dated around 3500 B.C. in Egypt. Because of that, Artemisia's commercially used name is wormwood (115).

Many *Artemisia* spp. have shown potential to be used against a number of different parasites such as malaria (116,117) and *Eimeria* in chickens and *Heterakis gallinarum* in turkey poults. Birds infected with *Eimeria* and treated with *Artemisia afra* had similar feed conversion to birds treated with toltrazuril (118) while chickens treated with *Artemisia annua* powder had significant reduction on oocysts shedding and lesion score compared to the control group (119). A crude aqueous extract of *Artemisia herba-alba* was compared to albendazole against *Heterakis gallinarum* infecting turkeys and showed no statistical differences in the reduction of egg output and worm burden (120).

The efficiency of *Artemisia absinthium* as an alternative treatment can be variable and, since it is a natural product, there are a lot of variables that can contribute to the different results. An investigation of the direct effect of aqueous and ethanolic extracts of wormwood *in vitro* in

adult worms showed significant paralysis and death caused by both extracts and, when these extracts were orally administrated (2.0 g kg^{-1} body weight) to small ruminants, there was a significant reduction on egg shedding that was also similar to the albendazole treated group (121). On the other hand, Mravčáková et al. (2020) found different results against the same worm in lambs. When evaluating the effect of wormwood, mallow, and a mix, they found an *in vitro* antihelminth effect demonstrated by the reduction on egg hatchability. However, this strong anthelmintic effect was not fully confirmed *in vivo* (122).

Artemisia absinthium can also have a synergic effect with other feed additives increasing animal performance. A study concluded that an inclusion of 4.5% with 0.60% of exogenous fibrolytic enzymes can contribute to better nutrient digestibility and feed intake which resulted in lower cost of wool production (123).

Artemisia absinthium has compounds that have been correlated with anti-helminth activities. These are terpenes, limonene, myrcene, α and β thujone, the sesquiterpenes, caryophellene, sabinyl acetate, chrysanthenyl and tannis (113,124). The mechanism of action of most of these compounds are still being characterized and, because of that, both *in vitro* and *in vivo* studies are needed for better understanding. What is known is that artemisia has an immuno-modulatory effect and can reduce proinflammatory mediators' expression and activating CD40 expression on dendritic cells (DCs) in mice (125). Likewise, there are *in vitro* studies showing that α and β thujone can potentially decrease juvenile (L3) larval motility and development of eggs against *Haemonchus contortus*.

One concern regarding *A. absinthium* is that it can be toxic if administered during long periods of time because of the presence of thujones or in higher doses (126). Some of the symptoms of intoxication include central nervous system disorders, hallucinations, vomiting and dizziness

(127). The mechanism behind neurological symptoms is due to the antagonism between thujone and the Gamma-aminobutyric acid (GABAA) receptor which can trigger an epileptic-like convulsion (128).

The effect of *A. absinthium* was tested along with another eight plant extracts against *Ascaridia galli* eggs *in vitro.* These methanolic extracts were compared to flubendazole. Although the flubendazole group was the one with the best results, all extracts significantly reduced the embryonic development when compared to the control group (129).

ii. Pumpkin seed

Pumpkins (*Cucurbita maxima*) are part of the family Cucurbitaceae. Pumpkin products stand out as they are used worldwide as a natural vermifuge, and its seeds are known to have anthelmintic properties when used in humans and animals. In the literature, pumpkin seeds have been shown to have anthelmintic efficacy in different hosts such as ostriches and mice. At a concentration of 1g/kg live weight orally administered, pumpkin seed meal was shown to be effective in the control of a mixed infection of ostrich nematodes (*Libyostrongylus douglassii*, *C. struthionis* and *Libyostrongylus dentatus*) resulting in reduction of egg shedding (130). A study done in naturally infected mice with *Aspiculuris tetraptera* compared water and ethanol extract of pumpkin seeds orally administered with ivermectin and showed a percentage of efficacy of 81, 85 and 91% when compared with the negative control, respectively (131).

Pumpkin seed also was effective against *Heligmosoides bakeri*, a human parasite, both *in vitro* and *in vivo*. The study compared hot water extract, cold water extract and ethanol extract and showed that all extracts were effective against larval stages and the ethanol extract also reduced egg hatchability and worm motility (132).

Pumpkin seeds have few active components such as flavonoids, terpenes, saponins and phydroxybenzoic acid that can affect the immune response, but the most studied ones are cucurbitine and cucurbitacin (133). *In vitro* studies of these substances have shown they can affect survival of larvae and have inhibitory properties against worm motility. The results of a study published in 2009 testing the anthelmintic of *Curcubita pepo* against *Hymenolepis nana* (popularly known as dwarf tapeworm and capable of infecting both humans and rodents) showed that pumpkin seed remedies not only damaged worm eggs but were also able to reduce egg shedding. This can potentially contribute to a reduction of contamination of the environment (134) and control of the parasite. Pumpkin seeds also have high levels of fiber which can affect intestinal motility and might assist in the expulsion of adults (135).

Recent studies evaluated the effect of pumpkin seeds on *A. galli.* The results of Acorda et al. (2019) indicate that pumpkin seeds have moderate efficiency in the reduction of worm counts when compared to mebendazole (136). Another study done with native chickens naturally infected with a mixed infection of *A. galli, Heterakis* and *Raillietina* also showed significant reduction on worm burner and egg shedding numbers (137).

A study published in 2018 with pumpkin seeds ethanolic extract was done *in vitro* and *in vivo*. On the former, the extract had similar effects to fenbendazole and, on the later, worms extracted from chickens treated with pumpkin had the same mortality rate as the ones from the positive control group for 48 h. The extract concentration was 2000 mg/kg while the positive control was 100 mg/kg of fenbendazole. The authors also pointed out that the efficacy was time and concentration dependent and that future studies are needed to determine optimal dosage (138).

Alternative production systems are continuing growing which can results and increasing on challenges with endoparasites. In order to confirm this, studies are still needed evaluating and

quantifying the prevalence of parasites in all different productions. *A. galli* is one of these endoparasites and its control can be challenging with urges for a better understanding of the immune response as well the parasite's impact on the intestinal microbiota, and the investigation of alternative treatments.

REFERENCES

1. Hughes BO, Black AJ. Battery cage shape: Its effect on diurnal feeding pattern, eggshell cracking and feather pecking. *British Poult Sci.* 17:327–336; 1976. doi:10.1080/00071667608416283.

2. Tauson R. Health and production in improved cage designs. *Poult Sci.* 77:1820–1827; 1998. doi:10.1093/ps/77.12.1820.

3. Thapa S, Thamsborg SM, Wang R, Meyling NV, Dalgaard TS, Petersen HH, Mejer H. Effect of the nematophagous fungus *Pochonia chlamydosporia* on soil content of ascarid eggs and infection levels in exposed hens. *Parasit & Vect.* 112018. doi:10.1186/s13071-018-2898-1.

4. United Egg Producers. U.S. Egg Production and Hen Population. *Facts & Stats.* [accessed 2022 Oct 1]. https://unitedegg.com/facts-stats/; 2021.

5. Fatka J. Nevada joins list of states mandating cage-free eggs. *Farm Progress.* [accessed 2022 Oct 1]. https://www.farmprogress.com/farm-policy/nevada-joins-list-states-mandating-cage-free-eggs; 2021.

6. Human Farm Animal Care. *Egg Laying Hens: Human Farm Animal Care standards for production*. 1-44. [accessed 2022 Oct 1]. https://2gn8ag2k4ou3ll8b41b7v2qp-wpengine.netdna-ssl.com/wp-content/uploads/Std19.Layers.4H-1.pdf; 2018.

7. Sossidou EN, Dal Bosco A, Castellini C, Grashorn MA. Effects of pasture management on poultry welfare and meat quality in organic poultry production systems. *World's Poult Sci J.* 71:375–384; 2015. doi:10.1017/S0043933915000379.

8. Koelkebeck KW, Amoss MS, Cain JR. Production, physiological, and behavioral responses of laying hens in different management environments1. *Poult Sci.* 66:397–407; 1987. doi:10.3382/ps.0660397.

9. Jones BR. Role of comparative psychology in the development of effective environmental enrichment strategies to improve poultry welfare. *Inter J Comp Psych.* 15: 77-106; 2002.

10. Karsten HD, Patterson PH, Stout R, Crews G. Vitamins A, E and fatty acid composition of the eggs of caged hens and pastured hens. *Renew Agri and Food Sys.* 25:45–54; 2010. doi:10.1017/S1742170509990214.

11. Alcicek A, Bozkurt M, Cabuk M. The effect of a mixture of herbal essential oils, an organic acid or a probiotic on broiler performance. *South Afri J of Ani Sci.* 34:217–222; 2004. doi:10.10520/EJC94382.

12. Sadeghi E, Akhondzadeh Basti A, Noori N, Khanjari A, Partovi R. Effect of *Cuminum Cyminum* L. essential oil and *Lactobacillus Acidophilus* (a Probiotic) on *Staphylococcus Aureus* during the manufacture, ripening and storage of white brined cheese. *J of Food Proc and Preserv.* 37:449–455; 2013. doi:10.1111/j.1745-4549.2011.00664.x.

13. Jeni RE, Dittoe DK, Olson EG, Lourenco J, Seidel DS, Ricke SC, Callaway TR. An overview of health challenges in alternative poultry production systems. *Poult Sci.* 100:101173; 2021. doi:10.1016/j.psj.2021.101173.

14. Bestman M, Bikker-Ouwejan J. Predation in organic and free-range egg production. *Animals* 10:177; 2020. doi:10.3390/ani10020177.

15. Mashaly MM, Hendricks GL, Kalama MA, Gehad AE, Abbas AO, Patterson PH. Effect of heat stress on production parameters and immune responses of commercial laying hens1. *Poult Sci.* 83:889–894; 2004. doi:10.1093/ps/83.6.889.

16. Ebeid TA, Suzuki T, Sugiyama T. High ambient temperature influences eggshell quality and calbindin-D28k localization of eggshell gland and all intestinal segments of laying hens. *Poult Sci.* 91:2282–2287; 2012. doi:10.3382/ps.2011-01898.

17. Jansson DS, Nyman A, Vågsholm I, Christensson D, Göransson M, Fossum O, Höglund J. Ascarid infections in laying hens kept in different housing systems. *Avian Pathol.* 39:525–532; 2010. doi:10.1080/03079457.2010.527923.

18. Brener B, Tortelly R, Menezes RC, Muniz-Pereira LC, Pinto RM. Prevalence and pathology of the nematode Heterakis gallinarum, the trematode *Paratanaisia bragai*, and the protozoan *Histomonas meleagridis* in the turkey, *Meleagris gallopavo*. *Mem Inst Oswaldo Cruz* 101:677–681; 2006. doi:10.1590/S0074-02762006000600017.

19. Dauda J, Lawal JR, Bello AM, Mustapha M, Ndahi JJ, Biu AA. Survey on prevalence of gastrointestinal nematodes and associated risk factors in domestic turkeys (*Meleagris Gallopavo*) slaughtered in poultry markets in Bukuru – Jos, Plateau State, Nigeria. *Int J Inn Agri & Bio Res.* 4:27-36. 2016.

20. Snabel V, Permin A, Magwisha HB, Suo X, Varady M, Tomasovicova O. On the species identity of *Ascaridia galli* (Schrank, 1788) and *Ascaridia dissimilis* (Perez Vigueras, 1931): A comparative genetic study. *Helminth.* 38,4:221-226. 2001.

21. Kaufmann F, Daş G, Sohnrey B, Gauly M. Helminth infections in laying hens kept in organic free-range systems in Germany. *Livestock Sci.* 141:182–187; 2011. doi: 10.1016/j.livsci.2011.05.015.

22. Tarbiat B, Jansson DS, Höglund J. Environmental tolerance of free-living stages of the poultry roundworm *Ascaridia galli*. *Vet Parasitol.* 209:101–107; 2015. doi: 10.1016/j.vetpar.2015.01.024

23. Zloch A, Kuchling S, Hess M, Hess C. Influence of alternative husbandry systems on postmortem findings and prevalence of important bacteria and parasites in layers monitored from end of rearing until slaughter. *Vet Recor.* 182:350–350; 2018. doi:10.1136/vr.104632.

24. Jansson DS, Nyman A, Vågsholm I, Christensson D, Göransson M, Fossum O, Höglund J. Ascarid infections in laying hens kept in different housing systems. *Avian Pathol.* 39:525–532; 2010. doi:10.1080/03079457.2010.527923.

25. Wuthijaree K, Lambertz C, Gauly M. Prevalence of gastrointestinal helminth infections in free-range laying hens under mountain farming production conditions. *Br Poult Sci.* 58:649–655; 2017. doi:10.1080/00071668.2017.1379049.

26. Baptista, A. *Perfil parasitológico em frangos do campo* [dissertation]. [Lisboa (Portugal)]. Universidade Técnica de Lisboa; 2010.

27. Sossidou EN, Dal Bosco A, Elson HA, Fontes CMGA. Pasture-based systems for poultry production: implications and perspectives. *World's Poult Sci J.* 67:47–58; 2011. doi:10.1017/S0043933911000043.

28. Ferdushy T, Nejsum P, Roepstorff A, Thamsborg SM, Kyvsgaard NC. *Ascaridia galli* in chickens: intestinal localization and comparison of methods to isolate the larvae within the first week of infection. *Parasitol Res.* 111:2273–2279; 2012. doi:10.1007/s00436-012-3079-3.

29. Luna-Olivares LA, Kyvsgaard NChr, Ferdushy T, Nejsum P, Thamsborg SM, Roepstorff A, Iburg TM. The jejunal cellular responses in chickens infected with a single dose of *Ascaridia galli* eggs. *Parasitol Res.* 114:2507–2515; 2015. doi:10.1007/s00436-015-4450-y.

30. McDougald LR. Internal parasites. In: Swayne DE, Boulianne M, Logue CM, McDougald LR, Nair V, Suarez DL, editors. *Diseases of poultry*. Ames (IA): Wiley-Blackwell. p. 1157–1191; 2020.

31. Onyirioha, JNN. Effects of varying temperatures on the ex-uterine development and incubation period of eggs of *Ascaridia galli*. *New York Sci J.* 4, 61-63; 2011.

32. Hall, GO. A large roundworm Ascaridia lineata found in egg of fowl. *Poult Sci*. *24*(6), 496-498; 1945.

33. Hodasi JKM. A Note on *Ascaridia Galli* in a hen's egg from Manitoba. *Can J Comp Med Vet Sci.* 27:100–102; 1963.

34. Reid WM, Mabon JL, Harshbarger WC. Detection of Worm Parasites in chicken eggs by candling. *Poult Sci.* 52:2316–2324; 1973. doi:10.3382/ps.0522316.

35. Machado HHS, Lemos LDS, Almeida LGD. Ciclo erratico de *Ascaridia galli* (SCHRANK, 1788) em ovo de galinha. *Br Ani Sci.* 8:147-149; 2007.

36. Brar RS, Kumar R, Leishangthem GD, Banga HS, Singh ND, Singh H. *Ascaridia galli* induced ulcerative proventriculitis in a poultry bird. *J of Parasitic Dis.* 40:562–564; 2016. doi:10.1007/s12639-014-0509-4.

37. Hinrichsen LK, Labouriau R, Engberg RM, Knierim U, Sørensen JT. Helminth infection is associated with hen mortality in Danish organic egg production. *Vet Rec.* 179:196; 2016. doi:10.1136/vr.103614.

38. Stehr M, Sciascia Q, Metges CC, Gauly M, Daş G. Co-expulsion of *Ascaridia galli* and *Heterakis gallinarum* by chickens. *Int J for Parasitol.* 48:1003–1016; 2018. doi: 10.1016/j.ijpara.2018.05.014.

39. Tarbiat B, Jansson D, Wall H, Tydén E, Höglund J. Effect of a targeted treatment strategy against *Ascaridia galli* on egg production, egg quality and bird health in a laying hen farm. *Vet Parasitol.* 286:109238; 2020. doi: 10.1016/j.vetpar.2020.109238.

40. Gauly M, Duss C, Erhardt G. Influence of *Ascaridia galli* infections and anthelmintic treatments on the behaviour and social ranks of laying hens (Gallus gallus domesticus). *Vet Parasitol.* 146:271–280; 2007. doi: 10.1016/j.vetpar.2007.03.005.

41. Sherwin CM, Nasr MAF, Gale E, Petek M, Stafford K, Turp M, Coles GC. Prevalence of nematode infection and faecal egg counts in free-range laying hens: relations to housing and husbandry. *British Poult Sci.* 54:12–23; 2013. doi:10.1080/00071668.2012.757577.

42. Sharma N, Hunt PW, Hine BC, Sharma NK, Swick RA, Ruhnke I. Detection of *Ascaridia galli* infection in free-range laying hens. *Vet Parasitol.* 256:9–15; 2018. doi: 10.1016/j.vetpar.2018.04.009.

43. Vasilev I, Osikovski E, Bazhkov S, Kamburov P, Bankov I, Rupova L. On the pathogenesis of ascaridiosis in fowl. *Izvestiia;* 1973.

44. Walker TR, Farrell DJ. Energy and nitrogen metabolism of diseased chickens: Interaction of *Ascaridia galli* infestation and vitamin a status. *British Poult Sci.* 17:63–77; 1976. doi:10.1080/00071667608416251.

45. Degen WGJ, Daal N van, Rothwell L, Kaiser P, Schijns VEJC. Th1/Th2 polarization by viral and helminth infection in birds. *Vet Microbiol.* 105:163–167; 2005. doi: 10.1016/j.vetmic.2004.12.001.

46. Marcos-Atxutegi C, Gandolfi B, Arangüena T, Sepúlveda R, Arévalo M, Simón F. Antibody and inflammatory responses in laying hens with experimental primary infections of *Ascaridia galli*. *Vet Parasitol.* 161:69–75; 2009. doi: 10.1016/j.vetpar.2008.12.011.

47. Schwarz A, Gauly M, Abel H, Daş G, Humburg J, Rohn K, Breves G, Rautenschlein S. Immunopathogenesis of *Ascaridia galli* infection in layer chicken. *Dev Comp Immunol.* 35:774–784; 2011. doi: 10.1016/j.dci.2011.02.012.

48. Pleidrup JA, Norup LR, Dalgaard TS, Kaiser P, Permin A, Schou TW, Vadekær DF, Jungersen G, Sørensen P, Juul-Madsen HR. Cytokine gene expression profiles in chicken spleen and intestinal tissues during *Ascaridia galli* infection. *Vet Parasitol.* 206:317–321; 2014. doi: 10.1016/j.vetpar.2014.10.016.

49. Dalgaard TS, Skovgaard K, Norup LR, Pleidrup J, Permin A, Schou TW, Vadekær DF, Jungersen G, Juul-Madsen HR. Immune gene expression in the spleen of chickens experimentally

infected with *Ascaridia galli*. *Vet Immunol Immunopathol.* 164:79–86; 2015. doi: 10.1016/j.vetimm.2015.01.003.

50. McKee AS, Pearce EJ. CD25+CD4+ Cells Contribute to Th2 Polarization during Helminth Infection by Suppressing Th1 Response Development. *The J of Immunol.* 173:1224–1231; 2004. doi:10.4049/jimmunol.173.2.1224.

51. Pleidrup J, Dalgaard TS, Norup LR, Permin A, Schou TW, Skovgaard K, Vadekær DF, Jungersen G, Sørensen P, Juul-Madsen HR. *Ascaridia galli* infection influences the development of both humoral and cell-mediated immunity after Newcastle Disease vaccination in chickens. *Vaccine* 32:383–392; 2014. doi: 10.1016/j.vaccine.2013.11.034.

52. Arora P, Moll JM, Andersen D, Workman CT, Williams AR, Kristiansen K, Brix S. Body fluid from the parasitic worm *Ascaris suum* inhibits broad-acting pro-inflammatory programs in dendritic cells. *Immunology* 159:322–334; 2020. doi:10.1111/imm.13151.

53. Urban JF, Steenhard NR, Solano-Aguilar GI, Dawson HD, Iweala OI, Nagler CR, Noland GS, Kumar N, Anthony RM, Shea-Donohue T, et al. Infection with parasitic nematodes confounds vaccination efficacy. *Vet Parasitol.* 148:14–20; 2007. doi: 10.1016/j.vetpar.2007.05.006.

54. Stothard JR, Gabrielli A-F. Schistosomiasis in African infants and preschool children: to treat or not to treat? *Trends in Parasitol.*23:83–86; 2007. doi:10.1016/j.pt.2007.01.005.

55. Little MC, Bell LV, Cliffe LJ, Else KJ. The characterization of intraepithelial lymphocytes, lamina propria leukocytes, and isolated lymphoid follicles in the large intestine of mice infected with the intestinal nematode parasite *Trichuris muris*. *J Immunol.* 175:6713–6722; 2005. doi:10.4049/jimmunol.175.10.6713.

56. Grainger JR, Smith KA, Hewitson JP, McSorley HJ, Harcus Y, Filbey KJ, Finney CAM, Greenwood EJD, Knox DP, Wilson MS, et al. Helminth secretions induce de novo T cell Foxp3 expression and regulatory function through the TGF-β pathway. *J of Exp Med.* 207:2331–2341; 2010. doi:10.1084/jem.20101074.

57. Rahimian S, Daş G, Gauly M. Maternal protection against *Ascaridia galli*? *Vet Parasitol.* 233:43–47; 2017. doi: 10.1016/j.vetpar.2016.11.014.

58. Thapa S, Hinrichsen LK, Brenninkmeyer C, Gunnarsson S, Heerkens JLT, Verwer C, Niebuhr K, Willett A, Grilli G, Thamsborg SM, et al. Prevalence and magnitude of helminth infections in organic laying hens (*Gallus gallus domesticus*) across Europe. *Vet Parasitol.* 214:118–124; 2015. doi: 10.1016/j.vetpar.2015.10.009.

59. Christenson RO, Earle HH, Butler RL, Creel HH. Studies on the Eggs of *Ascaridia galli* and Heterakis gallinae. *Transactions of the American Microscopical Soc.* 61:191–205; 1942. doi:10.2307/3222847.

60. Anders P & Jorgen WH. Epidemiology diagnosis and control of poultry parasites. *FAO animal health manual*, 4:1020-5187; 1998.

61. Daş G, Hennies M, Sohnrey B, Rahimian S, Wongrak K, Stehr M, Gauly M. A comprehensive evaluation of an ELISA for the diagnosis of the two most common ascarids in chickens using plasma or egg yolks. *Par & Vect.* 10:187; 2017. doi:10.1186/s13071-017-2121-9.

62. Katakam KK, Nejsum P, Kyvsgaard NChr, Jørgensen CB, Thamsborg SM. Molecular and parasitological tools for the study of *Ascaridia galli* population dynamics in chickens. *Avian Pathol.* 39:81–85; 2010. doi:10.1080/03079451003599284.

63. Tarbiat B, Enweji N, Baltrusis P, Halvarsson P, Osterman-Lind E, Jansson DS, Höglund J. A novel duplex ddPCR assay for detection and differential diagnosis of *Ascaridia galli* and *Heterakis gallinarum* eggs from chickens feces. *Vet Parasitol.* 296:109499; 2021. doi: 10.1016/j.vetpar.2021.109499.

64. Feyera T, Ruhnke I, Sharpe B, Elliott T, Shifaw A, Walkden-Brown SW. Comparative therapeutic efficacies of oral and in-water administered levamisole, piperazine and fenbendazole against experimental *Ascaridia galli* infection in chickens. *Vet. Parasitol.* 298:109514; 2021. doi:10.1016/j.vetpar.2021.109514.

65. Angel C, Akhter N, Arijo A, Qureshi TA, Gandahi JA, Qazi IH. Comparative efficacy of ivermectin and Nigella sativa against helminths in Aseel chickens (*Gallus gallus domesticus*). *J of Helminth.* 93:533–538; 2019. doi:10.1017/S0022149X18000718.

66. Lacey E. The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles. *Inter J for Parasitol.* 18:885–936; 1988. doi:10.1016/0020-7519(88)90175-0.

67. Tarbiat B, Jansson DS, Moreno L, Lanusse C, Nylund M, Tydén E, Höglund J. The efficacy of flubendazole against different developmental stages of the poultry roundworm *Ascaridia galli* in laying hens. *Vet Parasitol.* 218:66–72; 2016. doi: 10.1016/j.vetpar.2016.01.012.

68. Tarbiat B, Jansson DS, Tydén E, Höglund J. Evaluation of benzimidazole resistance status in *Ascaridia galli*. *Parasitology* 144:1338–1345; 2017. doi:10.1017/S0031182017000531.

69. Roos MH, Boersema JH, Borgsteede FHM, Cornelissen J, Taylor M, Joost Ruitenberg E. Molecular analysis of selection for benzimidazole resistance in the sheep parasite *Haemonchus contortus*. *Mol and Bioch Parasitol.* 43:77–88; 1990. doi:10.1016/0166-6851(90)90132-6.

70. Collins JB, Jordan B, Baldwin L, Hebron C, Paras K, Vidyashankar AN, Kaplan RM. Resistance to fenbendazole in *Ascaridia dissimilis*, an important nematode parasite of turkeys. *Poult Sci* 98:5412–5415; 2019. doi:10.3382/ps/pez379.

