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Department of Animal and Veterinary Sciences - section of Gut and Host Health, Aarhus University

Use of Alternative Methods to Control Helminths in Organic Layers

The effect of propionic acid, coriander seed oil and pumpkin seed oil on *Ascaridia galli* in organic laying hens (*Gallus gallus domesticus*)



Master thesis in Agrobiography

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Use of Alternative Methods to Control Helminths in Organic Layers

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Master Thesis (45 ECTS) by

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Preface

This master thesis has been written to fulfil the requirements for a Master's degree in Agrobiolology at the Faculty of Technical Sciences, Aarhus University, Denmark. The thesis is based on an animal experiment that lasted over five weeks as a part of the EU Horizon 2020 project Ppilow. The thesis has been written during the period from 15th of November 2023 to 3rd of June 2024.

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Maj Dahl Ørndrup

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List of abbreviations

<i>A. galli</i>	<i>Ascaridia galli</i>
BALT	Bronchial-associated lymphoid tissue (BALT)
BW	Body weight
BWG	Body weight gain
BWL	Body weight loss
CALT	Conjunctival-associated lymphoid tissue
DM	Dry matter
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immune sorbent assay
EPG	Eggs per gram feces
FACS	Fluorescence-activated cell sorting
FI	Feed intake
FCR	Feed conversion ratio
GALT	Gut-associated lymphoid tissue
H/L-ratio	Heterophils/lymphocytes ratio
IBV	Infectious Bronchitis Virus
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgY	Immunoglobulin Y
IL	Interleukin
MALT	Mucosa-associated lymphoid tissue

MBL	Mannose-binding lectin
MD	Meckel's Diverticulum
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
OD	Optical density
PBS	Phosphate-buffered saline
PF	Post feed treatment
r	Spearman correlation coefficient
RT	Room temperature

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Abstract

The conditions in which organic hens live pose a great risk of infection with helminth since they are in contact with feces and have access to outdoor areas. The most common intestinal helminth in organic layer flocks in several countries is *Ascaridia galli* (*A. galli*). The aim of the study was therefore to investigate how helminth burden and immunocompetence in organic layers are affected by grains treated with propionic acid or fed with oils from coriander seeds and pumpkin seeds as feed additives. Seventy-two hens at 80 weeks old of the breed Dekalb White were used and they were naturally infected with *A. galli* before arrival at the facilities. The hens were fed with either a control diet, a diet with propionic acid treated grains or a diet with pumpkin seed oil and essential coriander seed oil. The experiment ran for 4 weeks before the layers were slaughtered. Samples were taken through the experiment and at slaughter. The treatments did not affect the health, welfare, and production parameters of the hens. In this experiment setup it was not possible to prove anthelmintic effects of pumpkin seed oil combined with essential coriander seed oil or grains treated with propionic acid due to large individual variations in worm burden of the naturally infected hens. However, effects were found on immunocompetence as the phagocytosis ability of leucocytes in the blood showed a significant higher activity for heterophils, lymphocytes, and thrombocytes isolated from the the hens fed the propionic acid diet.

1. Introduction

Consumers have changed their demands to their food over the last years with increased focus on naturalness and animal welfare (Marcos-Atxutegi et al., 2009). This has resulted in an increase of the number of laying hens kept in alternative production systems such as free-range and floor husbandry (Gauly et al., 2002). In these systems the hens are not separated from their feces which comes with a high risk of infection with helminth parasites such as *Ascaridia galli* (*A. galli*). A study has shown that *A. galli* is the most common intestinal helminth in organic layer flocks in Austria, Belgium, Denmark, Germany, Italy, The Netherlands, Sweden, and the United Kingdom (Thapa et al., 2015). The second most common is the cecal nematode *Heterakis gallinarum*. Infection with *A. galli* in laying hens have both economic losses and negative effect on the welfare (Permin et al., 1997, Ponnudurai and Chellappa, 2001). When the hen ingests the infective egg, the egg hatches, and the larvae penetrate the intestinal wall causing damages leading to reduced nutrient absorption and thereby reduced weight gain, and in worst case even increased mortality (Skallerup et al., 2005, Hinrichsen et al., 2016).

An infection with *A. galli* can be treated with anthelmintics like flubendazole and Fenbendazole (Tarbiat et al., 2016, Feyera et al., 2022a). Due to a high general focus on resistance these years alternatives to drugs are of high priority. In order to find these alternatives, it is necessary to understand the host-parasite relationship and optimize biosecurity, genetics or feeding. Biosecurity can target the highly persistent eggs in the environment where focus is on cleaning and disinfection between the flocks (Permin, 2003). This is possible via the all-in all-out principle. It is more difficult when looking at the outdoor area, but management here might not be necessary since outdoor access have shown a negative association on *A. galli* worm burden (Thapa et al., 2015). According to the genetics, different studies have shown that some chicken breeds are more resistant to *A. galli* infections than others (Schou et al., 2003, Gauly et al., 2002, Gauly et al., 2001). This could help to prevent *A. galli* infections if more resistant breeds were chosen in the organic layer production. Supplementation of organic acids to the feed has been suggested to have positive effects on combating intestinal worms. *In vitro* studies have shown that propionic acid can kill 85% of the larvae of *Strongyloides papillosus*, *Haemonchus contortus* and *Muellerius capillaris* (Boyko and Brygadyrenko, 2022). Others have studied plant products like garlic or extracts of pumpkin seeds, citrus peel, ginger, curcumin, or pomegranate peel (Abdel Aziz et al., 2018, Abdelqader et al., 2012, Kavindra and Shalini, 2000, Bazh and El-Bahy, 2013). They all show a lethal effect on *A. galli* worms

in vitro but with limited *in vivo* effect compared to the anthelmintic drug fenbendazole. More studies are therefore needed to find the proper dose and optimized delivery methods.

2. Objective

The objective of this study is to critically evaluate how helminth burden and immunocompetence in organic layers are affected by organic acids such as propionic acid or oils from coriander seeds and pumpkin seeds as feed additives.

3. Background

3.1. Organic egg production

The organic egg production has developed on the basis of consumers demand for more natural, sustainable and ethically produced food. The aim of organic egg production is to prioritize health and welfare of the chickens while minimizing the environmental impact of farming practices.