71. Höglund J, Jansson DS. Infection dynamics of *Ascaridia galli* in non-caged laying hens. *Vet Parasitol* 180:267–273; 2011. doi: 10.1016/j.vetpar.2011.03.031.

72. Tarbiat B, Jansson DS, Tydén E, Höglund J. Comparison between anthelmintic treatment strategies against *Ascaridia galli* in commercial laying hens. *Vet Parasitol.* 226:109–115; 2016. doi: 10.1016/j.vetpar.2016.07.006.

73. Tarbiat B, Jansson DS, Tydén E, Höglund J. Evaluation of benzimidazole resistance status in *Ascaridia galli*. *Parasitology* 144:1338–1345; 2017. doi:10.1017/S0031182017000531.

74. Tarbiat B, Jansson DS, Tydén E, Höglund J. Comparison between anthelmintic treatment strategies against *Ascaridia galli* in commercial laying hens. *Vet Parasitol.* 226:109–115; 2016. doi: 10.1016/j.vetpar.2016.07.006.

75. Sharma N, Hunt PW, Hine BC, Ruhnke I. The impacts of *Ascaridia galli* on performance, health, and immune responses of laying hens: new insights into an old problem. *Poult Sci.* 98:6517–6526; 2019. doi:10.3382/ps/pez422.

76. Andersen JP, Norup LR, Dalgaard TS, Rothwell L, Kaiser P, Permin A, Schou TW, Fink DR, Jungersen G, Sørensen P, et al. No protection in chickens immunized by the oral or intra-muscular immunization route with *Ascaridia galli* soluble antigen. *Avian Pathol.* 42:276–282; 2013. doi:10.1080/03079457.2013.783199.

77. Hayashi RM, Lourenço MC, Kraieski AL, Araujo RB, Gonzalez-Esquerra R, Leonardecz E, da Cunha AF, Carazzolle MF, Monzani PS, Santin E. Effect of feeding *Bacillus subtilis* spores to broilers challenged with Salmonella enterica serovar Heidelberg Brazilian strain UFPR1 on performance, immune response, and gut health. *Front Vet Sci.* 5:13; 2018. doi:10.3389/fvets.2018.00013.

78. Pineda-Quiroga C, Borda-Molina D, Chaves-Moreno D, Ruiz R, Atxaerandio R, Camarinha-Silva A, García-Rodríguez A. Microbial and functional profile of the ceca from laying hens affected by feeding prebiotics, probiotics, and synbiotics. *Microorganisms* 7:123; 2019. doi:10.3390/microorganisms7050123.

79. Roto SM, Rubinelli PM, Ricke SC. An introduction to the avian gut microbiota and the effects of yeast-based prebiotic-type compounds as potential feed additives. *Front in Vet Sci.* 22015.

80. Stanley D, Hughes RJ, Moore RJ. Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. *Appl Microbiol Biotechnol.* 98:4301–4310; 2014. doi:10.1007/s00253-014-5646-2.

81. Xiao Y, Xiang Y, Zhou W, Chen J, Li K, Yang H. Microbial community mapping in intestinal tract of broiler chicken. *Poult Sci.* 96:1387–1393; 2017. doi:10.3382/ps/pew372.

82. Bhogoju S, Nahashon S, Wang X, Darris C, Kilonzo-Nthenge A. A comparative analysis of microbial profile of Guinea fowl and chicken using metagenomic approach. *PLOS ONE* 13:e0191029; 2018. doi: 10.1371/journal.pone.0191029.

83. Houlden A, Hayes K, Bancroft A, Worthington J, Wang P, Grencis R, Roberts I. Chronic *Trichuris muris* infection in c57bl/6 mice causes significant changes in host microbiota and metabolome: effects reversed by pathogen clearance. *PLOS ONE* 10:e0125945; 2015. doi: 10.1371/journal.pone.0125945.

84. Reynolds LA, Finlay BB, Maizels RM. Cohabitation in the intestine: interactions between helminth parasites, bacterial microbiota and host immunity. *J Immunol.* 195:4059–4066; 2015. doi:10.4049/jimmunol.1501432.

85. Brisbin JT, Gong J, Sharif S. Interactions between commensal bacteria and the gut-associated immune system of the chicken. *Ani Health Res Rev.* 9:101–110; 2008. doi:10.1017/S146625230800145X.

86. Dahl C, Permin A, Christensen JP, Bisgaard M, Muhairwa AP, Petersen KMD, Poulsen JSD, Jensen AL. The effect of concurrent infections with *Pasteurella multocida* and *Ascaridia galli* on free range chickens. *Vet Microbiol.* 86:313–324; 2002. doi:10.1016/s0378-1135(02)00015-9.

87. Okulewicz A, Złotorzycka J. Connections between Ascaridia galli and the bacterial flora in the intestine of hens. *Angew Parasitol.* 26:151–155; 1985.

88. Brookes A. Necrotic enteritis in broiler breeder chickens associated with *Ascaridia galli*. Animal Health Laboratory. Guelph: University of Guelph. 2020. AHL Newsletter.

89. Wei S, Morrison M, Yu Z. Bacterial census of poultry intestinal microbiome. *Poult Sci.* 9:671-683; 2013. doi:10.3382/ps.2012-02822.

90. Dumonceaux TJ, Hill JE, Hemmingsen SM, Van Kessel AG. Characterization of intestinal microbiota and response to dietary virginiamycin supplementation in the broiler chicken. *Appl Environ Microbiol.* 72:2815–2823; 2006. doi:10.1128/AEM.72.4.2815-2823.2006.

91. Johnson JS, Spakowicz DJ, Hong B-Y, Petersen LM, Demkowicz P, Chen L, Leopold SR, Hanson BM, Agresta HO, Gerstein M, et al. Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nat Commun.* 10:5029; 2019. doi:10.1038/s41467-019-13036-1.

92. Stackebrandt E, Goebel BM. Taxonomic Note: A Place for DNA-DNA Reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J of Syst and Evol Microbiol.* 44:846–849; 1994. doi:10.1099/00207713-44-4-846.

93. Drancourt M, Bollet C, Carlioz A, Martelin R, Gayral JP, Raoult D. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *J Clin Microbiol.* 38:3623–3630; 2000. doi:10.1128/JCM.38.10.3623-3630.2000.

94. Gong J, Si W, Forster RJ, Huang R, Yu H, Yin Y, Yang C, Han Y. 16S rRNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: from crops to ceca. *FEMS Microbiol Ecol.* 59:147–157; 2007. doi:10.1111/j.1574-6941.2006.00193.x.

95. Hall M, Beiko RG. 16S rRNA Gene Analysis with QIIME2. In: Beiko RG, Hsiao W, Parkinson J, editors. Microbiome analysis: methods and protocols. New York, NY: Springer. p. 113–129; 2018. (Methods in Molecular Biology). doi:10.1007/978-1-4939-8728-3_8.

96. Khan S, Moore RJ, Stanley D, Chousalkar KK. The gut microbiota of laying hens and its manipulation with prebiotics and probiotics to enhance gut health and food safety Ercolini D, editor. *Appl Environ Microbiol.* 86:e00600-20; 2020. doi:10.1128/AEM.00600-20.

97. Jamil M, Aleem MT, Shaukat A, Khan A, Mohsin M, Rehman T ur, Abbas RZ, Saleemi MK, Khatoon A, Babar W, et al. Medicinal Plants as an alternative to control poultry parasitic diseases. *Life* 12:449; 2022. doi:10.3390/life12030449.

98. Roberts T, Wilson J, Guthrie A, Cookson K, Vancraeynest D, Schaeffer J, Moody R, Clark S. New issues and science in broiler chicken intestinal health: emerging technology and alternative interventions. *J of Applied Poult Res.* 24:257–266; 2015. doi:10.3382/japr/pfv023.

99. Revolledo L, Ferreira AJP, Mead GC. Prospects in Salmonella Control: Competitive exclusion, probiotics, and enhancement of avian intestinal immunity. *J of Applied Poul Res.* 15:341–351; 2006. doi:10.1093/japr/15.2.341.

100. Callaway TR, Edrington TS, Anderson RC, Harvey RB, Genovese KJ, Kennedy CN, Venn DW, Nisbet DJ. Probiotics, prebiotics, and competitive exclusion for prophylaxis against bacterial disease. *Ani Health Res Rev.* 9:217–225; 2008. doi:10.1017/S1466252308001540.

101. Rodriguez-Palacios A, Staempfli HR, Duffield T, Weese JS. Isolation of bovine intestinal *Lactobacillus plantarum* and *Pediococcus acidilactici* with inhibitory activity against *Escherichia coli* O157 and F5. *J Appl Microbiol.* 106:393–401; 2009. doi:10.1111/j.1365-2672.2008.03959. x.

102. Zhang WF, Li DF, Lu WQ, Yi GF. Effects of isomalto-oligosaccharides on broiler performance and intestinal microflora. *Poult Sci.* 82:657–663; 2003. doi:10.1093/ps/82.4.657.

103. Xu ZR, Hu CH, Xia MS, Zhan XA, Wang MQ. Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora, and morphology of male broilers. *Poult Sci.* 82:1030–1036; 2003. doi:10.1093/ps/82.6.1030.

104. Chang L, Ding Y, Wang Y, Song Z, Li F, He X, Zhang H. Effects of different oligosaccharides on growth performance and intestinal function in broilers. *Front in Vet Sci.* 9; 2022.

105. Li X, Qiang L, Liu, Xu C. Effects of supplementation of fructooligosaccharide and/or *Bacillus Subtilis* to diets on performance and on intestinal microflora in broilers. *Arch Anim Breed* 51:64–70; 2008. doi:10.5194/aab-51-64-2008.

106. Sarangi NR, Babu LK, Kumar A, Pradhan CR, Pati PK, Mishra JP. Effect of dietary supplementation of prebiotic, probiotic, and symbiotic on growth performance and carcass characteristics of broiler chickens. *Vet World.* 9:313–319; 2016. doi:10.14202/vetworld.2016.313-319.

107. Zhou Z, Nie K, Huang Q, Li K, Sun Y, Zhou R, Wang Z, Hu S. Changes of cecal microflora in chickens following *Eimeria tenella* challenge and regulating effect of coated sodium butyrate. *Exp Parasitol.* 177:73–81; 2017. doi: 10.1016/j.exppara.2017.04.007.

108. Józefiak D, Kaczmarek S, Bochenek M, Rutkowski A. A note on effect of benzoic acid supplementation on the performance and microbiota population of broiler chickens. *J Anim Feed Sci.* 16:252–256; 2007. doi:10.22358/jafs/66746/2007.

109. Józefiak D, Kaczmarek S, Rutkowski A. The effects of benzoic acid supplementation on the performance of broiler chickens. *J of Anim Physiol and Anim Nutr*. 94:29–34; 2010. doi:10.1111/j.1439-0396.2008.00875. x.

110. Remmal A, Achahbar S, Bouddine L, Chami N, Chami F. In vitro destruction of *Eimeria* oocysts by essential oils. *Vet Parasitol.* 182:121–126; 2011. doi: 10.1016/j.vetpar.2011.06.002.

111. Shafi G, Hasan TN, Syed NA, Al-Hazzani AA, Alshatwi AA, Jyothi A, Munshi A. *Artemisia absinthium* (AA): a novel potential complementary and alternative medicine for breast cancer. *Mol Biol Rep.* 39:7373–7379; 2012. doi:10.1007/s11033-012-1569-0.

112. Kim DK, Lillehoj HS, Lee SH, Jang SI, Lillehoj EP, Bravo D. Dietary *Curcuma longa* enhances resistance against *Eimeria maxima* and *Eimeria tenella* infections in chickens. *Poult Sci.* 92:2635–2643; 2013. doi:10.3382/ps.2013-03095.

113. Irum S, Ahmed H, Mukhtar M, Mushtaq M, Mirza B, Donskow- Łysoniewska K, Qayyum M, Simsek S. Anthelmintic activity of *Artemisia vestita* Wall ex DC. and *Artemisia maritima L.* against *Haemonchus contortus* from sheep. *Vet Parasitol.* 212:451–455; 2015. doi: 10.1016/j.vetpar.2015.06.028.

114. Swaggerty CL, Bortoluzzi C, Lee A, Eyng C, Pont GD, Kogut MH. potential replacements for antibiotic growth promoters in poultry: interactions at the gut level and their impact on host immunity. *Adv Exp Med Biol.* 1354:145–159; 2022. doi:10.1007/978-3-030-85686-1_8.

115. Padosch SA, Lachenmeier DW, Kröner LU. Absinthism: a fictitious 19th century syndrome with present impact. *Subst Abuse Treat Prev Policy.* 1:14; 2006. doi:10.1186/1747-597X-1-14.

116. Mueller MS, Karhagomba IB, Hirt HM, Wemakor E. The potential of *Artemisia annua L.* as a locally produced remedy for malaria in the tropics: agricultural, chemical and clinical aspects. *J of Ethnopharmacol.* 73:487–493; 2000. doi:10.1016/S0378-8741(00)00289-0.

117. Ferreira JFS, Luthria DL, Sasaki T, Heyerick A. Flavonoids from *Artemisia annua L.* as antioxidants and their potential synergism with artemisinin against malaria and cancer. *Molecules* 15:3135–3170; 2010. doi:10.3390/molecules15053135.

118. Naidoo V, McGaw LJ, Bisschop SPR, Duncan N, Eloff JN. The value of plant extracts with antioxidant activity in attenuating coccidiosis in broiler chickens. *Vet Parasitol*. 153:214–219; 2008. doi: 10.1016/j.vetpar.2008.02.013.

119. del Cacho E, Gallego M, Francesch M, Quílez J, Sánchez-Acedo C. Effect of artemisinin on oocyst wall formation and sporulation during *Eimeria tenella* infection. *Parasitol Int.* 59:506–511; 2010. doi: 10.1016/j.parint.2010.04.001.

120. Seddiek SA, Ali MM, Khater HF, El-Shorbagy MM. Anthelmintic activity of the white wormwood, *Artemisia herba-alba* against *Heterakis gallinarum* infecting turkey poults. *J Med Plants Res*. *5*:3946-3957. 2011.

121. Tariq KA, Chishti MZ, Ahmad F, Shawl AS. Anthelmintic activity of extracts of *Artemisia absinthium* against ovine nematodes. *Vet Parasitol* 160:83–88; 2009. doi: 10.1016/j.vetpar.2008.10.084.

122. Mravčáková D, Komáromyová M, Babják M, Urda Dolinská M, Königová A, Petrič D, Čobanová K, Ślusarczyk S, Cieslak A, Várady M, et al. Anthelmintic activity of wormwood

(*Artemisia absinthium L.*) and Mallow (*Malva sylvestris L.)* against *Haemonchus contortus* in Sheep. *Animals* 10:219; 2020. doi:10.3390/ani10020219.

123. Beigh YA, Ganai AM, Sofi AH, Haq Z. Effect of feeding wormwood herb (*Artemisia absinthium Linn.*) added total mixed ration on wool production and quality in sheep. *J of Natural Fib.* 0:1–13; 2021. doi:10.1080/15440478.2021.1993404.

124. Váradyová, Zora. Ovicidal and larvicidal activity of extracts from medicinal plants against *Haemonchus contortus*. *Exp Parasitol*. 195:71–77; 2018.

125. Shahnazi M, Azadmehr A, Hajiaghaee R, Mosalla S, Latifi R. Effects of *Artemisia Absinthium L.* extract on the maturation and function of dendritic cells. *Jundishapur J Nat Pharm Prod.* 102015. doi:10.17795/jjnpp-20163.

126. Lachenmeier DW. Wormwood (*Artemisia absinthium L.*)—A curious plant with both neurotoxic and neuroprotective properties? *J of Ethnopharmacol.* 131:224–227; 2010. doi: 10.1016/j.jep.2010.05.062.

127. Duke JA. *Handbook of Medicinal Herbs.* 2nd ed. Routledge & CRC Press; 2002.

128. Behrens M, Brockhoff A, Kuhn C, Bufe B, Winnig M, Meyerhof W. The human taste receptor hTAS2R14 responds to a variety of different bitter compounds. *Biochem and Biophys Res Commun.* 319:479–485; 2004. doi: 10.1016/j.bbrc.2004.05.019.

129. Poulopoulou I, Horgan MJ, Siewert B, Siller M, Palmieri L, Martinidou E, Martens S, Fusani P, Temml V, Stuppner H, et al. In vitro evaluation of the effects of methanolic plant extracts on

the embryonation rate of *Ascaridia galli* eggs. *Vet Res Commun.* 1:11; 2022. doi:10.1007/s11259-022-09958-9.

130. Feitosa TF, Vilela VLR, Athayde ACR, Braga FR, Dantas ES, Vieira VD, de Melo LRB. Anthelmintic efficacy of pumpkin seed (*Cucurbita pepo Linnaeus*, 1753) on ostrich gastrointestinal nematodes in a semiarid region of Paraíba State, Brazil. *Trop Anim Health Prod.* 45:123–127; 2013. doi:10.1007/s11250-012-0182-5.

131. Erol A, Hamit C kun. Evaluation of the anthelmintic activity of pumpkin seeds (*Cucurbita maxima*) in mice naturally infected with *Aspiculuris tetraptera*. *J. Pharmacognosy Phytother.* 7:189–193; 2015. doi:10.5897/JPP2015.0341.

132. Grzybek M, Kukula-Koch W, Strachecka A, Jaworska A, Phiri AM, Paleolog J, Tomczuk K. Evaluation of anthelmintic activity and composition of pumpkin (*Cucurbita pepo L.*) seed extracts—*in vitro* and *in vivo* studies. *Inter J of Mol Sci.* 17:1456; 2016. doi:10.3390/ijms17091456.

133. Salehi, Sharifi-Rad, Capanoglu, Adrar, Catalkaya, Shaheen, Jaffer, Giri, Suyal, Jugran, et al. Cucurbita plants: from farm to industry. *Appl Sci.* 9:3387; 2019. doi:10.3390/app9163387.

134. Bayati NYA-, AL-Aubaidi IK, AL-Haidari SHJ. Anticestodal activity of the crude aqueous extract of pumpkin seed (*Cucurbita pepo*) against the dwarf tapeworm (*Hymenolepis nana*) in mice. 8; 2009.

135. Lima DF de, Brainer MM de A, Fabino RF, Silva BC da, Godoy MM de, Fabino Neto R, Morgado HS. Potencial antihelmíntico de sementes de abóbora (*Cucurbita mochata*) em equinos. *BJAER* 3:952–965; 2020. doi:10.34188/bjaerv3n3-016.

136. A. Acorda J, C. Mangubat IYE, P. Divina B. Evaluation of the in vivo efficacy of pumpkin (*Cucurbita pepo*) seeds against gastrointestinal helminths of chickens. *Turk J Vet Anim Sci.* 43:206–211; 2019. doi:10.3906/vet-1807-39.

137. Hellawi H, Ibrahim OMS. Evaluation of anthelmintic activity of n-hexane extract of *Cucurbita maxima* and *Azadirachta indica* pulp seed against *Ascaridia galli* in vitro. :8; 2021.

138. Abdel Aziz AR, AbouLaila MR, Aziz M, Omar MA, Sultan K. In vitro and in vivo anthelmintic activity of pumpkin seeds and pomegranate peels extracts against *Ascaridia galli*. *Beni-Suef Univ J of Basic and Appl Sci* 7:231–234; 2018. doi: 10.1016/j.bjbas.2018.02.003.

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Regular Article—

III. A SURVEY OF COCCIDIA AND NEMATODES IN PASTURED POULTRY IN THE STATE OF GEORGIA

Maria Tereza Bethonico Terra,[A] Wilmer J. Pacheco,[A] Mark Harrison,[B] Brigid A. McCrea,[C] and Rüdiger Hauck[ADE]

[A]Department of Poultry Science, Auburn University, Auburn, AL 36849

[B]White Oak Pastures, Bluffton, GA 29824

[C]Alabama Cooperative Extension System, Auburn, AL, 36849

[D]Department of Pathobiology, Auburn University, Auburn, AL 36849

[E]Corresponding author. E-mail: ruediger.hauck@auburn.edu

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ABSTRACT

The aim of this study was to evaluate and quantify the parasitological challenge in pastured poultry production in the state of Georgia. Over the course of 1-year, fecal samples from six turkey flocks, 10 broiler flocks, and 13 layer flocks were collected on a pastured farm in 2-wk intervals to determine counts of *Eimeria* oocysts and nematode eggs. Average coccidia counts were 10,198 oocysts per gram of feces (OPG) in broiler flocks, 1470 OPG in layer flocks, and 695 OPG in turkey flocks. The means in broiler and turkey flocks were higher at their first week on pasture. Counts in broilers and layers were significantly higher in spring than in winter and summer. Coccidia counts in broilers were lower than published numbers in conventionally reared poultry, indicating the rotation system of the pastures might effectively reduce the infection pressure. Next-generation sequencing of PCR products showed the presence of most described *Eimeria* spp. in

broilers, layers, and turkeys. In addition, operational taxonomic units (OTUs) x, y, and z were found. The frequency of species was similar for broilers and layers, with the exception that *Eimeria praecox* and OTU z were more common in layers. In layer flocks, the average count of roundworm eggs per gram of feces (EPG) was 509 EPG with 80% of the samples being positive. The mean counts had no clear pattern related to age. There was an increase of EPG with the increase of temperatures during spring and summer with the peak at midfall. Worm eggs from laying hens were identified as *Ascaridia galli.* The seasonal differences suggest that higher temperatures might result in an increase of egg survival and sporulation in the environment.

Key words: Epidemiology; organic production; chickens; coccidia; diagnosis

Abbreviations: COI = cytochrome oxidase I; EPG = eggs per gram; NCBI = National Center for Biotechnology Information; NGS = next generation sequencing; OPG = oocyst per gram; OTU = operational taxonomic unit.

INTRODUCTION

There is an increasing market demand for poultry meat and eggs that are produced in alternative and sustainable systems. These systems claim to be better for the environment, i.e., to be more sustainable and to provide better animal welfare since they allow the birds to exercise natural behavior, such as scratching and foraging for feed on the ground (1). However, one downside of this type of rearing is that the birds come into contact with their excreta, which increases their chances of getting infected with endoparasites such as coccidia and roundworms (2). In addition, treatment and prophylaxis of parasitic diseases in organic flocks with conventional drugs is not permissible (3), making parasitic infections a potential problem.

Eimeria spp., commonly referred to as coccidia, have a life cycle that is completed without intermediate hosts, and the birds get infected by ingesting oocysts that were shed in feces (4). They

are the causative agents of coccidiosis and frequently infect broilers and turkeys in commercial flocks (4,5). This disease has a major impact on the cost of production of broilers (6). Usually, coccidia are not a concern in layers flocks since conventional systems raise these animals in cages, separated from their droppings.

Helminthic parasites, such as *Ascaridia* and *Heterakis*, are commonly found in alternative systems in the production of chickens and turkeys (7,8,9,10,11). They infect the gastrointestinal tract of poultry species, can decrease egg production, and negatively affect animal welfare by partially or totally obstructing the lumen (12). Severity of infection can be correlated with increased mortality, and it is not rare to find birds infected with more than one worm species (13). Eggs of roundworms shed in feces are tenacious in the environment, and birds are infected after oral intake (14).

Pastured systems are a type of alternative system that may provide the animals with mobile houses moved every 2 or 3 days to a new patch on the pasture to avoid overgrazing of the pasture, thus assisting in the recovery of the vegetation. While these systems offer little or no biosecurity, the often-lower stocking density and the frequent change of location can reduce the concentration of feces on the ground and consequently decrease the infection pressure for some diseases (14). Yet these systems are rare compared to conventional production in the United States, and there is little literature evaluating and quantifying the prevalence of parasites in pastured production. The aim of the project was to systematically investigate the occurrence of coccidia and nematodes in broilers, layers, and turkeys in this type of system.

MATERIALS AND METHODS

Farm. Turkeys of various genetic lines and Royal Red and Robust White broilers are brooded until at least 4 weeks of age in heated rooms with outdoor access. When exactly birds are moved to pasture depends on the weather. Hyline Brown layer pullets are acquired close to the onset of egg production at around 17 weeks of age. Some broiler flocks are kept together with guineafowl (*Numida meleagris*). On pasture, birds are provided with mobile structures for shelter and nest boxes for the laying hen flocks. Flocks and structures are moved to a new area twice a week. The pasture fields are covered with a mix of forages. Typically, after a flock is moved from one pasture area, the area can be used to raise other species such as cattle, sheep, or pig, but no poultry for at least 1 yr. In addition to forage, animals are fed a formulated mash diet without anticoccidials and have access to grit. Broilers are slaughtered at 10 to 12 weeks of age with an approximate weight of 3.6 kg; turkeys are harvested at 21 to 23 weeks of age with an approximate weight of 14 kg. Layers are kept to an age of 18 mo. Birds are not vaccinated against coccidiosis.

Sample collection and parasitological investigation. A total of eight broiler flocks, eight turkey flocks, and four laying hen flocks were evaluated during the study (Fig. 3.1). Fecal samples were collected in Whirl-Pak bags (Whirl-Pak, Madison, WI) using plastic spoons during the morning at 2-wk intervals between March 2019 and March 2020. During each visit, two pooled fecal samples of at least 20 individual droppings each were collected per flock and stored at 4 C until they were processed within the next 4 days.

Samples were mixed thoroughly in the bag; 1g of feces then was mixed with 29 ml of saturated sodium solution and poured through a tea strainer. The filtered suspension was loaded into a McMaster chamber, and parasites were counted under a microscope (15).

Identification of *Eimeria* spp. Ten coccidia positive samples from broilers, 13 samples from layers, and six samples from turkeys were selected for identification of *Eimeria* spp. using next-generation sequencing (NGS) of PCR amplicons as previously described (16). Briefly, oocysts were purified to reduce fecal contamination and concentrated (17). DNA was extracted from the oocysts using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Partial 18S rRNA and cytochrome oxidase I (COI) genes were amplified by nested PCR using the Q5 High-Fidelity Kit (New England Biolabs, Ipswich, MA). Primers to amplify the COI gene of *Eimeria* spp. of turkeys were modified from the published primers to amplify the COI gene of *Eimeria* spp. of chickens (Table 3.1). PCR products were checked by agarose gel electrophoresis and sent to the University of Illinois at Chicago DNA Services Facility for NGS. Sequences of coccidia from broilers and layers were analyzed by comparing them to reference sequences using QIME 2.0 as previously described (16). Sequences of coccidia from turkeys were analyzed by comparing them to reference sequences that were designed for this study. All clustering was performed at 97% identity for both genes. Sequences that had a homology of less than 97% to one of the reference sequences were compared with homolog sequences available from the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/BLAST/) using the BLAST search algorithm (19).

Identification of worms. Worm eggs from one positive sample from layers were used for experimental infection of chickens. Larvae were purified from the intestinal wall, and a fragment of the COI gene was amplified using primers with a broad specificity (18) and the Q5 High-Fidelity Kit. The PCR product was purified using the Qiaquick PCR purification kit (Qiagen) and submitted to the Massachusetts General Hospital DNA Core Facility for forward and reverse Sanger sequencing. The sequence was uploaded to GenBank (accession number MT776400). The BLAST

search algorithm (19) was used to compare the obtained sequence with homolog sequences available from the NCBI server.

Statistical analyses. Data were analyzed as a one-way ANOVA using the GLM procedure of JMP software (20). The mean values were compared using Tukey's highest significant difference procedure with statistical significance considered at $P \leq 0.05$ unless otherwise indicated.

RESULTS

Detection of coccidia oocysts. Broiler flocks had the highest overall mean coccidia counts with 10,198 oocysts per gram of feces (OPG). The mean count in samples collected in winter inside brooders from one 4-wk-old flock and one 5-wk-old flock was 12,250 OPG. The mean count in the six flocks of the same age on pasture was 16,575 OPG. Overall, the mean count at 4 and 5 weeks of age was 15,493 OPG with a trend toward decreasing in the following weeks ($P = 0.080$) (Fig. 3.2A).

For layer flocks, the average coccidia count was 1470 OPG, and there were two distinct peaks at 23 and 27 of age with 5275 and 5875 OPG, respectively. There was no clear pattern of oocyst shedding related to age (Fig. 3.2B)

For turkey flocks, the average coccidia count was 695 OPG, and there was a peak at weeks 5 and 6 with 2950 and 2800 OPG, respectively (Fig. 3.2C). This resulted in statistical differences between different ages ($P = 0.0345$). The mean count in samples collected in brooders in winter from one 5-wk-old flock, two 7-wk-old flocks, and one 9-wk-old flock was 1700 OPG, while the average count of birds between 4 and 9 weeks raised on pasture was 1214 OPG.

Coccidia counts in broilers and layers were significantly higher during spring with mean counts of 23,950 ($P = 0.001$) and 2195 OPGs ($P = 0.010$), respectively, compared to winter and summer. However, season did not influence coccidia counts in turkeys (Fig. 3.3).

Detection of roundworm eggs. Roundworm eggs were detected in all four layer flocks. Out of the 82 times when a layer flock was sampled, at least one of the two or three samples was positive 66 times, which represents 80% positivity during year of the study. The mean count of roundworm eggs was 509 eggs per gram (EPG) without a clear pattern with age (Fig. 3.4A). However, there was an increase in roundworm egg counts that followed the increase in average daily environmental temperatures during spring and summer with a significantly higher peak reaching at midfall ($P = 0.001$) (Fig. 3.4B). In turkeys, only one sample had 50 EPG present during spring at 21 weeks of age, while all broiler samples were negative for roundworm eggs.

Identification of *Eimeria* spp. in chicken flocks. Ten samples from broilers and 13 samples from layers were examined with NGS to determine *Eimeria* species. All seven unambiguously described species and operational taxonomic units (OTUs) were detected in broilers as well as in layers. *Eimeria mitis/E. mivati*, *Eimeria maxima,* and *Eimeria acervulina* were the most encountered species. The frequency of species was similar for broilers and layers, with the exception that *Eimeria praecox* and OTU z were more common in layers (Fig. 5). Samples taken at different timepoints from the same two broiler flocks and two layer flocks were included. The species composition in the flocks evaluated did not change significantly over time.

The percentage of reads accounted for by the described *Eimeria* spp. of chickens was between 0 and 99% for the 18S rRNA gene and between 72% and 99% for the COI gene. Some sequences that had a homology of less than 97% to the reference sequences still had their highest homology to sequences of chicken coccidia. In other samples, reads had a high homology to *Eimeria* sp. ex *Numida meleagris, Eimeria innocua*, *Eimeria anseris, Eimeria gazella*, or *Eimeria megabubonis*. In addition, homologies to sequences of the free-living alveolates *Colpodella* spp. and uncultured organisms were common.

Identification of *Eimeria* spp. in turkey flocks. *In silico* evaluation of amplicon NGS for *Eimeria* spp. from turkeys based on 42 18S partial rRNA gene sequences and 19 partial COI gene sequences of turkey *Eimeria* spp. retrieved from GenBank showed the general suitability of the method. However, the evaluation also showed that not all species could be differentiated (data not shown). *Eimeria meleagridis*, *Eimeria gallopavonis*, and/or *Eimeria adenoides* were detected in four of the six samples analyzed. Other detected species were *Eimeria meleagrimitis* and *Eimeria dispersa*, while *E. innocua* was not detected (Fig. 3.6). In two samples, no sequences of turkey coccidia were present.

The percentage of reads accounted for by the described *Eimeria* spp. of turkeys was between 0 and 93% for 18S rRNA gene reads and between 1% and 68% for the COI gene reads. Sequences with a homology of less than 97% to the reference sequences had their highest homology either to *Eimeria* spp. of other hosts, including chickens, or further related or uncultured parasites. For example, no coccidia sequences were detected in the samples from T2 and T6, but reads aligned to an uncultured alveolate clone, an uncultured apicomplexan, and uncultured fungus (KF219457.1) as well as *Babesia* sp., *Filobasidium* sp., and *Penaeus vannamei*. Some COI sequences had their highest homology to turkey *Eimeria* spp.

Identification of roundworms. Adult worms found in chickens 9 wk after the experimental infection were morphologically identified as *Ascaridia galli*. The COI sequence obtained from larvae had a homology of 98%–100% to *A. galli* sequences.

DISCUSSION

***Coccidia* shedding.** For context, the oocysts count in broilers, layers, and turkeys need to be compared with counts in conventional systems. Haug *et al.* (21) were able to identify that 42.1% of broilers flocks investigated had more than 50,000 OPG with an average of 31,879 OPGs, which

is approximately three times higher than the counts in this study. Jenkins *et al.* (12) reported counts mostly below 20,000 OPG with peaks of more than 100,000 OPG. Borgonovo *et al.* (22) reported counts mostly below 10,000 OPG with a maximum of 68,000 OPG.

There are no published numbers of coccidia counts in commercial layers in the last 50 yr, and only one more than 40-yr-old publication quantifying coccidia oocysts in turkey flocks. A survey done in four turkey farms had peak oocysts counts higher than 9000, reaching numbers close to 40,000 in litter samples of 4-to-6-wk-old flocks (23).

The counts of coccidia observed in broilers were lower than previously published numbers (12,21,22), even though flocks evaluated in this study did not receive feed anticoccidials. Coccidia counts were higher in younger birds in brooders, due to the lack of rotation and possibly a faster sporulation of oocysts. In addition, the rotation system of the pastures might effectively reduce the infection pressure. The lower counts observed in older birds were likely due to developed immunity against *Eimeria* sp. (4).

The higher counts in spring compared to winter and summer suggest that coccidia might be sensitive to high and low environmental temperatures. There were no seasonal differences seen in coccidia recovery from turkey flocks. This might be related to turkeys' lower susceptibility to *Eimeria* sp., resulting in generally lower oocyst counts that are less affected by the infection dose.

Worms shedding and identification. *Ascaridia galli* are the most common worms found in conventionally reared laying hens (24). Worm eggs counts were similar to published numbers on free-range layer flocks. While this study had a mean of 509 EPG, Thapa *et al.* (25) reported a mean of 576 EPG in all investigated organic layer flocks and 808 EPGs on farms with pasture rotation systems in a prevalence study of helminth infection in organic production across Europe. Interestingly, there was no statistical difference in the egg counts between free-range farms with

or without rotation systems in the cited study. On the other hand, another study with free-range birds reported an average of 41.8 EPG in mobile system type and 41.4 EPG in all free-range birds (26). The tenfold difference between the results reported by Thapa *et al.* (25), this study, and results reported by Sherwin (26) might be related to different counting methods. In Thapa *et al.* (25) and in this study, a McMaster chamber was used, while Sherwin *et al.* (26) used a FLOTAC device.

The *A. galli* data suggest that this parasite is a challenge mainly in older birds, as it was consistently observed in layer flocks with only one positive sample of roundworm eggs in one turkey flock.

Embryonation of *A. galli* eggs in the environment requires between 14 to 30 days, and egg shedding will start after 5 to 6 weeks after the infection (27). Consequently, if the peak of EPGs happened in midfall, birds were likely infected 7 to 10 weeks earlier. Therefore, the seasonal difference suggests that higher temperatures in summer might result in an increase of egg survival and embryonation in the environment.

Eggs of other chicken helminths such as *Heterakis*, *Raillietina*, *Trichostrongylus*, and *Syngamus* were not observed, even though it is common to find more than one type of helminthic species in alternative production systems (24,25). Possible reasons are that critical intermediate hosts were lacking or that other parasites were present but not detected. Since coccidia were our primary target, flotation was used and flotation might not be the most suitable method for tapeworm or trematode eggs. Moreover, only one sample with roundworm eggs was tested by experimental infection. Presence of *Heterakis* in other samples cannot be excluded, because both worms have eggs with similar morphology, and the sampling process included cecal droppings as well.

Identification of *Eimeria* spp. in chicken flocks. The identification of *Eimeria* spp. will help to choose control and preventive measures such as vaccine selection. It is common to see birds infected with more than one *Eimeria* spp. (12,21). *Eimeria acervulina, E. maxima, Eimeria tenella*, *E. mitis*, and *E. mivati*, which we detected frequently in this study, are commonly present in conventionally reared broilers, while *Eimerica brunetti* and *Eimerica necatrix* are more commonly present in older birds (12,21). However, the latter two species were rare even in layers. The parasitological profiles of broilers and layers differed as *E. praecox* and OTU z were more common in layers. The reasons are unclear; *E. praecox* is usually regarded as more common in younger birds, while there is no information about the epidemiology of OTU z.

Detection of sequences with a homology of less than 97% to the reference sequences, but with their highest homology to sequences of chicken coccidia, indicate a large heterogeneity within the species or even the presence of yet undescribed species. In addition, the presence of *Eimeria* spp. from other hosts are the consequence of cross-contamination between animals of different species and reflects the limited biosecurity in pastured systems.

Identification of *Eimeria* spp. in turkey flocks. Our findings are similar to a study in commercial turkey flocks in Canada in which the most common species were *E. meleagrimitis* followed by *E. gallopavonis* and *E. meleagridis*, while the species that was the least isolated was *E. dispersa* (28).

Our method is not able to distinguish *E. meleagridis*, *E. gallopavonis*, and *E. adenoides* by their partial 18S rRNA gene sequence. While the partial COI gene product is supposed to differentiate *E. gallopavonis* from *E. meleagridis* or *E. adenoidis*, *E. gallopavonis* was found only in samples in which NGS of the partial COI gene showed presence of *E. adenoides* and/or *E. meleagridis*. The difficulties in differentiating these three species were not a surprise since other

authors believe that *E. adenoeides* is most probably identical to either *E. meleagridis* or *E. gallopavonis* (4). Moreover, these species are found in the same locations in the gastrointestinal track, and their oocysts have similar shapes and sizes (29). While the agreement of the 18S and adapted COI genes in the majority of the samples gives confidence in the results, it is also clear that the method needs to be thoroughly validated for turkey coccidia with known strains, and that other regions of the 18S rRNA gene might be more suitable.

In conclusion, this study was able to identify and quantify the prevalence of coccidia and nematodes in broilers, turkeys, and layers raised on a pastured farm in Georgia. Moreover, seasonal differences suggest that environmental conditions might influence the cycle of *Eimeria* species and *A. galli*. Further research is needed to better understand the dynamic of these parasites in this type of production system and to evaluate potential prevention methods.

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REFERENCES

1. Sossidou EN, Dal Bosco A, Castellini C, Grashorn MA. Effects of pasture management on poultry welfare and meat quality in organic poultry production systems. *World's Poult Sci J.* 71:375–384; 2015. doi:10.1017/S0043933915000379.

2. Jansson DS, Nyman A, Vågsholm I, Christensson D, Göransson M, Fossum O, Höglund J. Ascarid infections in laying hens kept in different housing systems. *Avian Pathol.* 39:525–532; 2010. doi:10.1080/03079457.2010.527923.

3. Electronic Code of Federal Regulations (eCFR). Electronic Code of Federal Regulations (eCFR), Title 7, Part 205, Subpart B, § 205.105 Allowed and prohibited substances, methods, and ingredients in organic production and handling.

4. Vrba V, Pakandl M. Coccidia of turkey: from isolation, characterization and comparison to molecular phylogeny and molecular diagnostics. *Int J Parasitol.* 44:985–1000; 2014. doi:10.1016/j.ijpara.2014.06.004.

5. Hauck R. Interactions between parasites and the bacterial microbiota of chickens. *Avian Dis.* 61:428–436; 2017. doi:10.1637/11675-051917-Review.1.

6. Prescott JF, Parreira VR, Mehdizadeh Gohari I, Lepp D, Gong J. The pathogenesis of necrotic enteritis in chickens: what we know and what we need to know: a review. *Avian Pathol.* 45:288–294; 2016. doi:10.1080/03079457.2016.1139688.

7. Brener B, Tortelly R, Menezes RC, Muniz-Pereira LC, Pinto RM. Prevalence and pathology of the nematode *Heterakis gallinarum*, the trematode *Paratanaisia bragai*, and the protozoan *Histomonas meleagridis* in the turkey, *Meleagris gallopavo*. *Mem Instituto Oswaldo Cruz* 101:677–681; 2006. doi:10.1590/S0074-02762006000600017.

8. Dauda J, Lawal JR, Bello AM, Mustapha M, Ndahi JJ, Biu AA. Survey on prevalence of

gastrointestinal nematodes and associated risk factors in domestic turkeys (*Meleagris gallopavo*) slaughtered in poultry markets in Bukuru—Jos, Plateau State, Nigeria. *Int J Innov Agric Biol Res.* 4(4):27–36; 2016.

9. Hinrichsen LK, Labouriau R, Engberg RM, Knierim U, Sørensen JT. Helminth infection is associated with hen mortality in Danish organic egg production. *Vet Record* 179:196–196; 2016. doi:10.1136/vr.103614.

10. Hinrichsen LK, Riber AB, Labouriau R. Associations between and development of welfare indicators in organic layers. *Animal* 10:953–960; 2016. doi:10.1017/S1751731115003018.

11. Zloch A, Kuchling S, Hess M, Hess C. Influence of alternative husbandry systems on postmortem findings and prevalence of important bacteria and parasites in layers monitored from end of rearing until slaughter. *Vet Record* 182:350–350; 2018. doi:10.1136/vr.104632.

12. Jenkins MC, Parker C, Ritter D. Eimeria oocyst concentrations and species composition in litter from commercial broiler farms during anticoccidial drug or live Eimeria oocyst vaccine control programs. *Avian Dis.* 61:214–220; 2017. doi:10.1637/11578-010317-Reg.1.

13. Stehr M, Sciascia Q, Metges CC, Gauly M, Daş G. Co-expulsion of Ascaridia galli and Heterakis gallinarum by chickens. *Int J Parasitol.* 48:1003–1016; 2018. doi:10.1016/j.ijpara.2018.05.014.

14. Sossidou EN, Dal Bosco A, Elson HA, Fontes CMGA. Pasture-based systems for poultry production: implications and perspectives. *World's Poult Sci.* J67:47–58; 2011. doi:10.1017/S0043933911000043.

15. Conway DP, McKenzie ME. Basic procedures and example protocols for testing anticoccidial drugs. In: Conway DP, McKenzie ME. editors. *Poultry coccidiosis*. Ames (IA): Blackwell Publishing Professional. p. 49–64; 2007.

16. Hauck R, Carrisosa M, McCrea BA, Dormitorio T, Macklin KS. Evaluation of next-generation amplicon sequencing to identify Eimeria spp. of chickens. *Avian Dis.* 63:577–583; 2019. doi:10.1637/aviandiseases-D-19-00104.

17. Hafeez MA, Shivaramaiah S, Dorsey KM, Ogedengbe ME, El-Sherry S, Whale J, Cobean J, Barta JR. Simultaneous identification and DNA barcoding of six Eimeria species infecting turkeys using PCR primers targeting the mitochondrial cytochrome c oxidase subunit I (mtCOI) locus. *Parasitol Res.* 114:1761–1768; 2015. doi:10.1007/s00436-015-4361-y.

18. Katakam KK, Nejsum P, Kyvsgaard NChr, Jørgensen CB, Thamsborg SM. Molecular and parasitological tools for the study of Ascaridia galli population dynamics in chickens. *Avian Pathol.* 39:81–85; 2010. doi:10.1080/03079451003599284.

19. Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL. NCBI BLAST: a better web interface. *Nucleic Acids Res.* 36:W5–9; 2008. doi:10.1093/nar/gkn201.

20. SAS Institute Inc. *JMP*, version 14.0 Cary, (NC): SAS Institute Inc. https://www.jmp.com/; 2018year.

21. Haug A, Gjevre A-G, Skjerve E, Kaldhusdal M. A survey of the economic impact of subclinical Eimeria infections in broiler chickens in Norway. *Avian Pathol.* 37:333–341; 2008. doi:10.1080/03079450802050705.

22. Borgonovo F, Ferrante V, Grilli G, Pascuzzo R, Vantini S, Guarino M. A data-driven prediction method for an early warning of coccidiosis in intensive livestock systems: a preliminary study. *Animals* 10:747; 2020. doi:10.3390/ani10040747.

23. Long PL, Millard BJ. Coccidiosis in turkeys: evaluation of infection by the examination of turkey broiler litter for oocysts. *Avian Pathol.* 6:227–233; 1977. doi:10.1080/03079457708418230.

24. Wuthijaree K, Lambertz C, Gauly M. Prevalence of gastrointestinal helminth infections in free-range laying hens under mountain farming production conditions. *Br Poult Sci.* 58:649–655; 2017. doi:10.1080/00071668.2017.1379049.

25. Thapa S, Hinrichsen LK, Brenninkmeyer C, Gunnarsson S, Heerkens JLT, Verwer C, Niebuhr K, Willett A, Grilli G, Thamsborg SM, et al. Prevalence and magnitude of helminth infections in organic laying hens (*Gallus gallus domesticus*) across Europe. *Vet Parasitol.* 214:118–124; 2015. doi: 10.1016/j.vetpar.2015.10.009.

26. Sherwin CM, Nasr MAF, Gale E, Petek M, Stafford K, Turp M, Coles GC. Prevalence of nematode infection and faecal egg counts in free-range laying hens: relations to housing and husbandry. *Br Poult Sci.* 54:12–23; 2013. doi:10.1080/00071668.2012.757577.

27. McDougald LR. Internal parasites. In: Swayne DE, Boulianne M, Logue CM, McDougald LR, Nair V, Suarez DL, editors. *Diseases of poultry*. Ames (IA): Wiley-Blackwell. p. 1157–1191; 2020.

28. Imai RK, Barta JR. Distribution and abundance of Eimeria species in commercial turkey flocks across Canada. *Can Vet*. J60:153–159; 2019.

29. Cervantes HM, McDougald LR, Jenkins MC. Coccidiosis. In: Swayne DE, Boulianne M, Logue CM, McDougald LR, Nair V, Suarez DL, editors. *Diseases of poultry*. Ames (IA): Wiley-Blackwell. p. 1193–1217; 2020.

Table 3.1. Primers used to identify *Eimeria* spp. and roundworms.

Target species and gene	Direction	Primer sequence	Reference
Eimeria spp. of chickens, COI gene (outer primers)	Forward	ACA ATA GCT GTH YTA GGT GTW ACA	16
	Reverse	CTA CTC CTG TRG TAC CHC CW	16
Eimeria spp. of chickens, COI gene (inner primers)	Forward	ACA CTG ACG ACA TGG TTC TAC AGC TGC HTT TAA YGG TGA YCC	16
	Reverse	TAC GGT AGC AGA GAC TTG GTC TTT TCT ARR CCW ACD GTC ATC	16
Eimeria spp. of turkeys, COI gene (outer primers)	Forward	ACA ATH GCT GTH YTA GGT GTH ACM	This study
	Reverse	CTA CHC CAG TAG TAC CWC CW	This study
Eimeria spp. of turkeys, COI gene (inner primers)	Forward	ACA CTG ACG ACA TGG TTC TAC AGC CGC WTT YAA YGG TGA TCC	This study
	Reverse	TAC GGT AGC AGA GAC TTG GTC TTT TCT ARR CCH ACT GTC ATC	This study
Eimeria spp., 18S rRNA gene (outer primers)	Forward	CGG GTA ACG GGG AAT TAG GG	16
	Reverse	TAC GAA TGC CCC CAA CTG TC	16
Eimeria spp., 18S rRNA gene (inner primers)	Forward	ACA CTG ACG ACA TGG TTC TAC AAT TGG AGG GCA AGT CTG GTG	16
	Reverse	TAC GGT AGC AGA GAC TTG GTC TTG CTG CAG TAT TCA GGG CRA	16
Ascaridia spp., COI gene	Forward	ATT ATT ACT GCT CAT GCT ATT TTG ATG	18
	Reverse	CAA AAC AAA TGT TGA TAA ATC AAA GG	18

Figure 3.1. Sampling periods and ages of broiler (B1–B8), layer (L1–L4), and turkey (T1–T8) flocks included in the study.