Production of eggs and types of production

Unit:

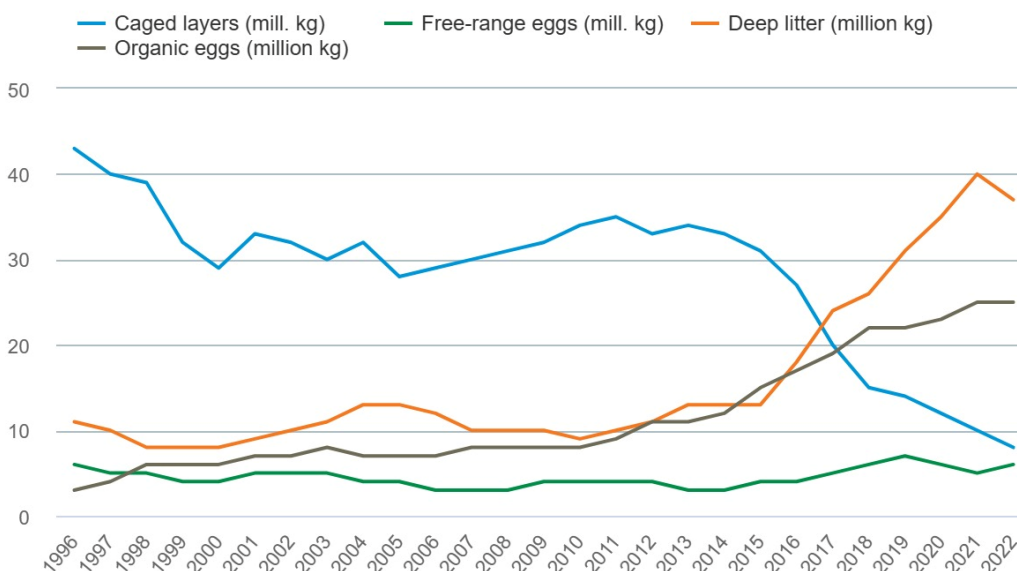


Figure 1: Egg production for the different types of production in Denmark from 1996-2022 (Danmarks statistik, 2024).

Figure 1 shows the different egg production systems in Denmark and the production of the different systems from the year 1996-2022. The organic egg production has increased in the entire period and especially from 2010 to 2022 (8 to 25 million kg) (Danmarks statistik, 2024). This increase can be attributed to the ban of conventional cages in 2012. Today, the organic egg production is the second biggest egg production system in Denmark after deep litter egg production.

The organic egg production is based on organic farming standards with a set of rules from the EU. An organic certification can be obtained by following the organic standards set by EU and regular inspections ensure compliance with the standards (EU, 2018/848). These standards involve regulations on the management, facilities, diet, and medical treatment. For example the rules from the EU states that the chickens shall have access to open air at least one third of their life and they are in general provided with more space (EU, 2018/848). The outdoor area should provide vegetation, shade, and protection from predators. In organic egg production, it is allowed to have flock sizes of 3000 hens/pen with restrictions of maximum six hens per m² in the indoor facilities and at least 4 m²/hen in the outdoor area (EU, 2018/848). Also, the chicken shall be provided with certified organic feed including roughage. This means that feed are grown without the use of synthetic pesticides, herbicides or fertilizer (EU, 2018/848). In case of medical treatment the withdrawal period is twice as long as in the conventional production and at least 48 hours (EU, 2018/848).

Infections with *A. galli* occur in all types of production systems, where the layers have access to the litter (Permin et al., 1999). Cases of infection with *A. galli* has increased since the ban on conventional battery cages in 2012 (Höglund et al., 2023). Hence, there is an increase in research into sustainable strategies to control *A. galli* infections.

3.2. *Ascaridia galli*

3.2.1. Morphology

A. galli is a common intestinal parasite in the small intestine of domestic and wild birds. It is a roundworm that belongs to the phylum Nematoda under the group helminths. The adult worm is yellow-white in color and semitransparent. It is the largest parasite found in birds where the adult male worm can reach a length from 42 to 76 mm and the female can be a little longer from 72 to 108 mm (Ramadan and ABOUZNADA, 1992). The head of the worm has an oral opening which is surrounded by three trilobed lips (Figure 2A). On the dorsal lip are two papillae and on the subventral lips is one papillae on each. Neck papillae occurs on the sides of the body near the anterior end (Ramadan and ABOUZNADA, 1992). The sexes can be distinguished from each other by looking at

the tail in the posterior end where the male has a tail with a little “hair” in the end females do not have this (Figure 2ABC).

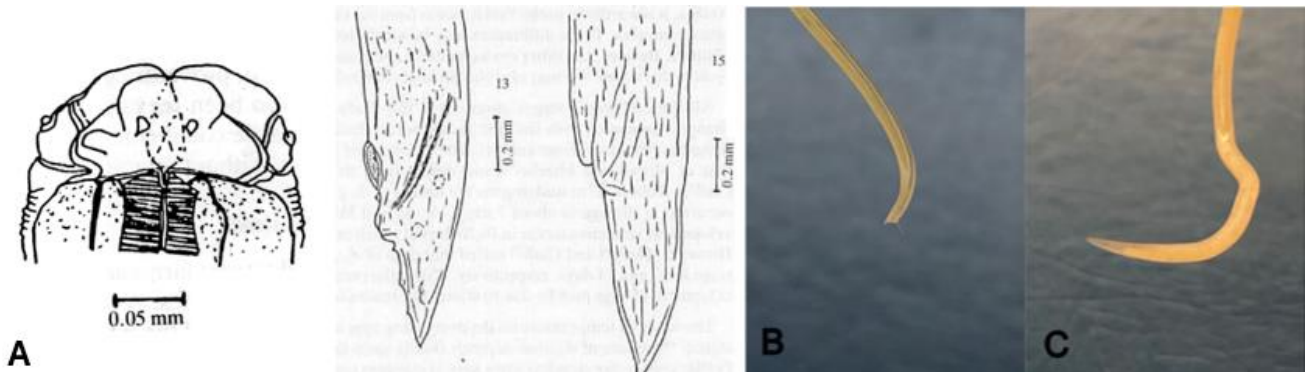


Figure 2: A) Drawings of first the head and then the posterior end of 25 days old *A. galli* worms; the left tail is a male and the right tail is a female (Ramadan and ABOUZNADA, 1992). B) Picture of the posterior end of a male from another experiment (Højmark, 2021). C) Picture of the posterior end of a female from another experiment (Højmark, 2021)