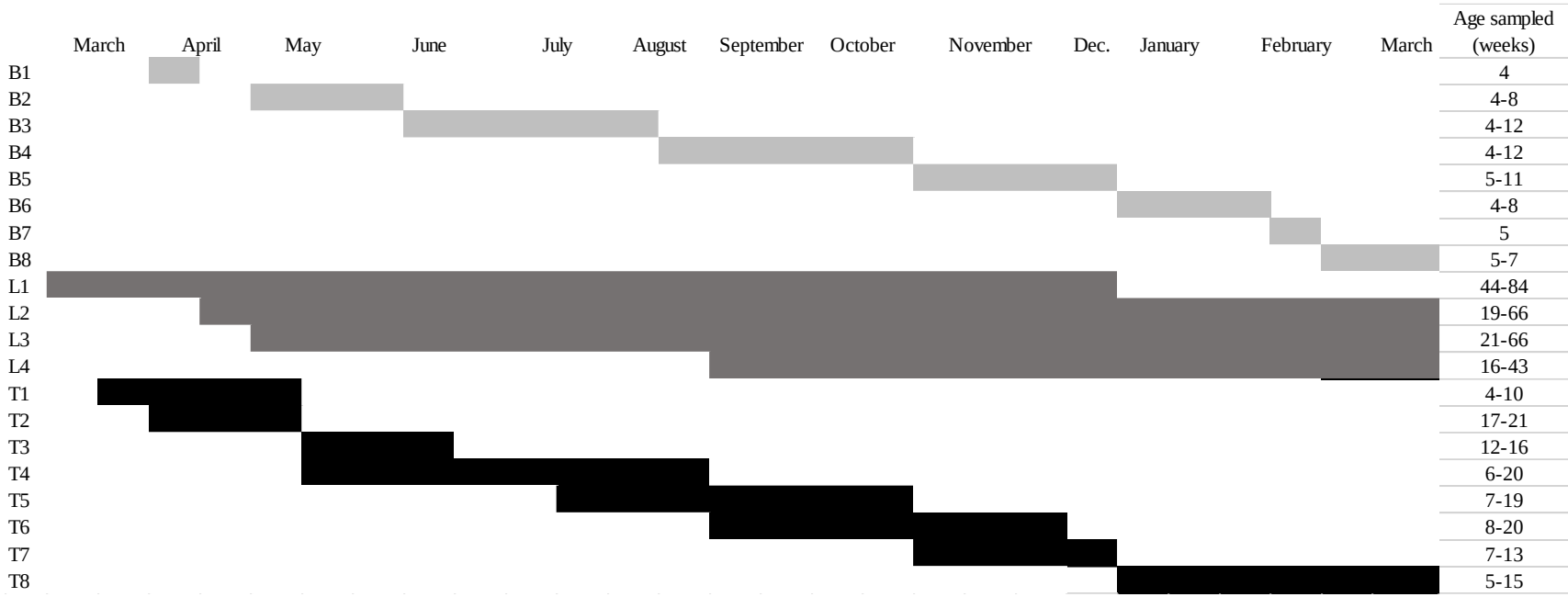
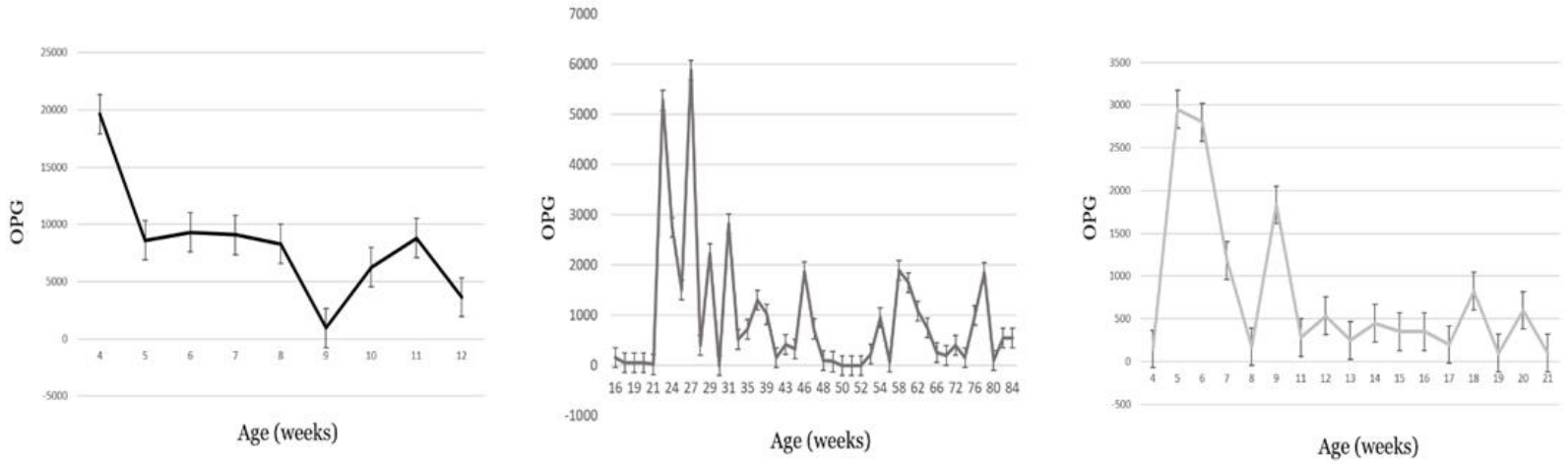
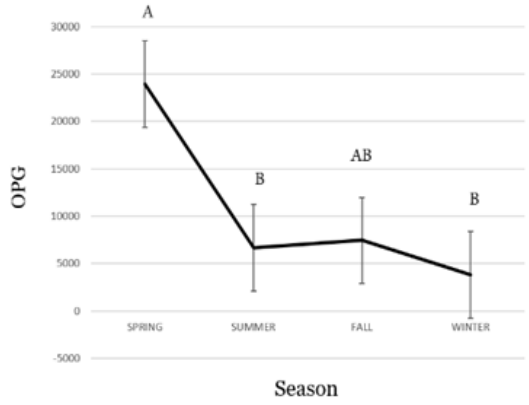


Figure 3.2. Mean counts of coccidia oocysts per gram (OPG) in feces of broiler (A), layer (B), and turkey (C) flocks organized according to age of the birds. Error bars show the standard error.

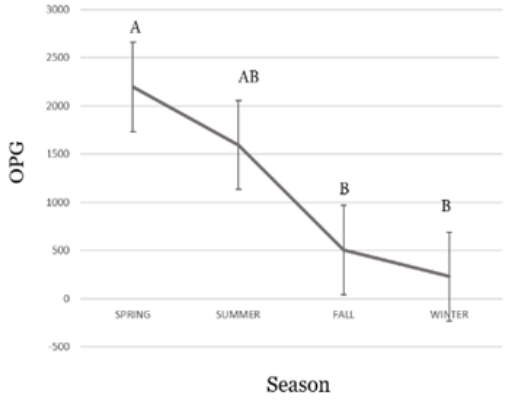


A B C

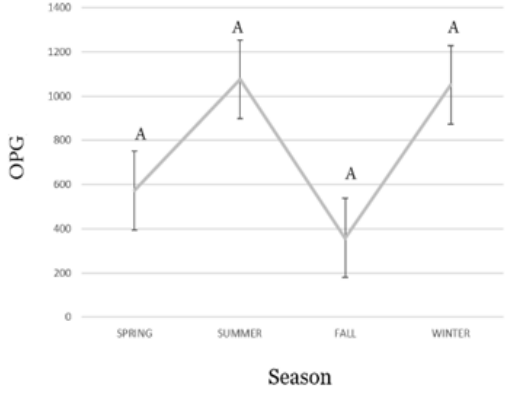
Figure 3.3. Mean counts of coccidia oocysts per gram (OPG) in feces of broiler (A), layer (B), and turkey (C) flocks in each season. Error bars show the standard error.



A



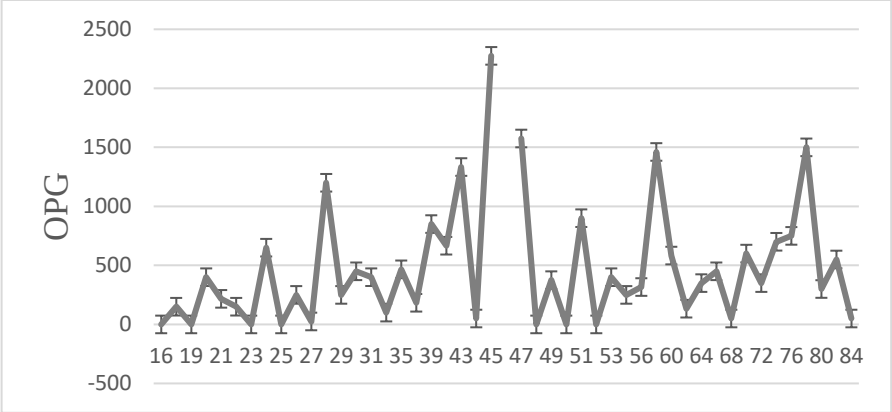
B



C

Figure 3.4. Mean counts of roundworm eggs per gram (EPG) in feces of layers organized according to bird's week of age (A) and for each season (B). Error bars show the standard error.

A)



B)

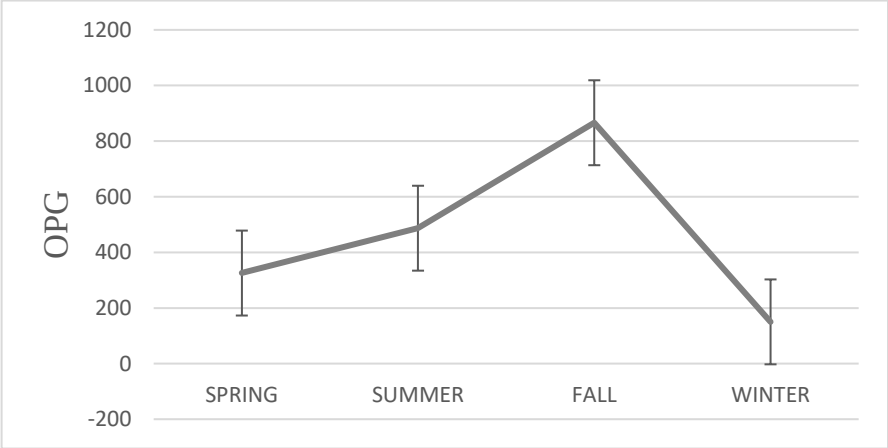


Figure 3.5. *Eimeria* spp. identified by next-generation sequencing of partial COI and 18S rRNA genes in fecal samples from four broiler flocks (B1–B4) and layer flocks (L1–L4). Several samples of flocks B3 and B4 taken at different time points were included. EA: *Eimeria acervulina*; EB: *Eimeria brunetti*; EMax: *Eimeria maxima*; EMit: *Eimeria mitis*; EMiv: *Eimeria mivati*; EN: *Eimeria necatrix*; ET: *Eimeria tenella*; Eprae: *Eimeria praecox*; +: detected; −: not detected, na: no amplicon obtained. Sequencing this part of the 18S rRNA gene cannot distinguish between ET and EN. Sequencing this part of the COI gene cannot distinguish between EMit and EMiv.

Flock	EA		EB		EMax		Emit	EMiv	EMit/ EMiv	EN/ ET	EN	ET	Eprae		OTU X		OTU Y		OTU Z	
	18S	COI	18S	COI	18S	COI	18S	18S	COI	18S	COI	COI	18S	COI	18S	COI	18S	COI	18S	COI
B1	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B2	-	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	+
B3a	+	+	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
B3b	+	na	+	na	+	na	+	+	na	+	na	na	-	na	-	na	-	na	-	na
B3c	-	-	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
B3d	-	-	-	-	-	-	+	+	-	+	-	+	-	-	-	-	-	-	-	-
B3e	-	-	-	+	-	+	+	+	+	-	-	-	+	-		-	-	-	-	-
B4a	-	+	-	-	+	+	-	-	-	+	-	-	-	-	-	+	-	+	-	+
B4b	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
B4c	-	-	-	-	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-
Total positive	5		4		8		8			7			1		1		1		2	
L1a	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	-
L1b	+	+	+	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-
L1c	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-
L2a	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+
L2b	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L2c	+	+	-	-	-	-	+	+	+	+	-	-	+	+	-	-	-	-	-	-
L2d	+	-	+	-	+	+	+	-	+	-	-	-	+	-	-	-	+	-	+	-
L2e	+	+	-	-	+	-	-	+	+	-	-	-	+	-	-	+	-	-	+	-
L2f	-	na	-	na	-	na	-	-	na	+	-	na	-	na	-	na	-	na	+	na
L2g	-	na	-	na	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L2h	-	-	-	-	-	+	+	+	+	+	-	-	+	+	-	-	-	-	-	-
L3	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+
L4	-	na	-	na	-	na	+	+	na	+	-	na	+	na	-	na	-	na	-	na
Total positive	7		3		5		9			4			7		1		2		6	

Figure 3.6. *Eimeria* spp. identified by next-generation sequencing of partial COI and 18S rRNA genes in fecal samples from four turkey flocks (T1–T6). Several samples of flocks T1 and T5 taken at different time points were included. EA: *Eimeria adenoides*; EG: *Eimeria gallopavonis*; EM: *Eimeria meleagridis*; +: detected; −: not detected, na: no amplicon obtained. Sequencing this part of the 18S rRNA gene cannot distinguish between EA, EG, and EM. Sequencing this part of the COI gene cannot distinguish between EA and AM.

Flock	EM, EA, EG	EM, EA	E. dispersa		E. innocua		E. meleagrimitis		E. gallopavonis	
	18S	COItur	18S	COItur	18S	COItur	18S	COItur	18S	COItur
T1a	+	Na	-	na	-	na	-	na	-	na
T1b	+	na	-	na	-	na	+	na	-	na
T2	-	na	-	na	-	na	-	na	-	na
T5a	+	+	-	+	-	-	+	+	-	+
T5b	+	+	-	-	-	-	-	+	-	+
T6	-	na	-	na	-	na	-	na	-	na
Total positive	6		1		0		4		2	

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Original Article

IV. INFECTION WITH *ASCARIDIA GALLI* DOES NOT SIGNIFICANTLY ALTER INTESTINAL MICRIBIOTA AND IS CLEARED AFTER CHANGES IN THE EXPRESSION OF CYTOKINES

Maria Tereza Terra-Long,[A] Andrea Pietruska,[B] Brigid A. McCrea,[C] and Ruediger Hauck[A,B,D]

[A]Department of Poultry Science, Auburn University, Auburn, AL 36849

[B]Department of Pathobiology, Auburn University, Auburn, AL 36849

[C]Alabama Cooperative Extension System, Auburn, AL 36849

[D]Corresponding author. E-mail: ruediger.hauck@auburn.edu

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ABSTRACT

Because of the trend of cage-free egg production, infections with the nematode *Ascaridia galli* are receiving increased attention. The aim of this study was to establish a timeline for the influence of *A. galli* on the expression of key cytokines related to a parasitic immune response, and on the composition of the jejunal microbiota. Twenty-eight male layer- type birds were challenged at 24, 25, and 26 days of age. An additional 28 birds were kept as uninfected controls. Starting on Day 31, three birds of each group were euthanized every week until 8 wk postinfection (PI). The number of larvae isolated from the intestinal wall decreased over time, until no larvae were seen at 7 and 8 wk PI. At 5 wk PI, there was a numerical upregulation of all cytokines (TGF-β, IFN-γ, IL-4, IL-8, IL-10, IL-13) in the infected group, but this change was only statistically significant for IL-13. At this time point, larvae were expected to have developed into adults that would have shed eggs in the feces. However, no adult worms were seen and there was no egg

shedding. For the microbiota analysis, there were significant differences in the alpha diversity (Faith's phylogenetic diversity) between challenge and control groups, and the beta diversity analysis showed slight differences between samples, suggesting that the age of the birds was the main reason for the separation of groups. These findings suggest that the upregulation of all cytokines evaluated in Week 5 might be the reason for resolution of the infection. Possible explanations are that a high infection dose and the fact that birds were fed with a more nutritionally dense feed might have contributed to the birds' immune system clearing the infection before the worms were able to reach maturity.

Key words: Nematodes, roundworms, cytokine expression, microbiota, layers, immunology

Abbreviations: bp = base pair; NCBI = National Center for Biotechnology Information; PI = post infection

INTRODUCTION

Ascaridia galli is an intestinal roundworm of chickens that affects the jejunum and can decrease egg production and negatively affect animal welfare. The worm also produces toxins that reduce the capacity of the animal to absorb nutrients (Vasilev *et al*., according to Gauly *et al.* [1]). The severity of infection can be correlated with increased mortality (2). The parasite's life cycle is direct, and birds are infected by the ingestion of fecal matter with embryonated eggs containing L2 larvae. After ingestion, the eggs release the larvae, which will migrate and penetrate the mucosa of the jejunum and settle while developing into adults. This histotrophic phase can last up to 7 wk. Adults are free in the lumen and can be found from 4 wk. post infection (PI) on. Egg shedding is expected to start 5 wk. PI. These eggs contain first-stage larvae that need to mature within the egg for 14–30 days in the environment (3).

Helminth infections are known to induce TH2 helper cell–based cytokine responses in mammals (4) and birds (5). This can manifest itself in an upregulation of IL-13 and IL-4, as well as influence the expression of other cytokines related to immune modulation, such as IL-10 (6).

Ascaridia galli can also have a negative effect on the jejunal microbiota, decreasing the microbiota diversity (7) and making birds more susceptible to infections with bacterial pathogens such as *Escherichia coli* and *Pasteurella multocida* (8,9).

A better understanding of the immune response to early stages of infections as well as its impact on the intestinal microbiota will be useful to develop alternative prophylactic methods and assess their efficacy. The aim for this study was to establish a timeline of how *A. galli* changes the expression of key cytokines related to a parasitic immune response and the composition of the jejunal microbiota early after infection.

MATERIALS AND METHODS

Preparation of the inoculum. Worm eggs from a naturally infected layer flock were purified using a process similar to Tarbiat *et al.* (10). Briefly, the feces were flushed with tap water through sieves with pore sizes of 212, 90, and 38 μm. Five milliliters of retained material in the last sieve were added to 45 ml of saturated sodium chloride. The suspension was centrifuged (420 × *g*, 5 min) and the supernatant was flushed with tap water through a 38-μm sieve. The material retained containing the eggs was finally suspended with distilled water at a concentration between 200 and 250 eggs/ml. These eggs were embryonated for 20 days at room temperature with frequent aeration, after which the embryonation rate was about 72%. For the challenge, the concentration was adjusted to 500 embryonated eggs/dose.

Birds and experimental design. Fifty-six male layer type birds were purchased as 1-day-old chicks from a commercial hatchery (Ideal Poultry, Cameron, TX) and placed into eight battery

cages with seven birds each. Birds were fed with a standard broiler starter diet during the entire experiment. Birds in four cages were challenged by oral gavage with 500 embryonated *A. galli* eggs when 24, 25, and 26 days old. Starting on Day 31 of age, that is, 1 week PI, three birds of each group were euthanized (six birds per time point) every week. Eight weeks PI, all remaining birds were euthanized and sampled. Animal care and experimental procedures were performed in compliance with all federal and institutional animal use guidelines and approved by the Auburn University Institutional Animal Care and Use Committee (PRN 2019–3449).

Sampling. Birds were euthanized using carbon dioxide followed by cervical dislocation. The gastrointestinal tract was removed, and the jejunum was isolated. The jejunum was opened in a longitudinal direction. The contents were first inspected for presence of adult worms. Approximately 10 cm upstream of Meckel's diverticulum, 1 g of jejunal wall was collected and stored for 24 hr. in RNAlater (Qiagen, Hilden, Germany) at 4 C. After that, RNAlater was discarded, and the sample was stored at −80 C for determination of the relative gene expression.

All jejunal content was carefully collected using plastic spoons in order to minimize contamination with chicken DNA. This content was immediately placed on ice and then stored at −80 C for microbiome analysis. The rest of the wall was used for larval recovery right after the sample collection.

Larval recovery from the intestinal content and wall. Larvae were recovered after pepsin–HCl digestion method as described by Ferdushy *et al*. (11). Digestion buffer was prepared by mixing 1 L of 42 C tap water, 10 ml of HCl (37%) and 10 ml of a 1:10,000 pepsin solution (VWR, Radnor, PA). The jejunal wall was cut into small pieces of 0.5 cm and digested with 200 ml digestion buffer under constant stirring of 250 rpm at 38 C for 90 min or until full digestion of the tissue. After the digestion, the liquid was poured through 180- and a 25-μm sieve. The larvae

retained in the 25-μm sieve were washed with tap water and stored in 70% ethanol until counting under a stereo microscope. To confirm infection, DNA from larvae recovered 1 wk PI was isolated, and a 533–base pair (bp) fragment of the COI gene was amplified using the Q5 High-Fidelity Kit (Qiagen). The PCR product was purified using the Qiaquick PCR purification kit (Qiagen) and submitted to the Massachusetts General Hospital DNA Core Facility for forward and reverse Sanger sequencing. The BLAST search algorithm (12) was used to compare the obtained sequence with homolog sequences available from the National Center for Biotechnology Information (NCBI) database.

Fecal egg count. Starting 3 wk PI, around 20 g of feces per cage were collected weekly and investigated for *A. galli* eggs. The parasitological exam was done using 1 g of the homogenized feces and 29 ml of saturated sodium chloride solution that were poured through a strainer. A McMaster chamber was used for the evaluation of the filtrated suspension under the microscope.

Relative gene expression. RNA was extracted from 15 mg jejunal wall with the Qiagen RNeasy kit (Qiagen) according to the manufacturer's instructions. DNA was digested on the column during RNA extraction with the QIAGEN RNase-Free DNase Set (Qiagen). Total RNA was transcribed into cDNA using the Lunascript kit (New England Biolabs, Ipswich, MA). The cDNA was diluted 1:3 in RNAse-free water, and partial TGF-β, IFN-γ, IL-4, IL-8, IL-10, IL-13, GAPDH, and HMBS genes were amplified in duplicates by qPCR using the Fast SYBR Green Master Mix (Qiagen). Cycling parameters consisted of an initial 10 min at 95 C denaturation cycle; 40 cycles of denaturation for 30 sec at 95 C, annealing for 1 min at 60 C and extension at 72 C for 30 sec. Specificity of PCR products was confirmed by melting curve analysis. Primers and their efficiencies are listed in Table 1.

To obtain primer efficiencies a standard curve was calculated for each gene with a twofold dilution. The gene expression was calculated relative to the expression of two housekeeping genes (GAPDH and HMBS) and the average of the uninfected birds as calibrator as described by Vandesompele *et al.* (13) and Hellemans *et al.* (14).

Microbiome analyzes. DNA was extracted from 200 µl jejunal content using the QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's instructions. Between 400 and 450 bp of the bacterial 16S rRNA gene were amplified by PCR using primers 515F with linker CS1and 926R with CS2 (15) using the Taq PCR Master Mix Kit (Qiagen) as described by Hamilton *et al.* (16). PCR products were checked by agarose gel electrophoresis and submitted to the University of Illinois at Chicago DNA Services Facility for next-generation amplicon sequencing on the Illumina MiSeq. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (accession number: PRJNA830517).

For analysis, the metadata file was validated by KEEMEI (17) and demultiplexed paired-end sequence reads were imported into QIIME2 2021.4 (18). Dada2 was used for primer trimming, truncating the sequences at 240 bp forward 235 bp reverse and denoising. Relative abundance tables were obtained using Silva database (19) after training the classifiers with the sequences. Alpha diversity was measured by Faith's phylogenetic diversity metric, Shannon entropy, and Pielou's evenness. Beta diversity was determined by Bray-Curtis dissimilarity, Jaccard similarity index, weighted unnormalized UniFrac, and unweighted UniFrac. Principal component analysis was used to visualize differences.

Statistical analysis. Normality was checked by Shapiro's test and frequency histograms. Relative gene expression values were log2 transformed and analyzed by two-way ANOVA with time and treatment (challenged and nonchallenged) as factors using R (20). The mean values were

compared using Tukey's honestly significant difference test. In addition, one-way ANOVA was used for each time point in order to identify differences between treatments. Relative abundance of taxa was compared by Kruskal-Walli's test, as data were not normal. The other differences between time points and treatments in the microbiome were analyzed using QIIME2. For the alpha diversity metrics: Faith's phylogenetic diversity was analyzed with a two-way ANOVA, and Shannon entropy and Pielou's evenness were compared by Kruskal-Walli's test. For the beta diversity metrics: Jaccard index and weighted unnormalized UniFrac were analyzed using PERMANOVA, and unweighted UniFrac and Bray-Curtis were analyzed using the Adonis test. $P \leq 0.05$ was considered statistically significant and $0.05 < P < 0.10$ indicated trends.

RESULTS AND DISCUSSION

Larval recovery from the intestinal wall and fecal egg count. One week PI, the average number of larvae recovered from the jejunal wall was highest with 38.6 larvae per bird. The number decreased in the second week PI to 14.2 larvae per bird and remained at a similar level until the fourth week PI. During this time, all samples were positive for *A. galli* larvae. The last time point when all samples containing larvae was the fifth week PI with an average number of 7.3 per bird (Fig. 4.1). In the sixth week PI, only in one out of three samples larvae were found. No larvae were recovered from the control birds during the entire experiment. The gradual decrease of the larval counts in the infected group are not surprising because, after infection with *A. galli* eggs, larvae take about 5 wk to develop into adult worms and can be found in the jejunal wall for up to 7 wk. The gradual reduction of the number of larvae over time had already been observed in other studies (21). However, there was no egg shedding in feces during the experiment, and adult worms were not observed until the end of the experiment, which suggested that initially the infection was established, but the development of the worms was interrupted.

Relative gene expression. The relative gene expression of the cytokines is shown in Fig. 4.2. Two-way ANOVA showed that expression levels differed significantly between treatments for IL-13 and TGF-β. However, *post hoc* tests did not identify specific significant differences. Therefore, the expression levels were analyzed by one-way ANOVA for each time point. Expression of IL-13 was significantly upregulated in infected birds 1 wk. PI ($P = 0.008$), 2 wk. PI ($P = 0.007$), and 5 wk. PI ($P = 0.047$). Expression of TGF-β was significantly upregulated in infected birds 1 week PI ($P = 0.028$). A trend of upregulation in infected birds was observed for IL-13 3 wk. PI ($P = 0.081$) and for downregulation of IFN-γ 3 wk. PI ($P = 0.086$). Because there were some outliers and the sample size was reflecting the pilot character of the experiment with only three birds per group and week, there were not many statistically significant differences.

Numerically, the main differences happened at the first and fifth week PI for all cytokines. At Week 1 PI, there was an upregulation of IL-8, IL-13, and TGF-β. This can be explained by the immune response to the larval invasion of the jejunal wall that happens during the first week PI. As a result, there was an upregulation of proinflammatory cytokine (IL-8) and a Th2-response cytokine (IL-13). In addition, a regulatory cytokine (TGF-β) was upregulated.

Helminths are known to stimulate not only the Th2 response, but they can also impact Th1 responses by downregulating their expression, here represented by IFN-γ (8,22). This effect was seen 2, 3, and 4 wk. PI when expression of IL-13 was upregulated, while expression of IFN-γ was downregulated. The inhibition of Th1 response caused by helminths has been investigated in other species such as *Ascaris suum*, and studies suggested that proteins from these parasites could potentially have therapeutic applications on autoimmune diseases (23).

The number of larvae isolated in the wall was reduced with time and no adult worms were detected in the jejunum. The observed abrupt response by the immune system might have been

caused by the infection scheme used. Although a dose of 500 embryonated eggs is not considered high, three repeated doses might have resulted in higher numbers of larvae that stimulated a stronger immune system. In addition, Ikeme *et al*. (24) noticed that a higher challenge dose does not result in higher number of larvae and adults and that this effect might be explained by competition between larvae for resources and space.

Furthermore, through the entire experiment the birds were fed a broiler diet with a higher nutrition density than a typical layer diet. A broiler diet will have on average 22% of crude protein and 3100 kcal/kg of energy (25), and a diet for 6-wk-old pullets will have an average 17.5% of crude protein and 2900–3000 kcal/kg of energy (26). It is known that nutrition can affect the immune system and amino acids such as threonine, arginine, and glutamine could be potentially important on birds' recovery after an injury (27). Therefore, it is likely that the high infection dose in conjunction with a higher than needed supply of energy and protein might have helped the immune system to clear the infection. After the fifth week PI, when the infection was cleared, the differences in the expression of cytokines between challenged and nonchallenged groups were reduced.

Microbiome analyses. A total of 971,330 reads were analyzed. The most common bacterial phylum present in the jejunum of nonchallenged and challenged birds was Firmicutes representing, 56% and 57% of the relative abundance, respectively. This phylum is composed by orders such as Lactobacillales, Bacillales, or Clostridiales. Cyanobacteria were the second most abundant phylum (36% and 31.8%, respectively). Firmicutes and Cyanobacteria have been found among the most common phyla in the jejunum of chickens before (28,29). The significance of these bacteria for the chicken microbiota is unknown. In unchallenged birds, Proteobacteria accounted for 5% of the relative abundance. The phylum Proteobacteria include bacteria from the

orders Enterobacteriales, Pseudomonadales, and Betaproteobacteriales. In the challenged birds, the relative abundance of Proteobacteria was reduced to 3%. None of the other phyla had a relative abundance of more than 1% (Fig. 4.3).

At the level order, differences between treatments were seen in the first 3 wk. when the challenged birds had a higher proportion of *Lactobacialales* and between 4 and 5 wk. PI when the opposite occurred, but only between 4 and 5 wk. PI, the difference between challenged and nonchallenged was statistically significant ($P = 0.037$; Fig. 4.4). After 5 wk., when the infection was cleared, the microbiota from both groups became more similar to each other again. According to Amit-Romach (30), the relative abundance of *Lactobacillus* spp. tends to increase with age. The results show the same trend of increasing Lactobacilli in the control group, but not between 4 and 5 PI in the challenged group. Notably, the differences in the microbiota did not persist after the infection was cleared, unlike after infection with coccidia (16).

Faith's phylogenetic diversity metric measures the richness of a community and was used to quantify the phylogenetic alpha diversity. It showed that differences between samples were explained by weeks PI ($P = 0.001$) and by the interaction between weeks PI and treatments ($P = 0.02$). Challenged animals tended to show a lower alpha diversity than unchallenged birds in Weeks 1 and 2, and there was a statistically significant difference between the groups in Week 2 PI ($P = 0.005$). Also, there was a tendency of reduction of diversity as birds get older (Fig. 4.5). The ANOVA of Pielou's evenness and Shannon diversity index of the bacterial communities showed no significant differences between challenged and unchallenged groups (data not shown).

The principal component analysis based on Jaccard similarity index showed a clear separation of the data into two groups (Fig. 4.6). The main reason for this division was the age of the birds ($P = 0.002$). During Weeks 1–3 PI and Weeks 6– 8 PI, birds tended to cluster together

irrespective of their infection status. Moreover, there was a balanced distribution of challenged and nonchallenged communities confirming that infection status did not influence the differences and similarities between communities. The unweighted UniFrac also showed statistically significant differences between birds of different ages ($P = 0.015$). A Bray-Curtis dissimilarity test and weighted unnormalized UniFrac showed no significant differences.

CONCLUSIONS

The aim for this study was to establish a timeline how *A. galli* changes the expression of key cytokines and the composition of the jejunal microbiota. Three different phases of the immune response were seen: Weeks 1–3 PI, when there was a moderate stimulation of the immune system by worm larvae; Weeks 4 and 5 PI, when the immune system showed a severe reaction that resulted in complete elimination of the worms; and Weeks 6 and 7 PI, when the birds had a full recovery. This loosely coincided with differences in the microbiota, where some differences in the relative abundance of *Lactobacialales* and in the alpha diversity between infected and noninfected birds were seen until Week 5 PI.

The interaction between the immune system and the parasites seems straightforward: First, the infection stimulated the immune system, and in the second phase a severe immune answer cleared the infection. In contrast, it is open to speculation why the microbiota changed. Possible mechanisms are the leakage of nutrients from damaged intestinal walls as shown for coccidia or because of the changed immune system (31).

The major limitation of the experiment was the small sample size on each time point, reflecting its character as a pilot experiment. However, it is clear that 5 wk. PI was a crucial week in the pathogenesis of the infection and further experiments about the interaction between host, parasite, and the bacterial microbiota should focus on this time point.

REFERENCES

1. Gauly M, Homann T, Erhardt G. Age-related differences of *Ascaridia galli* egg output and worm burden in chickens following a single dose infection. *Vet Parasitol*. 128:141–148; 2005. doi: 10.1016/j.vetpar.2004.11.023.

2. Hinrichsen LK, Labouriau R, Engberg RM, Knierim U, Sørensen JT. Helminth infection is associated with hen mortality in Danish organic egg production. *Vet Rec*. 179:196; 2016. doi:10.1136/vr.103614.

3. McDougald LR. Internal parasites. In: Swayne DE, Boulianne M, Logue CM, McDougald LR, Nair V, Suarez DL, editors. *Diseases of poultry*. Ames (IA): Wiley-Blackwell. pp. 1157–1191; 2020.

4. Little MC, Bell LV, Cliffe LJ, Else KJ. The characterization of intraepithelial lymphocytes, lamina propria leukocytes, and isolated lymphoid follicles in the large intestine of mice infected with the intestinal nematode parasite *Trichuris muris*. *J Immunol*. 175:6713–6722; 2005. doi:10.4049/jimmunol.175.10.6713.

5. Degen WGJ, Daal N van, Rothwell L, Kaiser P, Schijns VEJC. Th1/Th2 polarization by viral and helminth infection in birds. *Vet Microbiol*. 105:163–167; 2005. doi: 10.1016/j.vetmic.2004.12.001.

6. Pleidrup JA, Norup LR, Dalgaard TS, Kaiser P, Permin A, Schou TW, Vadekær DF, Jungersen G, Sørensen P, Juul-Madsen HR. Cytokine gene expression profiles in chicken spleen and intestinal tissues during *Ascaridia galli* infection. *Vet Parasitol*. 206:317–321; 2014. doi: 10.1016/j.vetpar.2014.10.016.

7. Okulewicz A, Złotorzycka J. Connections between *Ascaridia galli* and the bacterial flora in the intestine of hens. *Angew Parasitol*. 26:151–155; 1985.

8. Dahl C, Permin A, Christensen JP, Bisgaard M, Muhairwa AP, Petersen KMD, Poulsen JSD, Jensen AL. The effect of concurrent infections with *Pasteurella multocida* and *Ascaridia galli* on free range chickens. *Vet Microbiol*. 86:313–324; 2002. doi:10.1016/s0378-1135(02)00015-9.

9. Wuthijaree K, Lambertz C, Gauly M. Prevalence of gastrointestinal helminth infections in free-range laying hens under mountain farming production conditions. *Br Poult Sci*. 58:649–655; 2017. doi:10.1080/00071668.2017.1379049.

10. Tarbiat B, Rahimian S, Jansson DS, Halvarsson P, Höglund J. Developmental capacity of *Ascaridia galli* eggs is preserved after anaerobic storage in faeces. *Vet Parasitol*. 255:38–42; 2018. doi: 10.1016/j.vetpar.2018.03.025.

11. Ferdushy T, Nejsum P, Roepstorff A, Thamsborg SM, Kyvsgaard NC. *Ascaridia galli* in chickens: intestinal localization and comparison of methods to isolate the larvae within the first week of infection. *Parasitol Res*. 111:2273–2279; 2012. doi:10.1007/s00436-012-3079-3.

12. Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL. NCBI BLAST: a better web interface. *Nucleic Acids Res*. 36:W5–9; 2008. doi:10.1093/nar/gkn201.

13. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 3:research0034.1; 2002. doi:10.1186/gb-2002-3-7-research0034.

14. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol*. 8:R19; 2007. doi:10.1186/gb-2007-8-2-r19.

15. Walters W, Hyde ER, Berg-Lyons D, Ackermann G, Humphrey G, Parada A, Gilbert JA, Jansson JK, Caporaso JG, Fuhrman JA, *et al*. Improved bacterial 16S rRNA gene (V4 and V4-5)

and fungal internal transcribed spacer marker gene primers for microbial community surveys. *mSystems* 1:e00009–15; 2016. doi:10.1128/mSystems.00009-15.

16. Hamilton M, Ma X, McCrea BA, Carrisosa M, Macklin KS, Zhang C, Wang X, Hauck R. Influence of *Eimeria* spp. Infection on chicken jejunal microbiota and the efficacy of two alternative products against the infection. *Avian Dis*. 64:123–129; 2020. doi:10.1637/0005-2086-64.2.123.

17. Rideout JR, Chase JH, Bolyen E, Ackermann G, González A, Knight R, Caporaso JG. Keemei: cloud-based validation of tabular bioinformatics file formats in Google Sheets. *GigaScience* 5:27; 2016. doi:10.1186/s13742-016-0133-6.

18. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, *et al.* Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol*. 37:852–857; 2019. doi:10.1038/s41587-019-0209-9.

19. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res*. 41:D590–596; 2013. doi:10.1093/nar/gks1219.

20. Team R. RStudio: integrated development for R. RStudio.

21. Ferdushy T, Luna-Olivares LA, Nejsum P, Roepstorff AK, Thamsborg SM, Kyvsgaard NC. Population dynamics of *Ascaridia galli* following single infection in young chickens. *Parasitology* 140:1078–1084; 2013. doi:10.1017/S0031182013000401.

22. Maizels RM, Yazdanbakhsh M. Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nat Rev Immunol*. 3:733–744; 2003. doi:10.1038/nri1183.

23. Arora P, Moll JM, Andersen D, Workman CT, Williams AR, Kristiansen K, Brix S. Body fluid from the parasitic worm *Ascaris suum* inhibits broad-acting pro-inflammatory programs in dendritic cells. *Immunology* 159:322–334; 2020. doi:10.1111/imm.13151.

24. Ikeme MM. Effects of different levels of nutrition and continuing dosing of poultry with *Ascaridia galli* eggs on the subsequent development of parasite populations. *Parasitology* 63:233–250; 1971. doi:10.1017/s0031182000079555.

25. Aviagen. Ross Broiler—Nutrition Specifications. 2019.

26. Hy-Line. W-36 Commercial Layers—Management Guide. https://www.hyline.com/filesimages/Hy-Line-Products/Hy-Line-Product-PDFs/W-36/36%20COM%20ENG.pdf last accessed 5/11/2022 2020.

27. Bortoluzzi C, Rochell SJ, Applegate TJ. Threonine, arginine, and glutamine: influences on intestinal physiology, immunology, and microbiology in broilers. *Poult Sci*. 97:937–945; 2018. doi:10.3382/ps/pex394.

28. Bhogoju S, Nahashon S, Wang X, Darris C, Kilonzo-Nthenge A. A comparative analysis of microbial profile of Guinea fowl and chicken using metagenomic approach. *PLOS ONE*13:e0191029; 2018. doi: 10.1371/journal.pone.0191029.

29. Xiao Y, Xiang Y, Zhou W, Chen J, Li K, Yang H. Microbial community mapping in intestinal tract of broiler chicken. *Poult Sci*. 96:1387–1393; 2017. doi:10.3382/ps/pew372.

30. Amit-Romach E, Sklan D, Uni Z. Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. *Poult Sci*. 83:1093–1098; 2004. doi:10.1093/ps/83.7.1093.

31. Hauck R. Interactions between parasites and the bacterial microbiota of chickens. *Avian Dis*. 61:428–436; 2017. doi:10.1637/11675-051917-Review.1.

32. Katakam KK, Nejsum P, Kyvsgaard NChr, Jørgensen CB, Thamsborg SM. Molecular and parasitological tools for the study of *Ascaridia galli* population dynamics in chickens. *Avian Pathol*. 39:81–85; 2010. doi:10.1080/03079451003599284.

33. Dalgaard TS, Skovgaard K, Norup LR, Pleidrup J, Permin A, Schou TW, Vadekær DF, Jungersen G, Juul-Madsen HR. Immune gene expression in the spleen of chickens experimentally infected with *Ascaridia galli*. *Vet Immunol Immunopathol*. 164:79–86; 2015. doi: 10.1016/j.vetimm.2015.01.003.

34. Wang L, Li L, Lv Y, Chen Q, Feng J, Zhao X. *Lactobacillus plantarum* restores intestinal permeability disrupted by Salmonella infection in newly-hatched chicks. *Sci Rep*. 8:2229; 2018. doi:10.1038/s41598-018-20752-z

35. Zhang J, Gao Y-Y, Huang Y-Q, Fan Q, Lu X-T, Wang C-K. Selection of housekeeping genes for quantitative gene expression analysis in yellow-feathered broilers. *Ital J Anim Sci*. 17:540–546; 2018. doi:10.1080/1828051X.2017.1365633.

Table 4.1. Primers used to identify roundworms, cytokines, and housekeeping genes.

Target gene	Forward primer	Reverse primer	Primer efficiency	Amplicon size (bp)	Reference
Nematode COI	ATT ATT ACT GCT CAT GCT ATT TTG ATG	CAA AAC AAA TGT TGA TAA ATC AAA GG	Not applicable	533	(32)
Chicken IL-8	GCA AGG TAG GAC GCT GGT AA	GCG TCA GCT TCA CAT CTT GA	1.01	106	(33)
Chicken IL-13	AAG GAC TAC GAG CCC CTC AT	GCA AGA AGT TCC GCA GGT AG	0.81	100	(33)
Chicken IL-10	AAG AGG AGC AAA GCC ATC AA	TGG CTT TGT AGA TCC CGT TC	0.87	73	(33)
Chicken IFN-γ	CAG ATG TAG CTG ACG GTG GA	CAT CGA AAC AAT CTG GCT CA	0.84	98	(33)
Chicken TGF-β	CGA CCT CGA CAC CGA CTA CT	CCA CTT CCA CTG CAG ATC CT	0.97	103	(33)
Chicken GAPDH	TGG AGA AAC CAG CCA AGT AT	GCA TCA AAG GTG GAG GAA T	1.07	145	(34)
Chicken HMBS	GAT GGA TCC GAT AGC CTG AA	GAT GTG CTT AGC TCC CTT GC	1.01	195	(35)

Figure 4.1. Average number of *Ascaridia galli* larvae per bird recovered by pepsin-HCl digestion of the jejunal wall of male layer-type birds challenged with *A. galli* (*n* = 3 per group and time point).

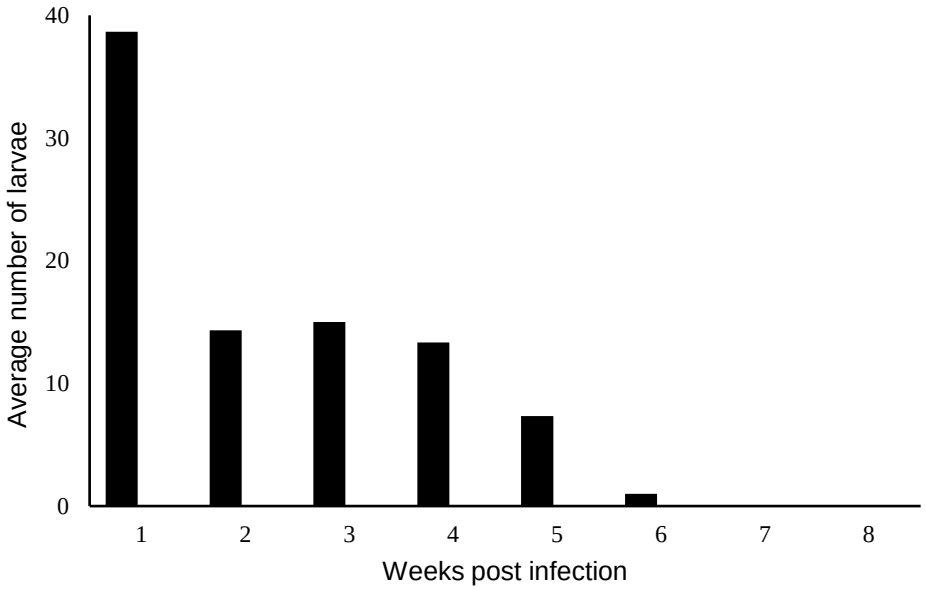


Figure 4.2. Relative gene expression of IL-8 (a), IL-13 (b), IFN-γ (c), TGF-β (d), and IL-10 (e) in the jejunum of male-layer-type birds on weeks in the jejunum 1–8 wk. PI. The gene expression was normalized by housekeeping genes (GAPDH and HMBS) and expressed relative to the average of the uninfected birds. Light red boxes represent birds challenged with embryonated eggs of *A. galli* while green boxes represent control birds (n = 3 per group and time point). *Indicate statistically significant differences between groups ($P \leq 0.05$). **Indicate trends ($0.05 < P < 0.10$).

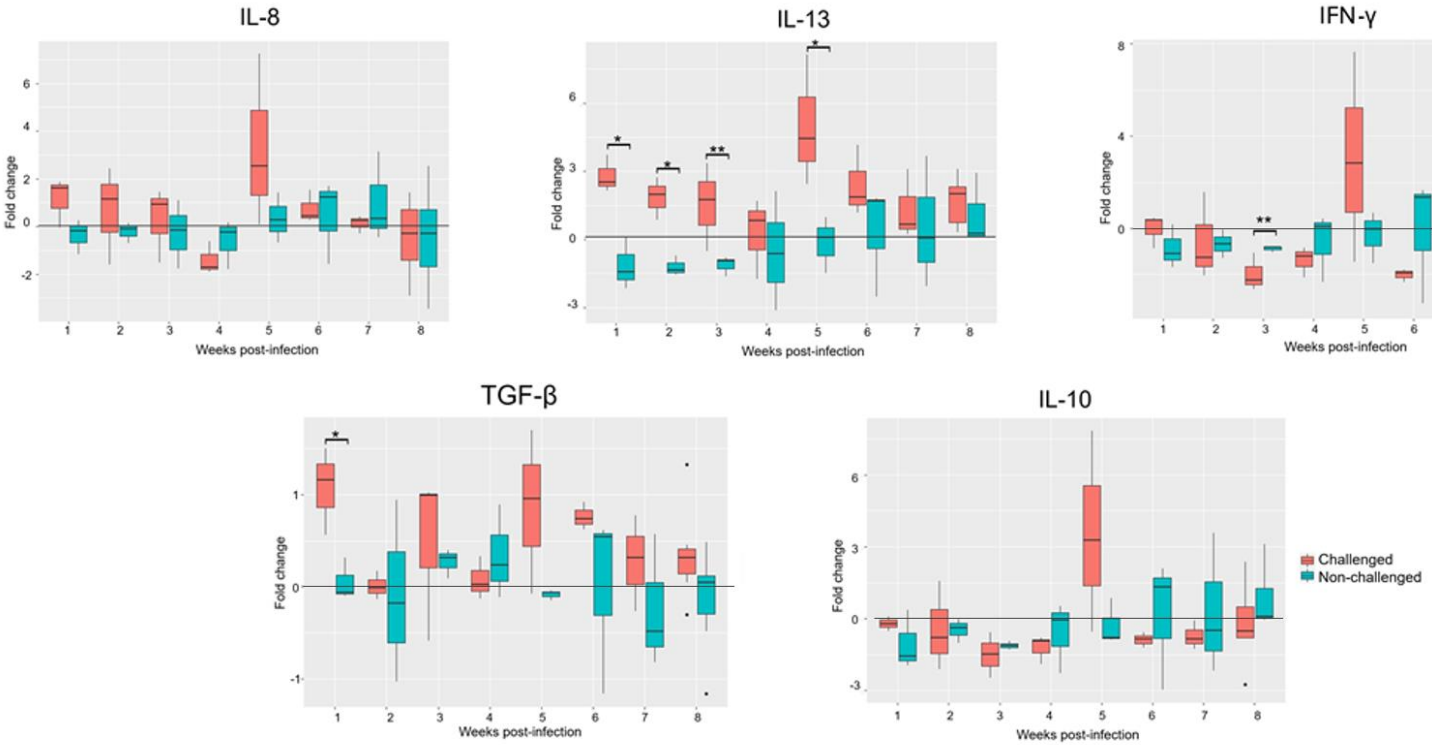


Figure 4.3. Average of the relative abundance of phyla isolated from jejunal content of male layer-type birds. The left column represents the control group and the right column animals challenged with *A. galli*. Three samples per group were collected weekly from Week 1 to Week 8 PI.

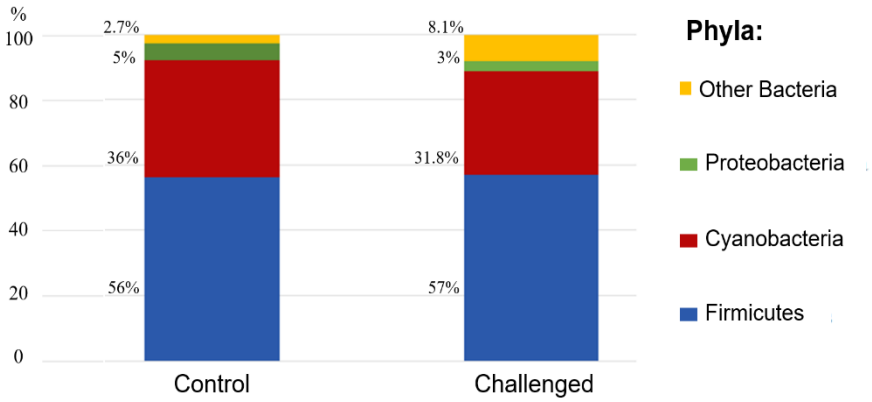


Figure 4.4. Average of the relative abundance of bacterial orders detected in the jejunal content of male layer-type birds. Challenged groups where infected with *A. galli*. Three samples per group were collected weekly from Week 1 to Week 8 PI.

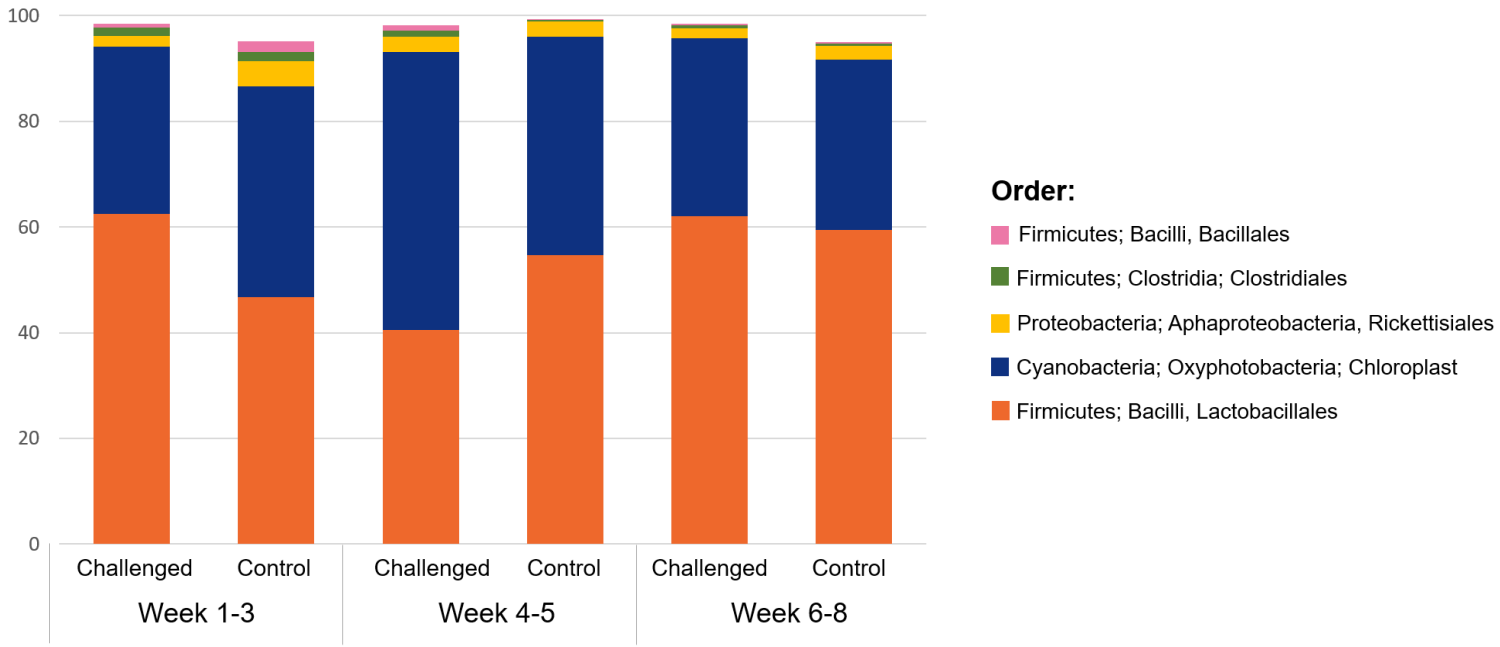


Figure 4.5. Faith's phylogenetic diversity metric (alpha diversity) of jejunal microbiota in the jejunum content of male layer-type birds. Orange boxes represent birds challenged with embryonated eggs of *A. galli* while blue boxes represent control birds. Three samples per group were collected weekly from Week 1 to Week 8 PI. *Indicate statistically significant differences between groups ($P \leq 0.05$).