3.2.2. Lifecycle

The life cycle of *A. galli* is direct and the birds are infected orally. Starting with the *A. galli* egg which gets excreted in the host feces (Figure 3 #1). The eggs are oval and enveloped with three layers. The inner layer is a permeable vitelline membrane, the next is a thick resistant covering and lastly a thin albuminous layer (Perry and Wharton, 2011). These layers make the eggs highly resistant to desiccation and allow long survival in the environment. The eggs will remain viable in the typical layer housing conditions where they can survive for several month. The eggs do not hatch in the environment but develop into eggs containing an infective larva ending with the third larval stage (L3) (Shohana et al., 2023). The different developmental stages of the egg are shown on Figure 4. The eggs can develop to the infective stage in a range of 15-35 °C, but prefer temperatures between 20 and 30 °C (Tarbiat et al., 2015). Oxygen and relative humidity above 70% are necessary for the eggs to develop (Tarbiat et al., 2015). The hen will ingest the eggs through contaminated water or feed but can also get it from earthworms which has ingested the infectious eggs (Perry and Wharton, 2011). The ingested egg with L3 larvae will hatch within 24 h in the proventriculus (Figure 3 #6), by stimulating factors such as temperature, pH and carbon dioxide levels (Tarbiat et al., 2015). In the first week of infection the larvae will settle in the jejunoileal section and migrate further down the intestine as the infection progresses where the larvae will penetrate the intestinal mucosa around 2 weeks after the egg is ingested by the hen (Ferdushy et al., 2012, Shohana et al., 2023) (Figure 3 #7). The following larval stage is histotrophic and can last 3-54 days whereafter it will reappear in the

intestinal lumen and become adult worms. It takes about five weeks for the worm to reach sexual maturity after which the female can excrete eggs in the small intestine for several months (Stehr et al., 2018).

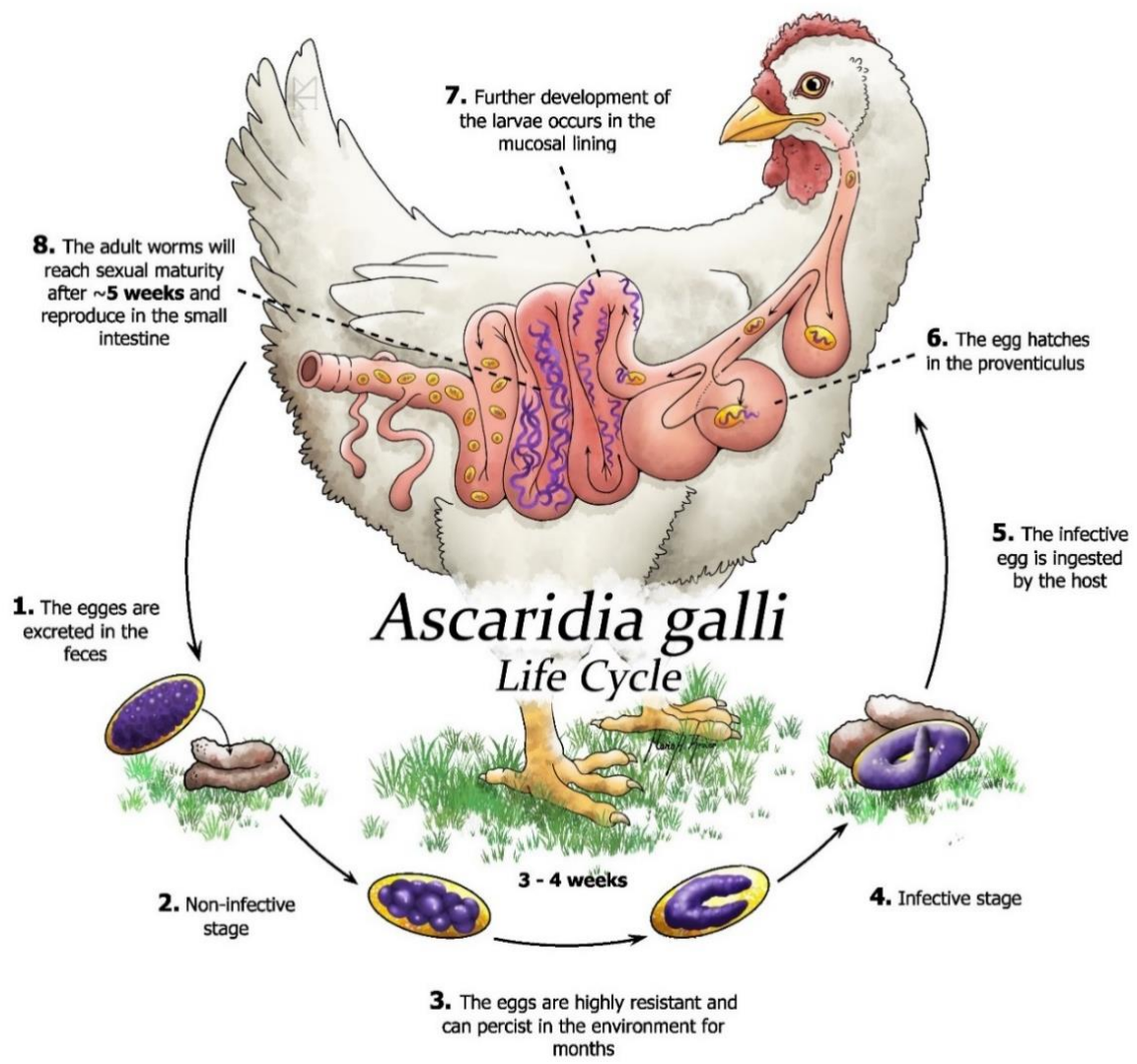


Figure 3: The direct lifecycle of *Ascaridia galli* in chickens starts with excreted eggs in the feces of the host. Then the eggs will embryonate in the environment before it is ingested by the hen. When the egg hatch the larvae will migrate to the intestine where it will mature and reproduce starting the cycle over again (Kroier, 2024).



Figure 4: Overview of the different developmental stages of *Ascaridia galli* eggs seen through a microscope. A) shows dead eggs. B) shows unembryonated eggs. C) shows different stages of developing eggs. D) shows the stage where it starts to have the shape of a worm. E) show a fully embryonated egg (Tarbiat et al., 2015).