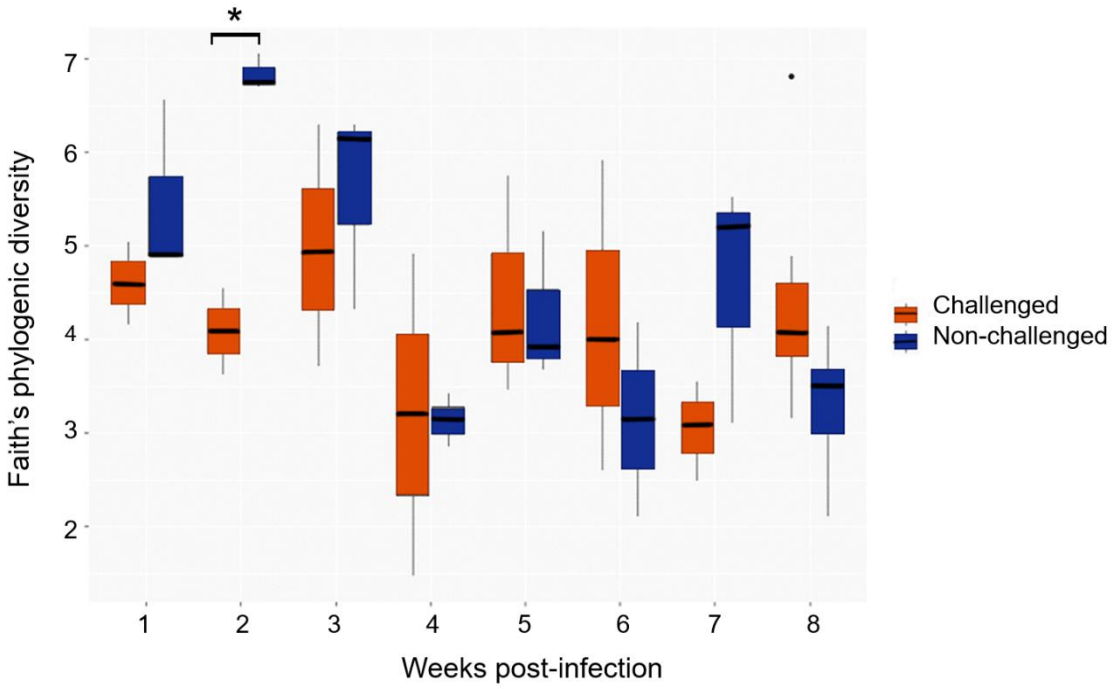
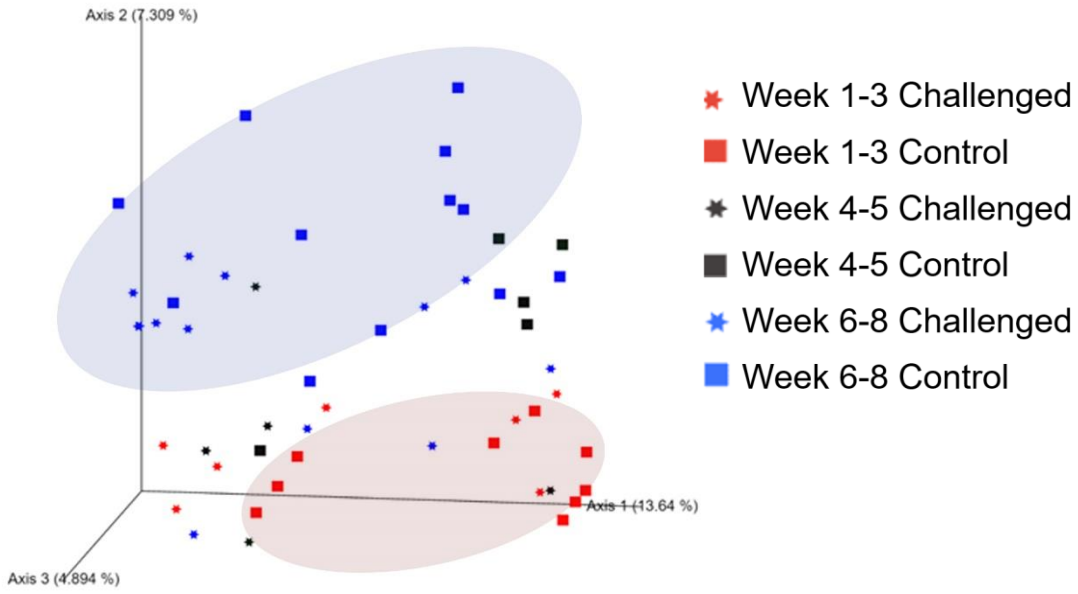


Figure 4.6. Principal component analysis of microbiota identified in the jejunum of content of male layer-type birds based on the Jaccard similarity index. Stars represent birds that were challenged with *Ascaridia galli* and squares represent control birds. Time point after infection is indicated by color. The age of the birds indicated by the shaded areas had a significant effect on the b diversity.



V. THE EFFECT OF ALTERNATIVE TREATMENTS *AGAINST ASCARIDIA GALLI* ON NUTRIENT DIGESTIBILITY, EGG PRODUCTION AND QUALITY, GENE EXPRESSION OF CYTOKINES, AND JEJUNAL MICROBIOTA IN INFECTED LAYING HENS

ABSTRACT

Ascaridia galli is a nematode commonly found in chickens in cage-free production systems. The aim of this study was to test if *Artemisia absinthium* (0.02% inclusion) and pumpkin seed (1% inclusion) can be used as treatments and alleviate potential negative effects caused by an *Ascaridia galli* infection on egg production and quality, nutrient digestibility, intestinal microbiota, and cytokine gene expression. Day-old layer-type birds were raised on the floor until 12 weeks of age when they were moved to cages. At 16 weeks, birds were divided into 3 groups. One group was left untreated, while artemisia and pumpkin seed were added to the feed. At 25 weeks, 8 birds from each treatment were necropsied, and half of the remaining birds were challenged with 250 embryonated *A. galli* eggs. Performance data was recorded daily. As soon as birds reached 50% of production, eggs were collected weekly for evaluation of quality. Fecal exams were conducted weekly to check for worm eggs. At 35 weeks, all birds were euthanized, and the jejunum was checked for adult worms. Digestibility analyses evaluated apparent ileal digestible energy (kcal/kg), crude protein (%), calcium (%) and phosphorus (%). Total RNA was extracted from jejunal wall for determination of expression of IL-8, INF- γ, IL-13, TGF-β4 and IL-10 genes by qPCR. Jejunal content was collected for investigation of the microbiota by 16S rRNA gene amplicon next generation sequencing. There were no differences in worm egg shedding and worm burden between treatments. For all nutrients, the digestibility was lower in birds challenged with *A. galli*. Birds fed with the control diet, when challenged, had lower calcium digestibility than

birds fed with either pumpkin or artemisia. However, there was no impact of infection status or treatment on egg quality and production. The jejunal microbiota of treated birds showed an increase on Lactobacillales. Our findings suggest none of the treatments were effective against *Ascaridia galli* at the used doses but had minor effects on alleviating the calcium digestibility reduction seen on challenged birds.

Keywords: Nematodes, roundworms, laying hens, pumpkin seed, artemisia, digestibility, egg quality, cytokine expression, microbiota,

INTRODUCTION

Ascaridia galli is a nematode commonly found in chickens in cage-free production systems (1). Adult worms are found free in the jejunum of chickens. They can compete with the host and the microbiome for nutrients. Because of that, infections with *A. galli* can potentially result in decrease of bird's performance reducing nutrient absorption and digestibility and negatively affecting egg production or quality (2). These parasites can also impair animal welfare by obstructing partially or totally the intestinal lumen (3).

In many countries, benzimidazoles are the only drug family of which compounds are approved to treat nematodes in commercial flocks of layers with no withdrawal period for eggs (4). The lack of drug options raises concerns regarding the possibility of resistance against benzimidazoles (5). The need of multiple treatments on the same flock was already described on chicken infected with *A. galli* (6). Additionally, many veterinarians also reported the need of multiple treatments against *Ascaridia dissimilis*, suggesting resistant worms in turkeys (7).

Over the last years, there has been an increase in market's demand for cage free egg production systems, which have shown high prevalence of *Ascaridia galli* (8). Moreover, not only are consumers more concerned with the use of drugs on food animals, but also organic productions

do not have the option of using these drugs. This situation requires research evaluating alternative treatments.

Two phytogenic products commonly used by backyard producers have shown potential to be used against other nematodes species in other hosts: *Artemisia absinthium* and pumpkin seed. *Artemisia absinthium* is commonly called wormwood and contains several compounds such as terpenes as limonene, myrcene, α and β thujone (9) that have shown anthelmintic properties. *A. absinthium* was effective in the control of *Haemonchus contortus* in sheep (10). Pumpkin seeds have a diversity of compounds with anthelmintic properties such as cucurbitacin, flavonoids, terpenes and saponins (11). Their potential of being used as an alternative to control nematodes has been shown in ostriches (12) and mice (13).

The aim of this study was to test if *Artemisia absinthium* and pumpkin seed can be used as treatments to alleviate potential negative effects caused by an *Ascaridia galli* infection on egg production and quality, nutrient digestibility, cytokine gene expression and intestinal microbiota.

MATERIALS AND METHODS

Birds and experimental design

A total of 96-one-day-old Hyline White pullets were raised on the floor until 12 weeks of age when they were moved to individual laying hen cages. At 16 weeks, birds were divided into 3 groups with different treatments that were mixed in the feed. Treatments were *Artemisia absinthium* (Starwest botanicals, Sacramento, California) and pumpkin seed powder (nuts.com, Cranford, NJ). Artemisia inclusion was 0.02% which is similar to what is used in sheep (9). Pumpkin seeds inclusion was 1%. The dose is based on anecdotal evidence by small flock owners.

One group was left untreated. As soon as birds reached 50% of egg production, eggs were collected weekly for evaluation of quality. At 25 weeks, 8 birds from each treatment were

necropsied **for comparisons between groups before challenge,** and half of the remaining birds in each treatment group was challenged by oral gavage with 250 embryonated eggs of *A. galli*, resulting in six groups (n = 12 birds per group). Fecal exams were conducted weekly to check for worm eggs. At 34 weeks, titanium dioxide was added (0.5%**)** to the feed for analysis of nutrient digestibility. At 35 weeks, birds were euthanized, and samples collected. Birds were fed diets formulated according to recommendations by the genetics' guideline. Animal care and experimental procedures were performed in compliance with all federal and institutional animal use guidelines and approved by the Auburn University Institutional Animal Care and Use Committee.

Preparation of the inoculum

Worm eggs from a naturally infected layer flock were purified using a process similar as described by Collins et. al (7). Briefly, feces collected in the flock were flushed with tap water through sieves with pore sizes of 212, 90 and 38 µm. Five milliliters of retained material in the last sieve were added to 45 mL of saturated sodium chloride solution. The suspension was centrifuged (420 g, 5 min) and the supernatant was flushed with tap water through a 38 µm sieve. The material retained containing the eggs was finally suspended with tap water. Eggs were stored in 50 ml centrifuge tubes with 10 ml of egg suspension and 1 ml of 0.5% formalin to prevent fungal growth. The eggs were embryonated for 21 days at room temperature with frequent aeriation, after which the embryonation rate was in average 67%. For the challenge, the concentration was adjusted to 250 embryonated eggs/dose.

Sampling

Birds were euthanized using carbon dioxide followed by cervical dislocation. The gastrointestinal tract was removed, and the jejunum and ileum were isolated. The jejunum was opened in a longitudinal direction. The content was first inspected for presence of adult worms; adult worms were collected in 70% alcohol. Approximately 10 cm upstream of Meckel's Diverticulum, 1 g of jejunal wall was collected and snap-frozen using dry ice. Sample was stored at -80ºC until determination of relative gene expression. All jejunal content was carefully collected using plastic spoons in order to minimize contamination with chicken DNA. The content was immediately placed on ice and then stored at -80ºC for microbiome analysis. Ileum digesta was collected in pools (n=3) by squeezing out ileal contents which was latter freeze dried for digestibility analysis.

Fecal egg counts

Starting at 20 weeks of age, about 20 g of feces (n = 4/group) were collected weekly and investigated for *A. galli* eggs. One gram of the homogenized feces and 29 ml of saturated sodium chloride solution were poured through a strainer. A McMaster chamber was used for counting eggs in the filtrated suspension under a microscope.

Nutrient analysis

Pools of ileal digesta (n = 4/group) were collected for evaluation of digestibility of crude protein (CP) (%), calcium (%), phosphorus (%) and apparent ileal digestible energy (AIDE, kcal/kg). To determine gross energy of feed, duplicate 0.75 g samples of feed were analyzed using an isoperibol oxygen bomb calorimeter (model no. 6400, Parr Instruments, Moline, IA) standardized with benzoic acid. Titanium dioxide concentration of feed and ileal digesta was

determined through procedures established by Short et al. (14). Crude protein (AOAC official method 990.03) and minerals (modified procedure as described by Wolf et al. (15) and CEM Application Notes for Acid Digestion were analyzed by a commercial laboratory (Dairy One Forage Laboratory, Ithaca, NY).

Apparent ileal digestibility of energy, protein, and minerals were determined using an equation adapted from Stein et al. (16):

$$\text{AID \%} = \{\frac{\text{AID \%} = \{[(\text{Nutrient/TiO2}) \text{ diet} - (\text{Nutrient/ TiO2}) \text{ digesta}]}{(\text{Nutrient/ TiO2}) \text{ diet}}\} \times 100$$

where (Nutrient/TiO_2) = ratio of protein, energy, or minerals to TiO_2 in diet or ileal digesta.

Percent gross energy digestibility values obtained from this equation were multiplied by the gross energy content of feed to determine apparent ileal digestible energy in units of kcal/kg (17).

Egg Production and Egg Quality

Eggs were collected, counted and the total weight per group was registered on a daily basis. The egg quality evaluation was done once per week in the morning with eggs collected the day before. For this analysis, each egg was weight and cracked. Albumen height was measured using a tripod micrometer (S-6428, B.C. Ames Company, USA). Next, the yolk color was analyzed using a Digital YolkFan^{TM} (DSM, Ontario, Canada). After that, the egg yolk was separated from the egg white and weighed. The eggshells were washed with tap water and dried at room temperature for 24 hours. On the next day, they were weight, and thickness was measured in three points along the equatorial area using a micrometer (B. C. Ames, Framingham, Massachusetts, USA). The final thickness was an average of these three measures. Egg white weight was calculated as the

difference between total egg weight and egg yolk plus eggshell weight. Haugh units were calculated using the formula:

$$HU = 100 \times \log(h + 7.57) - (1.7 \times W\ 0.37)$$

where "h" is the albumen height and "w" is the weight of the egg (18).

Relative gene expression

Relative gene expression was determined as described (19). In brief, RNA was extracted from 15 mg jejunal wall with the Qiagen RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was digested on the column during RNA extraction with the QIAGEN RNase-Free DNase Set (Qiagen). Total RNA was transcribed into cDNA using the Lunascript kit (New England Biolabs, Ipswich, MA). The cDNA was diluted 1:3 in RNAse-free water, and partial TGF-β, IFN-γ, IL-4, IL-8, IL10, IL13, GAPDH and HMBS genes were amplified in duplicates by qPCR using the Fast SYBR Green Master Mix (Qiagen). Cycling parameters consisted of an initial 10 min at 95 ºC denaturation cycle; 40 cycles of denaturation for 30 sec at 95ºC, annealing for 1 min at 60 ºC and extension at 72 ºC for 30 sec. Specificity of PCR products was confirmed by melting curve analysis. Primers and their efficiencies are listed in Table 1. The gene expression was calculated relative to the expression of two housekeeping genes (GAPDH and HMBS) and the average of the uninfected birds as calibrator as described by Vandesompele et al. (20) and Hellemans et al. (21).

Microbiome analyzes

The jejunal microbiota were determined as described (19). In brief, DNA was extracted from 0.2g jejunal content using the QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's instructions. Between 400 to 450 bp of the bacterial 16S rRNA gene were

amplified by PCR using primers 515F with linker CS1and 926R with CS2 (22) using the Taq PCR Master Mix Kit (Qiagen) as described by Hamilton et al. (23). PCR products were checked by agarose gel electrophoresis and submitted to the University of Illinois at Chicago DNA Services Facility for next-generation amplicon sequencing on the Illumina MiSeq. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database.

For analysis, demultiplexed paired- end sequence reads were imported into QIIME2 2022.2 (24). Dada2 was used for primer trimming, truncating the sequences at 245 bp forward 205 bp reverse and denoising. Relative abundance tables were obtained using Silva database (25) after training the classifiers with the sequences. Alpha diversity was measured by Faith's phylogenetic diversity metric, Shannon entropy and Pielou's evenness. Beta diversity was determined by unweighted UniFrac and Principal Component Analysis (PCoA) was used to visualize differences.

Statistical Analysis

Normality was checked by Shapiro's test and Frequency histograms. Relative gene expression values were log2 transformed. This as well as the digestibility of nutrients parameters were analyzed by two-way ANOVA for main effects treatment (control, artemisia and pumpkin), and infection status (challenged and non-challenged) and interactions using R (26). Mean values of all groups were compared by one-way ANOVA with Tukey's honestly significant difference test as post-hoc test. Egg production, egg quality and relative abundance of taxa and yolk color were compared by Kruskal-Wallis Test since data was not normally distributed with Fisher's least significant difference as Post hoc test. Other differences between infection status and treatments in the microbiome were analyzed using QIIME2. For the alpha diversity metrics: Faith's phylogenetic diversity, Shannon entropy and Pielou's evenness were analyzed by Kruskal-Wallis Test for each collection day. For beta diversity, unweighted UniFrac was analyzed using

PERMANOVA for each collection day. $p \leq 0.05$ was considered statistically significant and $0.05 < p < 0.10$ indicated trends.

RESULTS

Egg shedding and worm burden

At the time of infection until 4 weeks post infection (P.I.), *Eimeria* oocysts were identified in replicates of all groups. Shedding of worm eggs started 4 weeks post infection (P.I.) in the control and artemisia groups with an average of 150 and 25 eggs per gram (EPG), respectively. For the pumpkin seed treated group, shedding started only at 5 weeks P.I. Worm eggs were seen until the last day of collection for all groups as shown in Table 1. Average egg counts were 364 EPG in the control group, 596 EPG in the artemisia group and 521 EPG in the pumpkin seed group with no statistical significance difference between groups. The peak egg shedding in the control group was observed in weeks 7 and 8 P.I. with an average of 675 EPG, in week 8 P.I. with an average of 2100 EPG in the artemisia group and in week 9 P.I. with an average of 2050 EPG in the pumpkin seed group.

The average number of adult worms per bird at the end of the experiment was 2 for the control group, 5 for the artemisia group and 7 for the pumpkin seed group. Out of the 12 birds necropsied birds from the control or artemisia groups, 8 had adult worms while 9 out of 11 were positive in the pumpkin seed group. There were no statistical differences between the treatments regarding the worm burden.

Nutrient analysis

The nutrient digestibility analysis is shown in Table 3. Challenge with *A. galli* significantly affected nutrient digestibility of apparent ileal energy (p=0.0001), crude protein (p=0.013) and

phosphorus digestibility (p=0.041) with a reduction of 7 to 14%. In contrast, the comparison between diets did not show significant differences.

Ileal digestible energy was lower in challenged birds in the control (p=0.03) and artemisia groups (p=0.001), while this difference was not observed in the birds consuming pumpkin seed. For crude protein, the digestibility differed between infected and uninfected birds only in the artemisia group (p=0.03).

Calcium digestibility had a different pattern than what was seen with the other nutrients. Infection with *A. galli* did not significantly affect its digestibility, but different diets had a significant impact resulting in 30% lower digestibility on birds the fed control diet when compared to alternative treatments (p=0.016). More specifically, the comparison between challenged and non-challenged birds within groups showed that calcium digestibility was lower in challenged birds fed the control diet, but there were no differences between challenged and non-challenged birds that were fed with either artemisia or pumpkin seed on the concentrations used.

Egg Production and Egg Quality

The weekly egg production is presented in Figure 1. The control group was the first group to start laying eggs and maintained a slightly higher production until 23 weeks of age. There was a minor decrease in egg production of all groups two weeks after the challenge and again 5 weeks P.I. Differences between groups were not statistically significant.

All groups that were challenged with *A. galli* had a numerical lower egg production as shown in Table 4. Birds fed with artemisia had higher egg production than the other treatments, but differences between groups were not statistically significant. Table 5 shows the comparison between challenged and non-challenged groups in each treatment for egg quality parameters. None

of the parameters, which includes egg weight, albumen height, Haugh units, yolk color, eggshell weight, and eggshell thickness, had a statistical significance difference between diets or infection status.

Relative gene expression

The comparison of the gene expression of cytokines between treatments at the day of infection (25 weeks of age) is shown in Figure 2. There were no statistically significant differences between the treatments and the control for any of the cytokines evaluated.

The relative gene expression of the cytokines 35-week-old birds is shown in Figure 3. IL-8 had significant differences between treatments (p=0.001). Birds fed pumpkin seed had an upregulation of IL-8 while the artemisia group had a downregulation of this cytokine. For the IFN-γ, there was no difference between challenged and non-challenged animals within the treatments.

The upregulation of IL-13 was clear in all challenged birds across different treatments and the differences in gene expression was explained by both infection status (p=0.043) and the interaction between infection status and treatment (p=0.013). Only in the artemisia groups, challenged birds were significantly (p=0.012) different from non-challenged.

The expression of anti-inflammatory cytokines was the least impacted, as there were no differences in the expression of IL-10 in all groups. For TGF-β, there was a significant difference in gene expression between treatments (p=0.0498) explained by an upregulation in challenged birds treated with artemisia.

Microbiome analyzes

A total of 758,7680 reads were analyzed. The most common bacterial phylum identified in the jejunum of non-challenged and challenged were Firmicutes (between 94.9 and 95.5%)

followed by Cyanobacteria (between 4.5 and 3.6%), Proteobacteria (around 0.3%). Bacteroidetes was the fourth most abundance phylum in all birds, but there was a significant difference between infection status (p=0.009) where non-challenged birds the percentage was 0.4%, in challenged birds, this was reduced to 0.04% (Figure 4).

At the order level, there were no statistically significant differences between non-challenged and challenged groups (Figure 5). The most common orders found in the jejunum of birds were: Lactobacillales, chloroplasts, Clostridiales, and Rickettisiales. However, there were clear differences in the microbiota composition of birds fed with control diet and birds fed with either artemisia or pumpkin seed (Figure 4). Birds fed the alternative treatments had a lower proportion of chloroplasts (p=0.044) and higher proportion of Lactobacillales (p=0.064). The control group also had a higher proportion of Rickettisiales (p=0.025). Relative abundance of Clostridiales was significantly increased in older birds compared to with younger birds, especially in birds treated with pumpkin seed (p=0.045).

Neither artemisia nor pumpkin seed significantly impacted the alpha diversity in week 25 (results not shown). In week 35, neither infection status nor treatment had a significant influence on alpha-diversity (Figure 6).

The PCoA of the microbiota in week 35 based on unweighted UniFrac distance (Figure 7) showed that most of the birds treated with artemisia tended to cluster together and the PERMANOVA analysis showed that the distance between treatments was statistically significant (p=0.024). However, the differences between challenged and non-challenged were not significant.

DISCUSSION

The goal of this study was to test the impact of pumpkin seeds and *Artemisia absinthium* on potential negative effects by an *Ascaridia galli* infection on nutrient digestibility, egg

production and quality, as well as changes in cytokine gene expression and intestinal microbiota. To our knowledge, this is the first time that the impact of an infection with *Ascaridia galli* together with potential treatments with *Artemisia absinthium* and pumpkin seeds on digestibility of nutrients were evaluated. Calcium digestibility was the most affected with a reduction of close to 30% in infected non-treated birds followed by phosphorus with a reduction of 12%. With such reductions, it is intuitive to expect that the eggshell quality would be affected as well. However, neither eggshell weight nor thickness was reduced.