3.3. The immune system of the layer

The avian immune system is like that of mammals but has some differences, and it plays a vital role in protecting the bird from pathogens such as bacteria, viruses, fungi, and parasites. The immune systems consist of lymphatic vessels, lymphoid organs, and lymphoid tissue. The lymphoid organs are parted into primary lymphoid organs and secondary lymphoid organs and tissues, and they are illustrated on Figure 5. In the primary lymphoid organs, lymphocytes are generated, developed, and matured. The B cells mature in bursa of Fabricius and the T cells mature in thymus. The bursa of Fabricius is a special organ only found in birds and is located near the cloaca. The bursa of Fabricius starts to undergo involution after it has reached its maximum size at 8-10 weeks of age, and it is heavily involuted at 6-7 months of age. The involution of thymus starts at 3-4 month of age but

examination of changes suggest that the remaining cells in thymus retain their functional capacities (Ciriaco et al., 2003). Besides development and maturation of the lymphocytes the primary lymphoid organs are also where elimination of self-reactive B- and T cells takes place. In the secondary lymphoid organs and tissue, the mature lymphocytes interact with antigens and get activated and this happens in the lymph nodules, spleen, bone marrow, Harderian gland and mucosal-associated lymphoid tissues (MALT). Chickens do not have regular lymph nodes but have lymph nodules associated with the walls of the lymph vessels and in skin and lungs. MALT consists of gastrointestinal-associated lymphoid tissue (GALT), bronchial-associated lymphoid tissue (BALT), and conjunctival-associated lymphoid tissue (CALT). GALT are well-developed in birds and includes the lymphoid structures Meckel's Diverticulum, cecal tonsils and Peyer's patches (Lamichhane et al., 2014).

CHICKEN IMMUNE SYSTEM

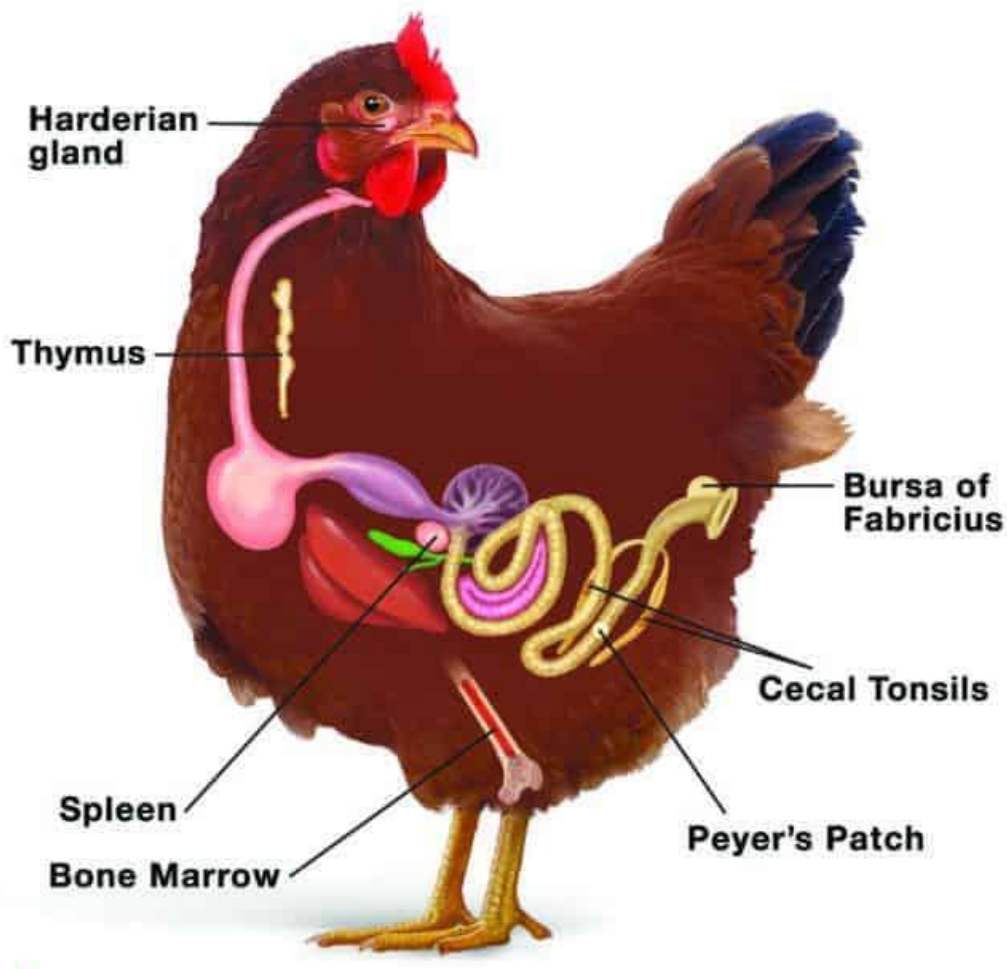


Figure 5: Illustration of lymphoid organs as part of the chicken immune system (Nutrena, 2021).

The immune system is parted in the innate and the adaptive immune system like in mammals. The innate immune system is the first line of defense and consist of physical barriers, cellular defense, and a complement system. These defense mechanisms can quickly recognize and neutralize potential threats without prior exposure or specific recognition of pathogens. The physical barriers are the skin and mucous membranes lining the respiratory, digestive, and reproductive tracts. They work to prevent the entry of pathogens into the body. The mucous membranes can secrete mucus that can trap pathogens and, in that way, prevent them from entering the body. The cellular defenses are the phagocytes and natural killer cells that can engulf and destroy pathogens that have managed to cross the physical barriers. The phagocytes are specialized white blood cells that can destroy pathogens through a process called phagocytosis. The avian heterophil is a granulocytic white blood cell which is a counterpart to the mammalian neutrophil. Heterophils are involved in phagocytic activity and have been shown to be the first present in case of an inflammation (Genovese et al., 2013). The avian thrombocytes are different in morphology from mammalian thrombocytes because they are nucleated, but they have similar functions. The humoral innate immune system covers pattern recognition receptors (PRRs), antimicrobial peptides, cytokines, and chemokines. PRRs can recognize specific molecular patterns associated with pathogens known as pathogen-associated molecular patterns (PAMPs). Examples of PRRs are the membrane bound Toll-like receptors (TLRs) and the soluble mannose-binding protein (MBL). When PRRs gets activated, they trigger the inflammatory response and recruit immune cells to the site of infection. Antimicrobial peptides can kill or inhibit the growth of pathogens and they can be found in mucus, tears, and other body fluids. Cytokines and chemokines are excreted by cells and are signaling molecules that can regulate the immune response by promoting inflammation, activating immune cells, and coordinating the recruitment of immune cells to sites of infection (Kaiser and Balic, 2015). Lastly the complement system is a group of proteins that helps antibodies and phagocytic cells to find and destroy pathogens more effectively. It can also result in killing of pathogens by formation of membrane attack complexes.

The adaptive immune system consists of humoral immunity and cell-mediated immunity. The humoral immunity consists of antibodies produced by B cells, that can recognize specific antigens on pathogens and mark them for destruction by other immune cells. Antibody production happens when a B cell gets exposed to a pathogen and thereby gets activated. The B cell will then proliferate, differentiate into plasma cells, and produce antibodies which in the chicken will be either IgM, IgY, or IgA. The IgY is an IgG homologue that chickens produce, and they are functionally equivalent but structurally different. IgY is the most common antibody found in the circulatory system of chickens

and the total amount of IgY in serum can be an indication of health status, fitness and nutritional state of the chicken (Kitaguchi et al., 2008). The cell-mediated immunity consists of T cells that can identify and kill infected cells in the host or help to coordinate the immune response by activating other immune cells, so the infected cell gets destroyed. The avian T cells are divided into two subpopulations TCR $\alpha\beta$ ⁺ T cells and TCR $\gamma\delta$ ⁺ T cells (Chen-lo et al., 2018). They can be further divided depending on the antigens on the cell surface. The TCR $\alpha\beta$ ⁺ T cells with CD4 antigens have helper cell functions and the ones with CD8 antigens have cytotoxic activity. The helper cells can further be divided into Th1 and Th2 depending on the cytokines they produce. In the chicken the TH2 cytokines interleukin (IL)-13 and IL-4 are both induced following infection with helminth pathogens like *A. galli* (Degen et al., 2005). TCR $\gamma\delta$ ⁺ T cells are innate-like T cells that do not recognize pathogens through MHC presentation. Instead they respond to non-peptide antigens like glycoproteins and other small molecule polypeptides (Fenzl et al., 2017). The chicken TCR $\gamma\delta$ T cells have a cytotoxic function.

Chickens also have a highly polymorphic major histocompatibility complex (MHC) which plays an important part in presentation of antigens to T cells. The MHC molecules will bind to antigen fragments from pathogens and present them on the surface of the cells and thereby allow T cells to recognize the pathogen. The MHC class I presents antigens from intracellular pathogens mainly to CD8⁺ T cells and the MHC class II present antigens from extracellular pathogens mainly to CD4⁺ T cells. The MHC class I are expressed on most cell types, but the MHC class II are only expressed on antigen-presenting cells such as dendritic cells, macrophages and B cells (Kaiser and Balic, 2015).

3.4. Effects of *A. galli* infection on the host

A. galli has a negative impact on the chicken when it gets infected. This applies both to health, welfare, and productivity. When the larvae penetrate the intestinal mucosa in their development process it causes damage to the mucosa and gives irritation and inflammation. Looking at the intestine histology during infection reveals severe traumatic lesion in the small intestinal wall (Marcos-Atxutegi et al., 2009). In the intestine the mucous membrane can be completely altered resulting in no villi and crypts. This is likely caused by migrating larvae and/or damages caused by the adult worms. In connection with this, there is also seen an intense cellular infiltration by lymphocytes, macrophages and eosinophils in the intestine mucous membrane (Shohana et al., 2023).

If the worm burden is high it is associated with loss of appetite, diarrhea, and mechanical intestinal obstruction. This can further lead to reduced nutrient absorption and depletion of fat reserves in the liver (Daş et al., 2010, Sharma et al., 2018a, Feyera et al., 2022b). Toxins from *A. galli* can influence the enzyme systems in the intestinal mucosa and in that way also interfere with the normal absorption of nutrients (Vasilev et al., 1973). This reduces the growth rate and egg production of the hen and increases the feed conversion ratio (FCR). In worst case scenario severe infections can lead to reduced weight gain and may lead to increased mortality (Skallerup et al., 2005, Hinrichsen et al., 2016). Furthermore, an infection with *A. galli* may also increase the risk of bacterial infections with *Escherichia coli* and *Pasteurella multocida* (Dahl et al., 2002, Permin et al., 2006).

A study done by Schwarz et al. (2011) demonstrated that an infection with *A. galli* resulted in a local gut-associated immune reactions. They found that different T cell populations infiltrated the intestinal epithelium and lamina propria. CD4⁺ T helper cells were found to accumulate with a two-fold increase in the epithelium in the parasite tissue phase in the duodenal mucosa. This was supported by another study finding an increase of intraepithelial CD4⁺ T helper cells in infected chickens with a peak at day 20 post infection (Ruhnke et al., 2017). Schwarz et al. (2011) suggest that the CD4⁺ intraepithelial lymphocytes may play a role in control of an *A. galli* infection by elimination of the larvae during the duodenal tissue stage. Others have found that the relative number of CD8 α ⁺ T cells decreased in the duodenum and jejunum 2 weeks after infection (Ruhnke et al., 2017). Also, TCR $\gamma\delta$ ⁺ T cells were shown to increase within 1-week post infection in the duodenum (Shohana et al., 2023).

In addition to the T cell response an increase in mRNA expression of the Th2 cytokines IL-4 and IL-13 was dominating the intestinal immune reactions (Schwarz et al., 2011, Shohana et al., 2023). This was supported by another study where it was the same cytokines that dominated the mRNA expression in splenic and ileal tissue that was upregulated during an infection with *A. galli* (Kaiser, 2007, Dalgaard et al., 2015).

Another finding was a development of specific IgY serum antibodies in infected birds, but there was no evidence that it had a protective role in the *A. galli* infection (Schwarz et al., 2011). In a study by Marcos-Atxutegi et al. (2009), they found high concentrations of the specific IgY anti-*A. galli* antibodies both in blood and egg yolk from infected laying hens during *A. galli* infection. An inflammatory reaction in the intestinal wall was also found, with appearance of an intense cellular infiltration in the mucous and submucous membrane. They found a higher IgY antibody response against embryonated eggs than adult antigens (Marcos-Atxutegi et al., 2009). This could be due to

the larvae invading the intestinal wall creating a stronger stimulation than the worms living in the lumen which stimulate predominantly antibody response of the IgA isotype. But others have found elevated levels of IgY in connection with *A. galli* infection (Norup et al., 2013, Ferdushy et al., 2014, Ruhnke et al., 2017). The increased level of any of the antibodies does not provide protection against the nematode and thereby do not prevent reinfection. They may however only affect larval growth (Andersen et al., 2013, Norup et al., 2013)

3.5. Treatment/Prevention of infection with *A. galli*

The most effective method to control *A. galli* is through treating the hen with an anthelmintic like flubendazole and Fenbendazole (Tarbiat et al., 2016, Feyera et al., 2022a). An infection can be important to treat since results indicate that flocks of laying hens kept on litter will remain infected and the prevalence of *A. galli* increase with age (Zloch et al., 2018). However, as with other drugs, it is important to have in mind the risk of resistance development in the worms. Therefore it is important to have focus on the choice of drug and use a restrictive and well-considered approach (Höglund et al., 2023).