The significant reduction in calcium and phosphorus digestibility did not result in a reduction in performance. This is not the first time that no changes in egg production were observed after an infection with *A. galli* (27,28). The low numbers of adult worms present in the intestine by the end of trial in all treatments and lower number of worm egg shedding on feces indicates that this was not a severe infection. In addition, there are different mechanisms in which a reduction of nutrient digestibility will be compensated in order to maintain homeostasis.

In the case of eggshell quality, a way to compensate for the reduction of the mineral digestibility is a recruitment of the mineral from bones (29,30). Even though at necropsy, bone quality was not tested, in the future, it would be interesting to evaluate if *A. galli* might have an impact on it. This is especially important considering that both alternative treatments maintained mineral digestibility levels.

Although an impact on overall bird's performance was not noticed, neither due to the challenge or the different treatments, it was interesting to see a minor decrease in egg production of the challenged groups 2 weeks after the challenge and again 5 weeks P.I. These two times coincide with the beginning and end of the histotrophic phase of the parasite (31). The larval

migration and attachment in the intestinal mucosa are known to stimulate the immune system and result in inflammation (32).

The effectiveness evaluation of the treatments against *A. galli* infection showed that they were not able to affect neither the establishment of the de disease nor reduce the number of positive samples for worms. In fact, 81% of birds treated with pumpkin seeds and 66% birds treated either with artemisia or control had adult worms in the intestine. Additionally, worm egg shedding in both treatments had higher peak number and a higher average than the control. A possible explanation for this small and negative difference is due to antinutritional compounds that can be found in both treatments such as tannin, trypsin inhibitor and phytic acid (33,34). However, the absence of statistically significant differences between the alternative treatments and the control suggests that they can be safely used without affecting egg production or quality.

The changes in the relative gene expression of cytokines were very subtle. This can be explained due to the fact that the adults are free in the lumen, in contrast to the larvae that are attached to the mucosa, which will result in a more severe inflammation (19,32). IL-13, a Th2 response related cytokine was the only cytokine that showed a more expressive upregulation on birds infected with *A. galli*. This agrees with previous results investigating the immune response against *A. galli* carrying larvae as well as adult worms (19,35,36).

Another possible explanation for the very small changes on cytokine gene expression is that in previous studies, the sampling time points were closer to the infection (35,37). The worm egg count showed a peak around 7- and 8-weeks P.I. and a considerable reduction in the following weeks in all groups. This change could be due to an immune response that affected the adult, and, at 10 weeks P.I., the remaining worms found at necropsy where not enough to stimulate a strong immune response.

Alternative treatments caused a modulation of the immune system. Birds treated with pumpkin had a significantly higher upregulation of IL-8 a pro-inflammatory cytokine. *Artemisia,* on the other seems to have an anti-inflammatory effect causing an upregulation of TGF-β in infected birds. The anti-inflammatory properties of *Artemisia absinthium* were previously demonstrated by Hadi et al. 2014 using carrageenan-induced paw edema in mice (38).

The most common bacterial phyla found in the jejunum agree to previous studies (39–41). The alpha diversity analysis showed that the infection status did not impact the richness or evenness within the communities. Our previous study showed that the larvae form of *A. galli* can affect the immune system and the microbiota. However, once the infection was cleared, these effects are not long lasting, and the challenged groups tended to have a similar microbiota than the non-challenged birds again (19). In the present study, the results suggest that, different from the larvae, the adults do not seem to have important effects on the intestinal microbiota, or rather that changes caused by the larvae are reversed once they develop into adult birds. Similar to the cytokine expression, testing at earlier time points might show more pronounced differences between the microbiota of infected and control birds.

The main differences seen in the microbiota at 35 weeks of age were due to the treatments. Different from what was seen in the immune response where the gene expression of all cytokines was only slight changed by the treatments, both treatments altered the relative abundance of taxa in the jejunal microbiota as seen in the analysis of the beta-diversity.

Lactobacillales were increased by both treatments. Lactobacillales produce acetate that is a source of energy for enterocytes and can increase mineral absorption and reduce attenuate inflammasome activation (42,43). The effect on acetate on mineral absorption could be an explanation of why challenged birds receiving the alternative treatments did not have a reduction

calcium digestibility. A study of the metabolome would be important to confirm this hypothesis. Clostridiales were also increased in birds treated with pumpkin seeds. Some *Clostridium* species, such as *Clostridium sporogenes* produce tryptamine that can improve host metabolism, improve mucosal barrier function as well as maintain host-microbe homeostasis (44,45).

Finally, it is important to point out that the simplest processing of the phytogenic treatments, i.e., just mixing powder in the feed, was done in this study in order to simulate what most backyard producers do. This means that all compounds present in both treatments with a potential antiparasitic effect were present in lower concentrations than in extracts. This can be the reason why not many differences were seen in our research, but their aqueous and alcoholic extracts did show a potential in other hosts (10,13,46,47). Coccidiosis was detected up to four weeks P.I., i.e., six weeks before the end of the trial. Since this is a self-limiting disease and it was detected in all groups, we do not expect a distortion of the results by it.

In conclusions neither treatment at the dosage used showed to be effective against *Ascaridia gall*. The infection did not affect egg production or egg quality during the period evaluate. However, the *A. galli* did impact on nutrient digestibility and treated birds had better calcium digestibility and higher relative abundance of Lactobacillales which could be the reason for this difference. Future studies investigating this mechanism and different doses are needed.

REFERENCES

1. Thapa S, Hinrichsen LK, Brenninkmeyer C, Gunnarsson S, Heerkens JLT, Verwer C, Niebuhr K, Willett A, Grilli G, Thamsborg SM, et al. Prevalence and magnitude of helminth infections in organic laying hens (*Gallus gallus domesticus*) across Europe. *Vet Parasitol.* 214:118–124; 2015. doi: 10.1016/j.vetpar.2015.10.009.

2. Tarbiat B, Jansson D, Wall H, Tydén E, Höglund J. Effect of a targeted treatment strategy against *Ascaridia galli* on egg production, egg quality and bird health in a laying hen farm. *Vet Parasitol.* 286:109238; 2020. doi: 10.1016/j.vetpar.2020.109238.

3. Torres A, Costa C, Pinto P, Santos H, Amarante A, Gómez S, Resende M, Martins N. An outbreak of intestinal obstruction by *Ascaridia galli* in broilers in Minas Gerais. *Braz J. Poult Sci.* 21: eRBCA-2019-1072; 2019. doi:10.1590/1806-9061-2019-1072.

4. Wuthijaree K, Lambertz C, Gauly M. Prevalence of gastrointestinal helminth infections in free-range laying hens under mountain farming production conditions. *Br Poult Sci.* 58:649–655; 2017. doi:10.1080/00071668.2017.1379049.

5. Tarbiat B, Jansson DS, Tydén E, Höglund J. Comparison between anthelmintic treatment strategies against *Ascaridia galli* in commercial laying hens. *Vet Parasitol.* 226:109–115; 2016. doi: 10.1016/j.vetpar.2016.07.006.

6. Höglund J, Jansson DS. Infection dynamics of *Ascaridia galli* in non-caged laying hens. *Vet Parasitol.* 180:267–273; 2011. doi: 10.1016/j.vetpar.2011.03.031.

7. Collins JB, Jordan B, Baldwin L, Hebron C, Paras K, Vidyashankar AN, Kaplan RM. Resistance to fenbendazole in *Ascaridia dissimilis*, an important nematode parasite of turkeys. *Poult Sci.* 98:5412–5415; 2019. doi:10.3382/ps/pez379.

8. Jansson DS, Nyman A, Vågsholm I, Christensson D, Göransson M, Fossum O, Höglund J. Ascarid infections in laying hens kept in different housing systems. *Avian Pathol.* 39:525–532; 2010. doi:10.1080/03079457.2010.527923.

9. Irum S, Ahmed H, Mukhtar M, Mushtaq M, Mirza B, Donskow- Łysoniewska K, Qayyum M, Simsek S. Anthelmintic activity of Artemisia vestita Wall ex DC. and *Artemisia maritima L.* against *Haemonchus contortus* from sheep. *Vet Parasitol* 212:451–455; 2015. doi: 10.1016/j.vetpar.2015.06.028.

10. Tariq KA, Chishti MZ, Ahmad F, Shawl AS. Anthelmintic activity of extracts of *Artemisia absinthium* against ovine nematodes. *Vet Parasitol.* 160:83–88; 2009. doi: 10.1016/j.vetpar.2008.10.084.

11. Salehi, Sharifi-Rad, Capanoglu, Adrar, Catalkaya, Shaheen, Jaffer, Giri, Suyal, Jugran, et al. Cucurbita plants: from farm to industry. *Appl Sci.* 9:3387; 2019. doi:10.3390/app9163387.

12. Feitosa TF, Vilela VLR, Athayde ACR, Braga FR, Dantas ES, Vieira VD, de Melo LRB. Anthelmintic efficacy of pumpkin seed (*Cucurbita pepo Linnaeus*, 1753) on ostrich gastrointestinal nematodes in a semiarid region of Paraíba State, Brazil. *Trop Anim Health Prod.* 45:123–127; 2013. doi:10.1007/s11250-012-0182-5.

13. Erol A, Hamit C kun. Evaluation of the anthelmintic activity of pumpkin seeds *(Cucurbita maxima*) in mice naturally infected with *Aspiculuris tetraptera*. *J Pharmacognosy Phytother.* 7:189–193; 2015. doi:10.5897/JPP2015.0341.

14. Short FJ, Gorton P, Wiseman J, Boorman KN. Determination of titanium dioxide added as an inert marker in chicken digestibility studies. *Animal Feed Sci and Tech.* 59:215–221; 1996. doi:10.1016/0377-8401(95)00916-7.

15. Peters J, Combs S, Hoskins B, Jarman J, Kovar J, Watson M, Wolf A, Wolf N. Recommended methods of manure analysis. *Univ of Wisconsin Coop Ext*. 2003.

16. Stein HH, Fuller MF, Moughan PJ, Sève B, Mosenthin R, Jansman AJM, Fernández JA, de Lange CFM. Definition of apparent, true, and standardized ileal digestibility of amino acids in pigs. *Livestock Sci* 109:282–285; 2007. doi: 10.1016/j.livsci.2007.01.019.

17. Gautier AE, Rochell SJ. Influence of coccidiosis vaccination on nutrient utilization of corn, soybean meal, and distillers dried grains with solubles in broilers. *Poult Sci.* 99:3540–3549; 2020. doi: 10.1016/j.psj.2020.03.035.

18. Eisen EJ, Bohren BB, McKean HE. The Haugh Unit as a measure of egg albumen quality. *Poult Sci.* 41:1461–1468; 1962. doi:10.3382/ps.0411461.

19. Terra-Long MT, Pietruska A, McCrea BA, Hauck R. Infection with *Ascaridia galli* does not significantly alter intestinal microbiota and is cleared after changes in the expression of cytokines. *Avian Dis.* 66:186-192; 2022. doi:10.1637/aviandiseases-D-22-00017.

20. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Gen Biol*. 15:1-12; 2002. doi:10.1186/gb-2002-3-7-research0034.

21. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Ge. Biol.* 8: R19; 2007. doi:10.1186/gb-2007-8-2-r19.

22. Walters W, Hyde ER, Berg-Lyons D, Ackermann G, Humphrey G, Parada A, Gilbert JA, Jansson JK, Caporaso JG, Fuhrman JA, et al. Improved bacterial 16S rRNA Gene (V4 and V4-5) and fungal internal transcribed spacer marker gene primers for microbial community surveys. *mSystems.* 1: e00009-15; 2016. doi:10.1128/mSystems.00009-15.

23. Hamilton M, Ma X, McCrea BA, Carrisosa M, Macklin KS, Zhang C, Wang X, Hauck R. Influence of *Eimeria spp*. infection on chicken jejunal microbiota and the efficacy of two alternative products against the infection. *Avian Dis.* 64:123–129; 2020. doi:10.1637/0005-2086-64.2.123.

24. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol.* 37:852–857; 2019. doi:10.1038/s41587-019-0209-9.

25. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41: D590-596; 2013. doi:10.1093/nar/gks1219.

26. Team R. RStudio: integrated development for R. RStudio.167-171; 2012.

27. Sherwin CM, Nasr MAF, Gale E, Petek M, Stafford K, Turp M, Coles GC. Prevalence of nematode infection and faecal egg counts in free-range laying hens: relations to housing and husbandry. *British Poult Sci.* 54:12–23; 2013. doi:10.1080/00071668.2012.757577.

28. Sharma N, Hunt PW, Hine BC, Sharma NK, Swick RA, Ruhnke I. Detection of *Ascaridia galli* infection in free-range laying hens. *Vet Parasitol.* 256:9–15; 2018. doi: 10.1016/j.vetpar.2018.04.009.

29. Whitehead CC. Overview of bone biology in the egg-laying hen. *Poult Sci.* 83:193–199; 2004. doi:10.1093/ps/83.2.193.

30. Hervo F, Narcy A, Nys Y, Létourneau-Montminy M-P. Effect of limestone particle size on performance, eggshell quality, bone strength, and in vitro/in vivo solubility in laying hens: a meta-analysis approach. *Poult Sci.* 101:101686; 2022. doi: 10.1016/j.psj.2021.101686.

31. Sossidou EN, Bosco AD, Elson HA, Fontes CMGA. Pasture-based systems for poultry production: implications and perspectives. *World's Poult Sci J.* 67:47–58; 2011. doi:10.1017/S0043933911000043.

32. Luna-Olivares LA, Kyvsgaard NChr, Ferdushy T, Nejsum P, Thamsborg SM, Roepstorff A, Iburg TM. The jejunal cellular responses in chickens infected with a single dose of *Ascaridia galli* eggs. *Parasitol Res.* 114:2507–2515; 2015. doi:10.1007/s00436-015-4450-y.

33. Fagbemi TN, Oshodi AA, Ipinmoroti KO. processing effects on some antinutritional factors and in vitro multienzyme protein digestibility (ivpd) of three tropical seeds: breadnut (*Artocarpus*

altilis), cashewnut (*Anacardium occidentale*) and fluted pumpkin (*Telfairia occidentalis*). *Pakistan J. of Nutrition.* 4:250–256; 2005. doi:10.3923/pjn.2005.250.256

34. Elinge, CM, Muhammad A, Atiku FA, Itodo AU, Peni IJ, Sanni OM, Mbongo AN. Proximate, mineral and anti-nutrient composition of pumpkin (*Cucurbitapepo L)* seeds extract. *Int J of Plant Res.* 2:146–150; 2012. doi: 10.5923/j.plant.20120205.02.

35. Pleidrup JA, Norup LR, Dalgaard TS, Kaiser P, Permin A, Schou TW, Vadekær DF, Jungersen G, Sørensen P, Juul-Madsen HR. Cytokine gene expression profiles in chicken spleen and intestinal tissues during *Ascaridia galli* infection. *Vet Parasitol.* 206:317–321; 2014. doi: 10.1016/j.vetpar.2014.10.016.

36. Dalgaard TS, Skovgaard K, Norup LR, Pleidrup J, Permin A, Schou TW, Vadekær DF, Jungersen G, Juul-Madsen HR. Immune gene expression in the spleen of chickens experimentally infected with *Ascaridia galli*. *Vet Immunol Immunopathol.* 164:79–86; 2015. doi: 10.1016/j.vetimm.2015.01.003.

37. Schwarz A, Gauly M, Abel H, Daş G, Humburg J, Rohn K, Breves G, Rautenschlein S. Immunopathogenesis of *Ascaridia galli* infection in layer chicken. *Dev & Comp Immunol.* 35:774–784; 2011. doi: 10.1016/j.dci.2011.02.012.

38. Hadi A, Hossein N, Shirin P, Najmeh N, Abolfazl M. Anti-inflammatory, and analgesic activities of *Artemisia absinthium* and chemical composition of its essential oil. *Int J Pharm Sci Rev.* 24: 237-244; 0214.

39. Xiao Y, Xiang Y, Zhou W, Chen J, Li K, Yang H. Microbial community mapping in intestinal tract of broiler chicken. *Poult Sci.* 96:1387–1393; 2017. doi:10.3382/ps/pew372.

40. Bhogoju S, Nahashon S, Wang X, Darris C, Kilonzo-Nthenge A. A comparative analysis of microbial profile of guinea fowl and chicken using metagenomic approach. *PLOS ONE* 13: e0191029; 2018. doi: 10.1371/journal.pone.0191029.

41. Khan S, Moore RJ, Stanley D, Chousalkar KK. The gut microbiota of laying hens and its manipulation with prebiotics and probiotics to enhance gut health and food safety Ercolini D, editor. *Appl Environ Microbiol.* 86: e00600-20; 2020. doi:10.1128/AEM.00600-20.

42. Xu M, Jiang Z, Wang C, Li N, Bo L, Zha Y, Bian J, Zhang Y, Deng X. Publisher correction: acetate attenuates inflammasome activation through GPR43-mediated Ca2+-dependent NLRP3 ubiquitination. *Exp Mol Med.* 51:1–1; 2019. doi:10.1038/s12276-019-0296-1.

43. Zhang J-M, Sun Y-S, Zhao L-Q, Chen T-T, Fan M-N, Jiao H-C, Zhao J-P, Wang X-J, Li F-C, Li H-F, et al. SCFAs-Induced GLP-1 secretion links the regulation of gut microbiome on hepatic lipogenesis in chickens. *Front. Microbiol.* 10:2176; 2019. doi:10.3389/fmicb.2019.02176.

44. Bansal T, Alaniz RC, Wood TK, Jayaraman A. The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation. *Proc Natl Acad Sci U.S.A.* 107:228–233; 2010. doi:10.1073/pnas.0906112107.

45. Roager HM, Licht TR. Microbial tryptophan catabolites in health and disease. *Nat Commun.* 9:3294; 2018. doi:10.1038/s41467-018-05470-4.

46. Bayati NYA-, AL-Aubaidi IK, AL-Haidari SHJ. Anticestodal activity of the crude aqueous extract of pumpkin seed (*Cucurbita pepo*) against the dwarf tapeworm (*Hymenolepis nana*) in mice. :8; 2009.

47. Shahnazi M, Azadmehr A, Hajiaghaee R, Mosalla S, Latifi R. Effects of *Artemisia absinthium* l. extract on the maturation and function of dendritic cells. *Jundishapur J Nat Pharm Prod*. 102015. doi:10.17795/jjnpp-20163.

48. Katakam KK, Nejsum P, Kyvsgaard NChr, Jørgensen CB, Thamsborg SM. Molecular and parasitological tools for the study of *Ascaridia galli* population dynamics in chickens. *Avian Pathol.* 39:81–85; 2010. doi:10.1080/03079451003599284.

49. Wang L, Li L, Lv Y, Chen Q, Feng J, Zhao X. *Lactobacillus plantarum* restores intestinal permeability disrupted by Salmonella infection in newly hatched chicks. *Sci Rep.* 8:2229; 2018. doi:10.1038/s41598-018-20752-z.

50. Zhang J, Gao Y-Y, Huang Y-Q, Fan Q, Lu X-T, Wang C-K. Selection of housekeeping genes for quantitative gene expression analysis in yellow-feathered broilers. *Italian J of Anim Sci.* 17:540–546; 2018. doi:10.1080/1828051X.2017.1365633.

Table 5.1: Primers used to identify roundworms, cytokines, and housekeeping genes

Target gene	Forward primer	Reverse primer	Annealing temperature	Amplicon size (bp)	Reference
COI	ATT ATT ACT GCT CAT GCT ATT TTG ATG	CAA AAC AAA TGT TGA TAA ATC AAA GG	50	533	(36)
GAPDH	TGG AGA AAC CAG CCA AGT AT	GCA TCA AAG GTG GAG GAA T	60	145	(37)
HMBS	GAT GGA TCC GAT AGC CTG AA	GAT GTG CTT AGC TCC CTT GC	60	195	(38)
IL-8	GCA AGG TAG GAC GCT GGT AA	GCG TCA GCT TCA CAT CTT GA	60	106	(39)
IL-13	AAG GAC TAC GAG CCC CTC AT	GCA AGA AGT TCC GCA GGT AG	60	100	(39)
IL-10	AAG AGG AGC AAA GCC ATC AA	TGG CTT TGT AGA TCC CGT TC	60	73	(39)
INF-γ	CAG ATG TAG CTG ACG GTG GA	CAT CGA AAC AAT CTG GCT CA	60	98	(39)
TGF-β	CGA CCT CGA CAC CGA CTA CT	CCA CTT CCA CTG CAG ATC CT	60	103	(39)

Table 5.2: Average number of worm eggs per gram of feces in birds challenged with *Ascaridia galli*. Birds were fed either a control diet, artemisia (0.02% inclusion in feed) or pumpkin seed (1% inclusion in feed). In week 25, birds were orally infected with *A. galli*.

Weeks P.I.	Control	Artemisia	Pumpkin
4	150	25	0
5	25	100	150
6	125	400	300
7	675	275	125
8	675	2100	525
9	525	975	2050
10	375	300	500
Average	364	596	521

Table 5 3: Nutrient digestibility. Birds were fed either a control diet, artemisia (0.02% inclusion in feed) or pumpkin (1% inclusion in feed). In week 25, birds were orally infected with *Ascaridia galli* (challenged group) or distilled water (non-challenged group). Nutrient digestibility was determined at the end of the experiment in week 35.

Treatment	Challenge	Apparent Ileal Energy digestibility (AIDE, kcal/kg)	Crude Protein digestibility (%)	Calcium digestibility (%)	Phosphorus digestibility (%)
Control	No	2962^{A}	63.66^{AB}	57.83^{AB}	53.38^{A}
Control	Yes	2722^{B}	54.23^{AB}	20.86^{B}	47.10^{A}
Artemisia	No	2927^{A}	66.64^{A}	59.38^{AB}	53.32^{A}
Artemisia	Yes	2548^{B}	51.49^{B}	56.98^{AB}	46.31^{A}
Pumpkin	No	2839^{AB}	59.06^{AB}	46.88^{A}	53.67^{A}
Pumpkin	Yes	2797^{AB}	61.76^{AB}	63.83^{AB}	47.27^{A}
SEM		33.58	1.55	4.00	1.29
Control		2825^{A}	58.27 ^{A}	39.34^{A}	50.24 ^{A}
Artemisia		2738 ^{A}	57.98 ^{A}	58.01^{B}	49.31 ^{A}
Pumpkin		2818 ^{A}	60.411 ^{A}	55.35 ^{B}	50.93 ^{A}
Non-Challenged		2902^{A}	62.71 ^{A}	54.70 ^{A}	53.48 ^{A}
Challenged		2702 ^{B}	55.82 ^{B}	48.20 ^{A}	46.83^{B}
p-value					
Treatment		0.202	0.666	0.016	0.629
Challenge		0.0001	0.013	0.157	0.041
Interaction		0.013	0.023	0.002	0.434

$^{A, B}$ Means followed by different letters are Superscripts indicate significance difference ($p \leq 0.05$) between groups.

Means represent 4 replicates of 3 birds per replicate for each of the 6 treatments (n=3).

Table 5.4: Average weekly egg production (%) of treatment groups in weeks 25 to 35. Birds were fed either a control diet, artemisia (0.02% inclusion in feed) or pumpkin seed (1% inclusion in feed). In week 25, birds were orally infected with *Ascaridia galli* (challenged group) or distilled water (non-challenged group).

	Non-challenged	Challenged
Control	96.0 %	94.8 %
Artemisia	97.8 %	96.7 %
Pumpkin	96.2 %	91.4 %

Table 5.5: Egg quality parameters of eggs collected from birds non-challenged and challenged with *Ascaridia galli* and fed with control, artemisia (0.02% inclusion in feed) or pumpkin seed (1% inclusion in feed) diets. In week 25, birds were orally infected with *A. galli* (challenged group) or distilled water (non-challenged group). The experiment ended in week 35.

Treatment	Challenge	Egg Weight (g)	Egg Shell Weight (g)	Egg Shell Thickness (mm)	Albumen Height (mm)	Haugh Units	Yolk Color
Control	No	59.84	5.85	0.412	9.31	95.59	5.87
Control	Yes	59.06	5.77	0.408	9.19	95.13	5.96
Artemisia	No	57.70	5.59	0.407	8.89	94.05	5.92
Artemisia	Yes	58.76	5.81	0.413	9.31	95.82	5.78
Pumpkin	No	59.25	5.66	0.402	9.54	97.06	5.92
Pumpkin	Yes	59.03	5.78	0.411	9.21	95.40	5.89
SEM		0.16	0.02	0.008	0.05	0.23	0.03
Control		59.45	5.81	0.410	9.25	95.36	5.92
Artemisia		58.24	5.70	0.410	9.11	94.95	5.85
Pumpkin		59.14	5.72	0.407	9.38	96.23	5.91
Non-Challenged		58.95	5.70	0.407	9.25	95.59	5.90
Challenged		58.95	5.79	0.410	9.23	95.45	5.88

Figure 5.1. Weekly egg production curves (%) of non-challenged birds (green) and birds challenged with *Ascaridia galli* (red). At week 25, birds were orally infected with *A. galli* (challenged group) or distilled water (non-challenged group). a) Birds fed the control diet, b) birds fed with artemisia (0.02% inclusion in feed), c) birds fed with pumpkin seed (1% inclusion in feed.

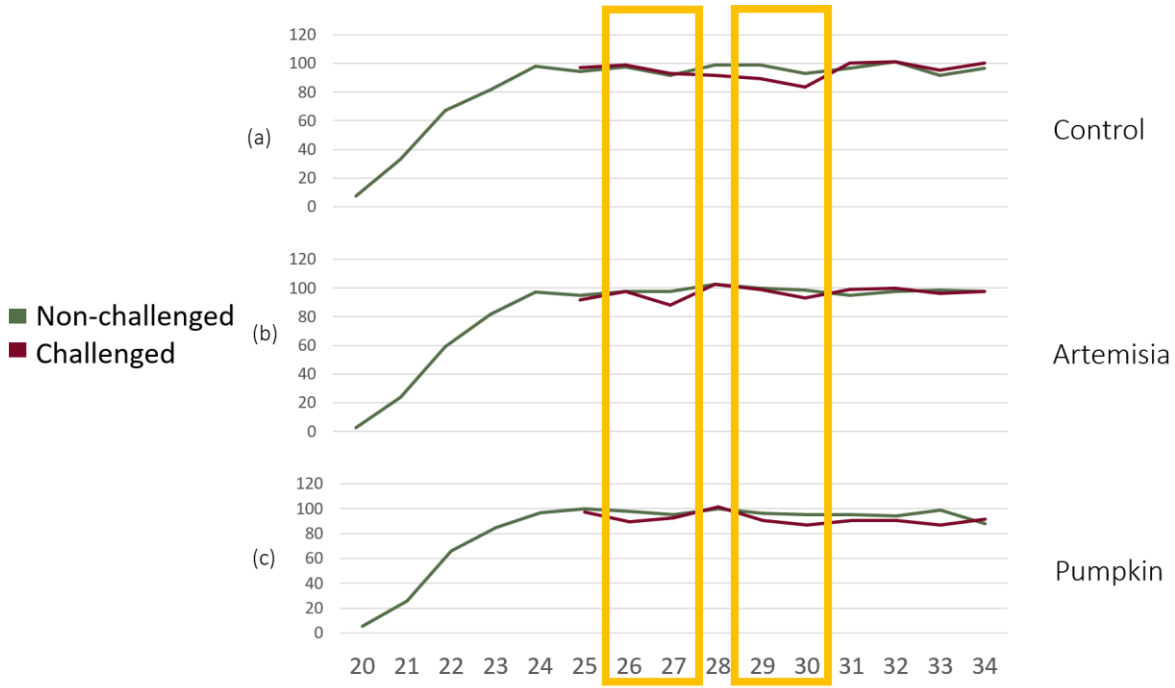


Figure 5.2. Relative gene expression of IL-8 (a), IFN-γ (b), IL-13 (c), IL-10 (e), and TGF-β (d) in the jejunum of layer hens at 25 weeks. The gene expression was normalized by housekeeping genes (GAPDH and HMBS) and expressed relative to the average of the control birds. Grey boxes represent birds from the control group, blue boxes represent birds treated with artemisia (0.02% inclusion in the feed) and orange boxed represent bird treated with pumpkin seed (1% inclusion in the feed).

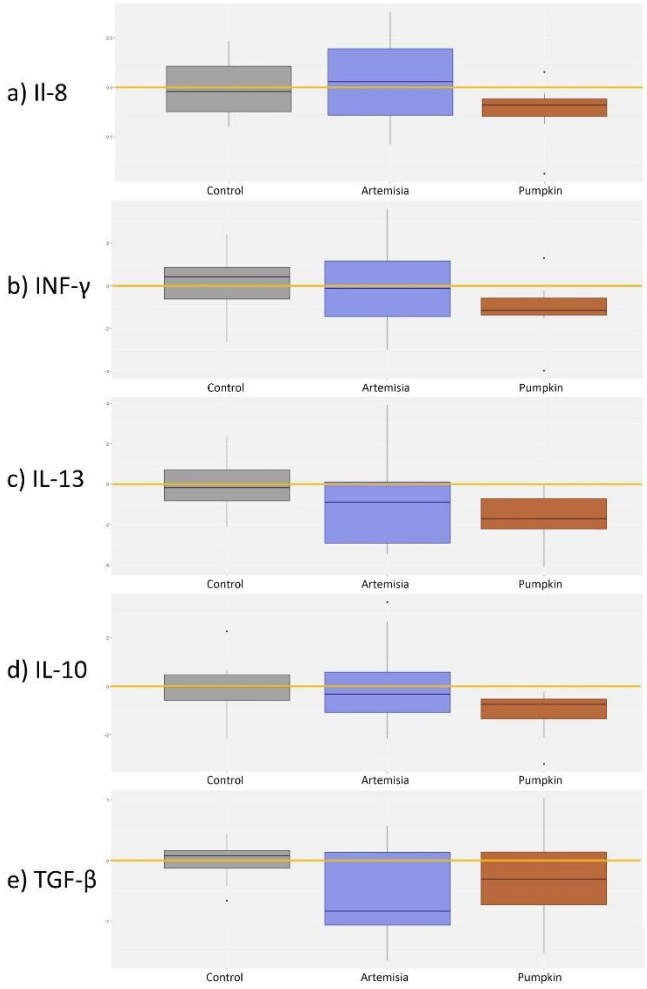


Figure 5.3. Relative gene expression of IL-8 (a), IFN-γ (b), IL-13 (c), IL-10 (e), and TGF-β (d) in the jejunum of layer hens at 35 weeks (10 weeks post infection). The gene expression was normalized by housekeeping genes (GAPDH and HMBS) and expressed relative to the average of the uninfected birds from the control group. Red boxes represent birds challenged with embryonated eggs of *Ascaridia galli* while green boxes represent control birds. Birds were fed a control diet, treated with artemisia (0.02% inclusion in the feed) or pumpkin seed (1% inclusion in the feed).

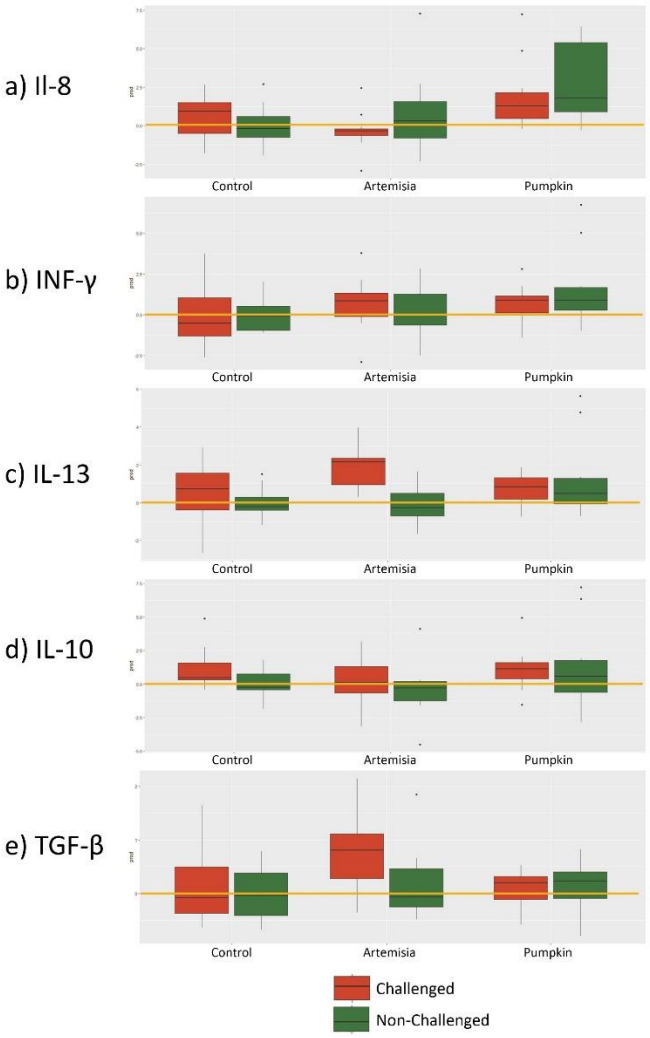


Figure 5.4. Average of the relative abundance of phyla detected in the jejunal content of layer hens at 25 (before challenge) and 35 weeks (10 weeks post infection). Challenged and Non-Challenged samples were grouped according to treatment: control, artemisia (0.02%).

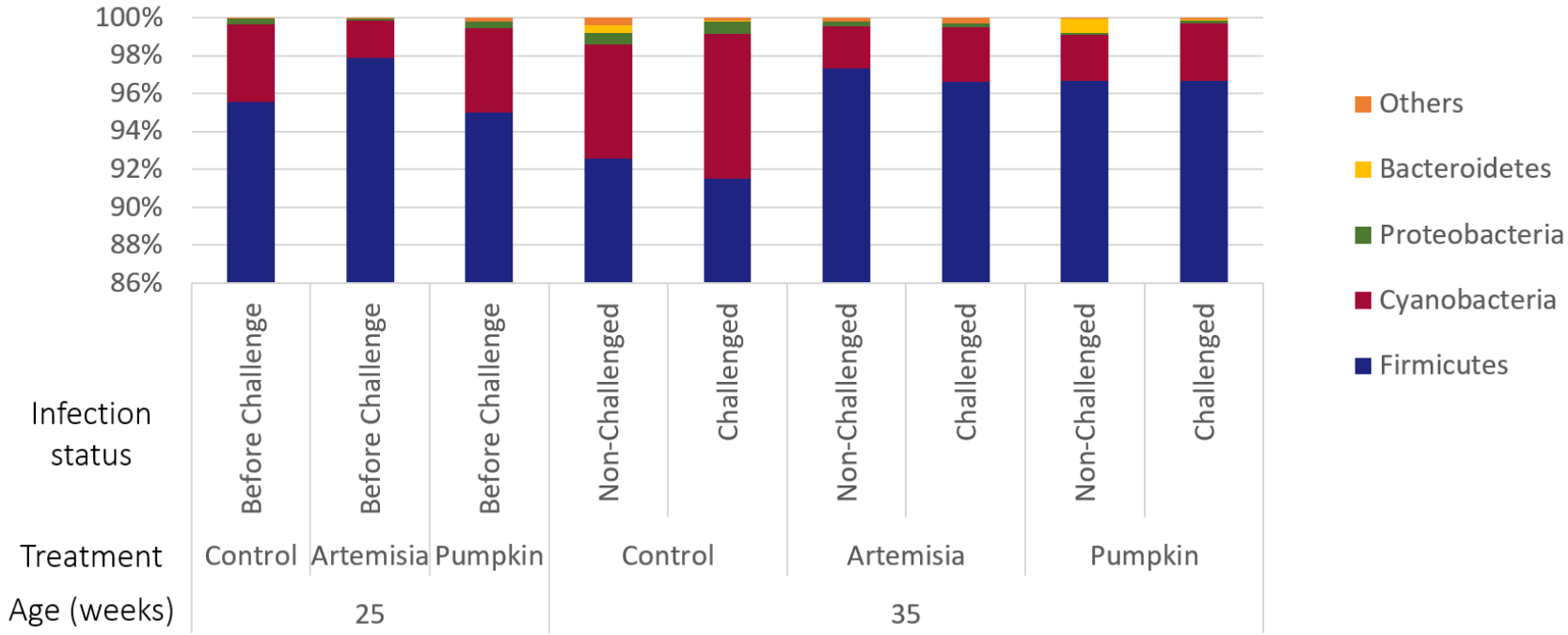


Figure 5.5. Average of the relative abundance of orders detected in the jejunal content of layer hens at 25 (before challenge) and 35 weeks (10 weeks post infection). Challenged and Non-Challenged samples were grouped according to treatment: control, artemisia (0.02%) or pumpkin seed (1%). Challenged groups where infected with *Ascaridia galli* in week 25.

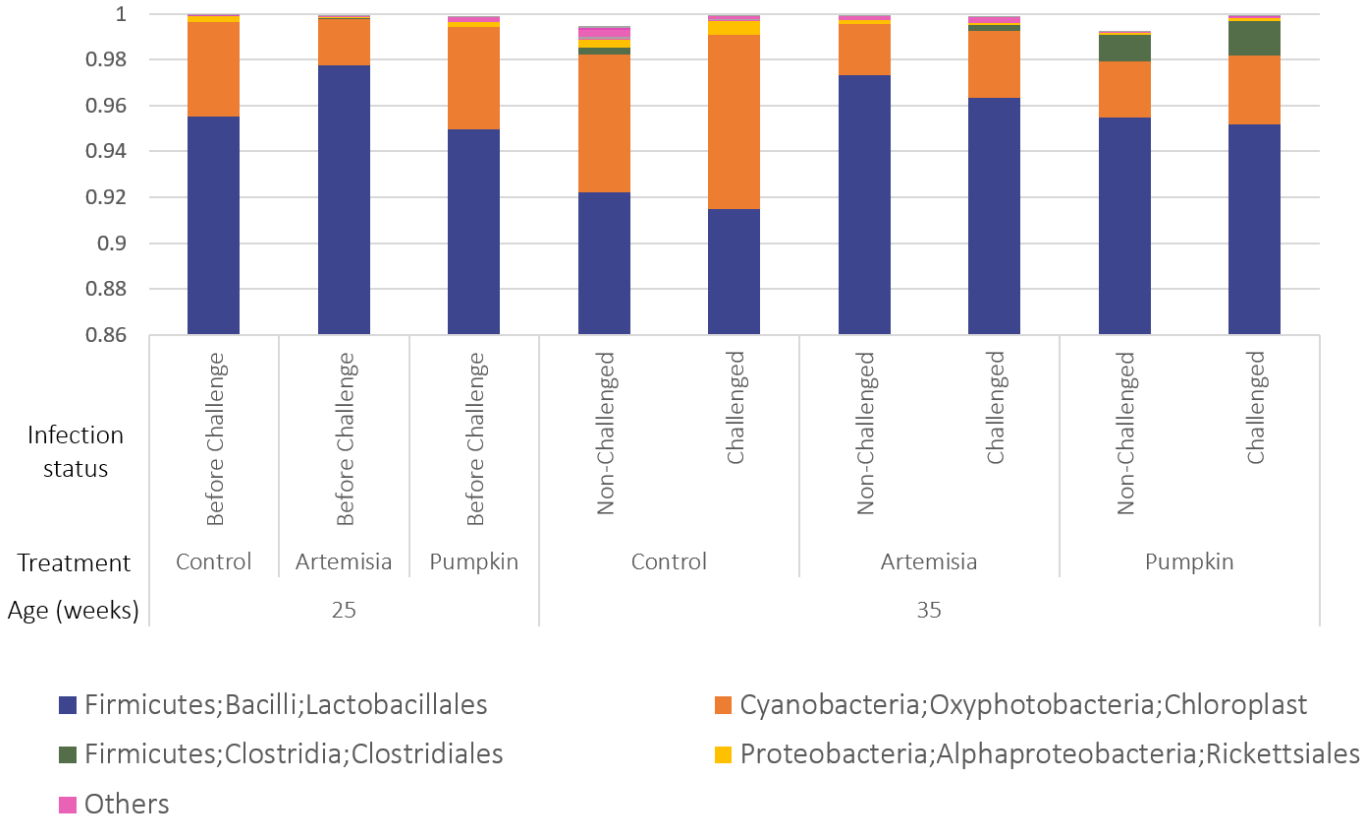


Figure 5.6. Pielou's evenness test (alpha diversity) of jejunal microbiota in the jejunal content of layer hens. The black boxes represent 25-week-old-birds before the challenge, the pink box represents non-challenged birds 35 weeks, and the red box represents challenged birds with *Ascaridia galli* at 35 weeks (10 weeks post-infection).

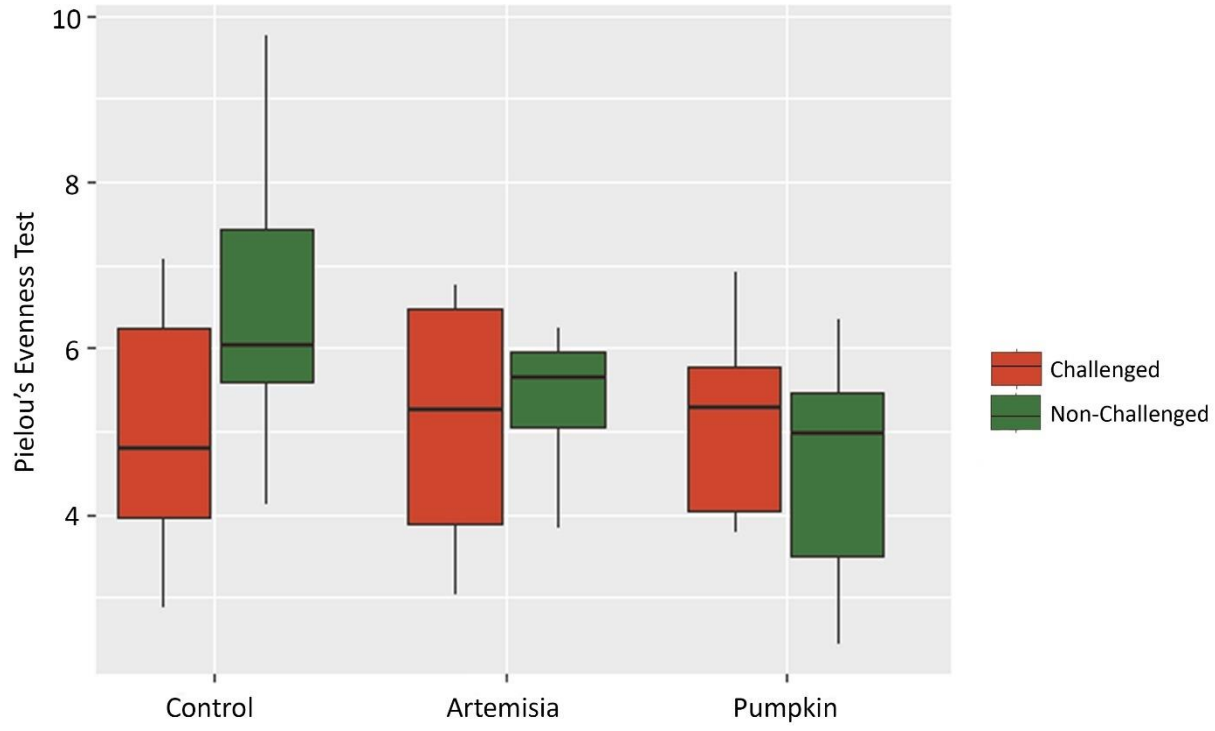
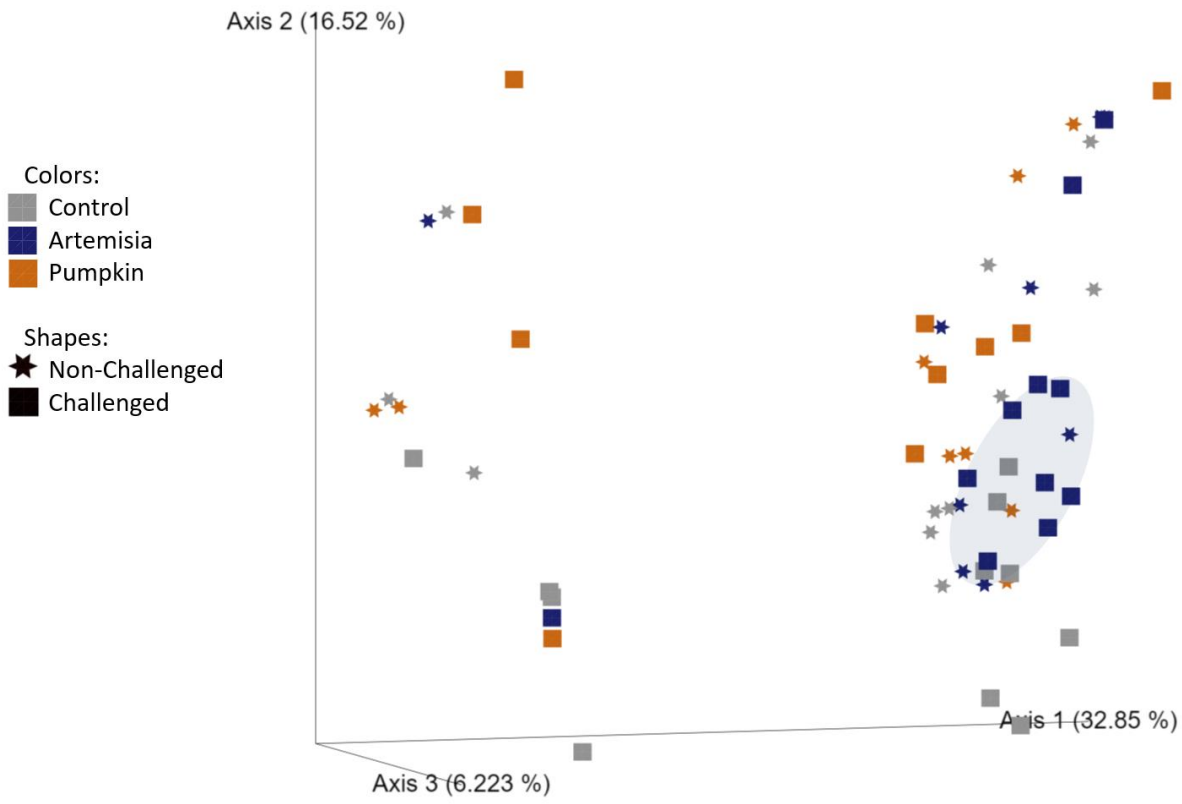


Figure 5.7. Principal Component Analysis of microbiota identified in the jejunum of content of layer hens based on the unweighted UniFrac index. Grey shapes represent control birds, blue shapes represent bids fed artemisia (0.02%), and orange shapes represent birds fed pumpkin seed (1%). Stars represents birds that were challenged with *Ascaridia galli* at 35 weeks (10 weeks post-infection) while squares represent non-challenged birds.



VI. CONCLUSIONS

The first study provided information about the prevalence of parasites in an alternative system and suggests this environment can the affect parasite challenge. Specifically, it tested for coccidia oocysts and round worm eggs in broilers, layers, and turkeys during various seasons. Higher temperatures seemed to be favorable for the worm egg survival. The data also showed that the rotation of pastures seems to be effective on the control of coccidiosis given the fact that oocyst counts were lower than what is found in conventionally reared broilers. However, this was not true for roundworms.

Not only were 80 percent of the layer samples positive for roundworms, which is considered a high prevalence, but also flock remained positive throughout different seasons and with different ages. This indicates the inefficiency of controlling roundworms by rotational systems and urges for other methods for controlling the disease. The next two chapters focused on this parasite, how it can modulate the immune system and the microbiota as well as investigated at alternatives that could be used on the control of such prevalent parasite.

Our second study investigated the immune response and changes in the intestinal microbiota at several time points after infection with *A. galli*. The observed changes were only due to the presence of larvae because the birds cleared the infection before the larvae developed into adult worms. Three different phases were seen in the immune response. First, there was a moderate stimulation the immune system in the very earlier stages of the worm's life cycle due to larval tissue invasion. Second, an upregulation of all evaluated cytokines was seen when the larvae as expected to develop into adults, which did not happen. This is most likely due to the immune response by the hens. Lastly, a full recovery by the host without differences in cytokine expressions

between challenged and non-challenged birds was observed. The changes in the jejunal microbiota showed a similar pattern as the immune response.

The reason why the immune system was able to fully clear the birds of the infection has not been fully elucidated. It is known that nutrition can be used as a tool to modulate the immune system and contribute to better intestinal health, so the nutrient-dense diet in this experiment might have improved the immune response. This hypothesis needs more research. The time-period 5 weeks P.I. was a crucial week in the pathogenesis of the infection. However, this experiment did not yield any data regarding to the immune and microbiota response to adult worms.

The last study evaluated two possible treatments for *A. galli* as well as the impacts of the infection with *A. galli* and these treatments on the cytokine gene expression and microbiota. Different from what was seen in the previous study, the modulation of the immune system was very subtle. This suggests that adult worms cause a lower inflammatory response than larvae. The absence of impact of the egg quality and production as well as the low number of adult worms recovered are other facts that show that this was a mild infection. However, there were significant differences on nutrient digestibility. These differences support the idea that *A. galli* can affect birds' physiology.

Regarding as the potential treatments for *A. galli*, neither *Artemisia absinthium* nor pumpkin seed showed a clear effect. Most noticeable, they supported the host to maintain calcium digestibility in challenged birds. The observed increase in Lactobacillales is a potential reason of this effect since they were previously correlated with the production of acetate with can increase mineral absorption. In order to confirm this hypothesis, studies such as metabolome analysis are needed.

Overall, this research demonstrated that nematodes have high prevalence on alternative production systems and tools for controlling the diseases are still needed. *A. galli* (mainly larvae) modulated both immune system and the jejunal microbiota. This study offers scientifically verified information to backyard producers as well as cage-free farmers that pumpkin seeds and *Artemisia absinthium* were not effective against *A. galli* at the concentrations used.