To avoid the use of anthelmintic drugs, one may focus on prevention. The non-cage systems provide favorable conditions for the *A. galli* eggs where they can accumulate and develop into infectivity in the litter indoors or the soil on the pasture. Here they can remain infective for at least a year (Farr, 1956). This is because it is difficult to maintain a high level of hygiene and *A. galli* eggs become infective within 14-21 days under optimal conditions (Tarbiat et al., 2015). Hence, focusing on biosecurity can be a solution for prevention. The layer production works with the principle all-in all-out and it is therefore possible to deep clean the stables between production cycles to avoid transmission from one cycle to another (Permin, 2003). However, this is only effective if boots, machines, and other equipment entering the barn are also cleaned. To reduce the majority of *A. galli* eggs the litter should be removed often (i.e. weekly or more) and the floor should be kept as dry as possible as most parasites require nearly 100% relative humidity to develop (Permin, 2003). Normally it is recommended to wash the inventory with high-pressure devices but this may improve the conditions for parasite egg development and survival and may help spreading the infective stages all over the pens (Permin, 2003). Another possibility is to use high temperatures such as flame-throwers since disinfectants are generally not active against parasite eggs. After the house has been cleaned and disinfected the last step is to lime-wash. This works against parasite eggs since the drying effect

of lime decreases the survival of the eggs and the pH level exceeds 8 which also decreases the survival of the eggs. After this the house should be left empty for 2-4 weeks before new animals are taken in (Permin, 2003).

Cleaning and disinfection are far more difficult with the outdoor area since it is not possible to clean. However, this may be less important as a study has shown a negative association between *A. galli* worm burden and pasture access time (Thapa et al., 2015). This could be because the EU regulations require a maximum stocking density of 6 hens per m² indoors and a maximum of one hen per four m² outdoor area (EU, 2018/848), and thereby the feces may potentially be spread over a larger area outdoors compared to indoors, thus theoretically decreasing the risk of animals becoming infected. However, feces may not be spread evenly over the pastures as the majority of hens have been shown to primarily forage on areas close to the henhouse (Hegelund et al., 2006, Heckendorn et al., 2009). The difference could also be because transmission pathways, survival, and development of *A. galli* eggs to infectivity may differ between the indoor and outdoor areas. The indoor areas may provide more favorable conditions (e.g. temperature) for egg development throughout the year (Tarbiat et al., 2015). *A. galli* eggs excreted outdoors during the winter months cannot develop to infectivity in colder climates where temperatures drop below 15 °C (Tarbiat et al., 2015). Also, *A. galli* eggs excreted outdoors in the summer month are likely to die of environmental factors such as direct sunlight (Brown, 1927, Bray and Lancaster, 1992) and desiccation (Caldwell and Caldwell, 1928, Seamster, 1950). However, there are still management initiatives that can help to reduce the worm burden. Rotational paddock management with a permanently used, small all-weather run with wood chips or gravel on the ground has been recommended due to it showing reduced fecal egg counts compared to unmanaged paddocks (Maurer et al., 2013).

Another way to reduce infections with *A. galli* can be through selective breeding for chicken lines with parasite resistance. Studies have shown that some chicken breeds were more resistant towards *A. galli* infections than others (Schou et al., 2003, Permin and Ranvig, 2001). Others suggest that it is possible to select for *A. galli* resistance within the chicken line meaning that chickens of the same line has different genetic resistance towards *A. galli* (Gauly et al., 2002). This could be due to genetic differences and studies have shown a connection between MHC haplotypes and susceptibility to parasitic infections (Schou et al., 2010b, Norup et al., 2013).

Another area of interest is to use different feeding strategies with e.g. antiparasitic feed supplementation to reduce *A. galli* infection. This can involve the addition of herbs, plants, or organic

acids to the diet. Examples of such studies are presented in the next section (3.4 Feed alternatives to anthelmintics against *A. galli*).

3.6. Feed alternatives to anthelmintics against *A. galli*

3.6.1. Organic acids

Organic acids are commonly used as food additives in food for humans for greater processing of the product (Boyko and Brygadyrenko, 2022). There is scientific literature on how these additives can be used in the food industry, but there is a lack of information on the effects on living organisms. This can be of interest for pest control in farm animals. Hence the additives can be an alternative to veterinary medicine. There are no studies where organic acids have been tested for *A. galli* control but studies on other nematodes have been performed.

An *in vitro* study has investigated the influence of 32 organic food additives on larvae of the nematodes *Strongyloides papillosus*, *Haemonchus contortus* and *Muellerius capillaris* found in ruminants. They found that propionic acid and 2-phenylphenol had strong nematocidal effects on the nematodes, where 1 % concentrations of the substances killed at least 85% of the larvae except for *H. contortus*, which were tolerant to 2-phenylphenol in 52% of the cases (Boyko and Brygadyrenko, 2022).

In another *in vitro* study, it has also been found that formic acid has indicated nematocidal properties against nematode larvae from *Strongyloides papillosus* and *Haemonchus contortus*. The lowest median lethal dose (LD50) was found to be 4.7 g/kg on average for the third-stage larvae of *S. papillosus*, 0.076 g/kg for the first- and second-stage larvae of *S. papillosus*, and 4.1 g/kg for the third-stage larvae of *H. contortus* (Boyko et al., 2019). In the current study the effect of grain preserved with propionic acid on *A. galli* was studied.

3.6.2. Plants

Throughout the years different plants and extracts of plants have been investigated for anthelmintic effects with varying success. *In vitro* tests have shown that different concentrations of garlic oil can cause mortality of *A. galli* worms (Kavindra and Shalini, 2000). The main active component in garlic is allicin and this has been tested *in vivo* on cockerels where the doses 225 µg of allicin per bird and

2.250 µg of allicin per bird were compared with the anthelmintic treatment flubendazole. None of the doses had a significant effect on the number of adult *A. galli* worms (Velkers et al., 2011).

Other studies have underpinned the difficulty in translating promising *in vitro* studies into successful *in vivo* studies. As another example, extracts from ginger and curcumin were found to have potential effects against *A. galli*. *In vitro* where study 100 mg of either ginger or curcumin methanolic extracts had a lethal effect on 85.8% and 80.8% respectively on *A. galli* after 48 h of exposure (Bazh and El-Bahy, 2013). The efficacy was statistically significant lower when it was tested *in vivo* where 100 mg of either ginger or curcumin methanolic extracts had a lethal effect on 58.3% and 45% respectively on *A. galli* after 48 h of exposure (Bazh and El-Bahy, 2013).

Extracts from pumpkin seeds (*Cucurbita pepo*) and pomegranate peels have been investigated in both *in vitro* and *in vivo* studies where they were compared with fenbendazole. In the *in vitro* study the highest mortality of *A. galli* worms on 73.3% and 85% was seen with the highest concentration of 75 mg/ml of pomegranate peel aqueous extracts and pumpkin seed ethanol extract respectively 36h after incubation (Abdel Aziz et al., 2018). This was not statistically significant different from 5 mg/ml fenbendazole with a mortality on 89.2%. In the *in vivo* study, 2000 mg/kg of pomegranate peel aqueous extracts and 2000 mg/kg of pumpkin seed ethanol extract were used and compared with 100 mg/kg fenbendazole. The results showed that the pumpkin seed extract had a statistically significant higher mortality than pomegranate peel extract after 48h after treatment (66.9% vs 61.9%) (Abdel Aziz et al., 2018). The mortality of pumpkin seed extract was not statistically significant from that of fenbendazole (66.9% vs. 73.5%) (Abdel Aziz et al., 2018). For both the *in vitro* and *in vivo* study the lethal effect of the extracts was improved with increasing treatment time from 3-36h and 12-48h respectively.

Another study on pumpkin (*Cucurbita pepo*) seeds has found similar results where chickens were feed with 2 g pumpkin seeds per bird per day compared with mebendazole. The pumpkin seed treatment showed a significant decrease in worm load (Acorda et al., 2019). Results indicated that compared to mebendazole, the pumpkin seeds were moderately effective in reducing worm counts of *Ascaridia* spp. and *Raillietina* spp and moderately effective in reducing egg output of the worms (Acorda et al., 2019).

A study on pumpkin seed powder supplemented for 7 days have shown a medium immunostimulation effect on phagocytosis in mice with the dose 3.8 g/kg body weight (BW) and 7.6 g/kg BW (Iwo et al., 2014). Also, it has shown a stimulating effect on the cellular immune response for the dose 7.6

g/kg BW. Furthermore, it was found that both doses of pumpkin seed powder showed a significant increase in lymphocyte count indicating a stimulating effect on lymphocyte proliferation (Iwo et al., 2014).

Extracts of citrus peels have also been studied both *in vitro* and *in vivo* and were compared with fenbendazole. In the *in vitro* study a concentration on 50 mg/ml of extracts of citrus peels was used against 0.5 mg/ml of fenbendazole. A statistically significant difference between the lethal effect of the two was found until 7h after exposure were extracts of citrus peels had the same effect as fenbendazole (Abdelqader et al., 2012). In the *in vivo* study, the efficiency in elimination of *A. galli* worms of the highest concentration of 1200 mg/kg⁻¹ was on 68.4 % which was lower than the efficiency for fenbendazole on 97.1 % (Abdelqader et al., 2012).

Extracts of coriander seeds has to my knowledge not been studied against *A. galli* but on other helminths. A study by Helal et al. (2020) has investigated the effects of coriander essential oil *in vitro* on infective larvae of *Haemonchus contortus*, *Trichostrongylus axei*, *Teladorsagia circumcincta*, *Trichostrongylus colubriformis*, *Trichostrongylus vitrinus* and *Cooperia oncophora*. A 2 % concentration of coriander essential oil showed a strong inhibitory effect against all species (inhibited mobility of 93 %) except for *C. oncophora*. Another study tested essential coriander seed oil *in vitro* on both eggs and larvae of *Haemonchus contortus*. It showed a 99 % inhibition on the eggs at a concentration of 2.5 mg/ml and a 97.8 % inhibition on the larvae development at a concentration of 10 mg/ml (Macedo et al., 2013). Coriander essential oil has to my knowledge not been tested *in vivo*.

Coriander leaf aqueous extract has been shown to have immunomodulatory effects in rats where it stimulated spleen lymphocyte proliferation significantly at concentrations ranging from 7.8 to 125 µg/ml (Gomez-Flores et al., 2010). Also, coriander leaf methanol extracts have shown to stimulate thymic lymphocyte proliferation significantly (Gomez-Flores et al., 2010).

The effect of a mixture of pumpkin seed oil and essential oil from coriander seeds on *A. galli* were studied in the current study.

4. Materials and methods

4.1. Animals

The experiment was completed with 72 layers of the breed Dekalb White which was 80 weeks old when arriving at the facilities. These were purchased from the egg producer Niels and Grethe, Hingevej 9, Hadsten. On arrival at bio-secure animal facilities at the Department of Animal Science the layers were randomly assigned to pens. Before they were put in a pen, they got tagged with a foot ring providing an individual number and were weighed. During the experiment, three layers were taken out of the experiment due to poor health conditions not related to the experimental diet.

4.2. Study design

The experiment consisted of 3 treatments and 4 repetitions pr. treatment (Table 1). The layers were divided into 12 pens in two different houses. In each pen, 6 layers were placed randomly. The pens were equipped with a ladder, two nest boxes, and wood shavings as litter material. The temperature in the facilities was 21 °C, and the light-dark cycle was set after daylight hours. The experiment ran for 33 days in total with 4 days to acclimatize before start of treatment.

Table 1: Study design overview.

Treatment #	Group	Feed	Number of pens	Number of layers per pen
1	1	Control	4	6
2	2	Coriander/pumpkin	4	6
3	3	Propionic acid	4	6
Total				72

Care of experimental animals and experimental procedures were carried out in accordance with the Ministry of Environment and Food of Denmark, the Danish Veterinary and Food Administration under Act 253 of 08/03/2013 and Act 12 of 07/01/2016. A license to conduct the animal experiment was obtained by the Danish Animal Experiments Inspectorate (license no. 2023-15-0201-01491).

4.3. Feed

The layers were fed with grains bought from Jan Volmar, Skærlund Skolevej 20, 7330 Brande. Grain for group 3 was preserved with 0.5 % propionic acid, and grain for group 1 or 2 was not preserved with propionic acid. In feed for group 2, essential coriander seed oil (Fischer Pure Nature APS, cat no. Koriander olie - 100ml) and cold pressed pumpkin (*Curcubita pepo*) seed oil (Bio Planete, Helsam, cat. No. 51820) was included. The composition of the experimental diets is shown in Table 2, and these were mixed at the feed factory. The supplementary feed was pressed into feed pellets by cold pressing (60 °C) and then mixed with chicken shells and calcium carbonate. The feedstuffs used were organic. During the first four days, all animals were allocated control feed. Water and feed were provided *ad libitum*.

Table 2: Feed composition in g/100 g of the three diets control, propionic acid, and coriander/pumpkin. The coriander/pumpkin diet contains less vegetable oil and fat, soy to compensate for the essential coriander seed oil and pumpkin seed oil.

Raw materials, g/100 g	Control Group 1	Propionic acid Group 2	Coriander/pumpkin Group 3
Untreated grain	44	0	44
Propionic acid (0.5%) treated grain	0	44	0
Hulled oats	11	11	11
Chicken shells, coarse	4.05	4.05	4.05
Calcium carbonate, feed chalk	4.5	4.5	4.5
Soy cake feed	23.87	23.87	23.87
Rapeseed cake feed	6.96	6.96	6.96
Rapeseed, low glucosinolate	2.92	2.92	2.92
Grass protein concentrate	1.29	1.29	1.29
Forblanding DA MIX Poultry (min-vit)	0.40	0.40	0.40
Vegetable oil and fat, soy	0.38	0.38	0.32
Sodium chloride	0.23	0.23	0.23

Monocalcium phosphate	0.18	0.18	0.18
Grass green flour, alfalfa	0.17	0.17	0.17
Sodium bicarbonate	0.09	0.09	0.09
Betafin S1	0.08	0.08	0.08
Pumpkin seed oil	0	0	0.026
Essential coriander seed oil	0	0	0.026
Total	100	100	100

4.4. Samples

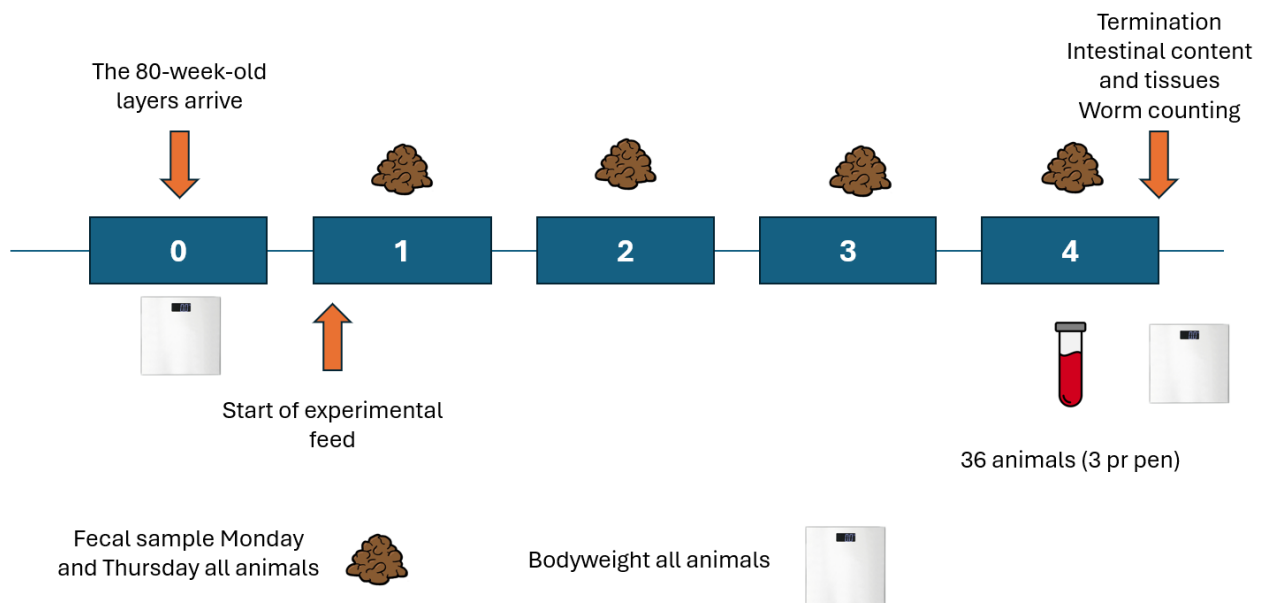


Figure 6: Overview of experimental setup. See the following text for more precise information about samplings.

4.4.1. Performance samples

4.4.1.1. Body weight

All layers were weighed at arrival (-4 days post feed treatment (pf.)) and on the day of slaughtering (day 28 and 29 pf.). The daily body weight gain (BWG) was calculated as a mean for each bird over the entire trial period. The termination of the experiment was carried out over two days, where the

layers that had blood sample taken were killed on the first day and the rest were killed on day two. Parameter including data from both slaughter days were counted as the same day for Figures and statistical calculations.

$$\text{Daily BWG} \left(\frac{g}{\text{day}} \right) = \frac{BW_{\text{Arrival}}(g) - BW_{\text{Slaughter}}(g)}{\text{Trial duration (days)}}$$

4.4.1.2. Feed consumption

Feed consumption was registered for each pen for the total period of 28 days. The daily feed intake (FI) per hen was calculated as a mean for each pen:

$$\text{Daily feed intake pr hen} = \frac{(\text{Feed given (g)} - \text{left overs (g)})}{\text{number of hens in the pen} \cdot \text{days}}$$

Also, the feed conversion ratio (FCR) was calculated as a mean for each pen:

$$\text{FCR} = \frac{\text{total egg weight (g)}}{(\text{Feed given (g)} - \text{left overs (g)})}$$

4.4.1.3. Egg production

Eggs from the hens were collected daily/every other day in egg trays belonging to each pen from the day they started the treatments. The total number of eggs were subsequently counted and weighed once a week. The egg production (%) was calculated as the total number of eggs from one week divided by 7 days divided by the number of hens in the pen times 100:

$$\text{Egg production (\%)} = \frac{\text{Total number of eggs}}{\frac{\text{Total number of hens in the pen}}{7}} * 100$$

The egg weight was also calculated per pen per week as the total weight of eggs divided by the number of eggs:

$$\text{Egg weight (g)} = \frac{\text{Total egg weight}}{\text{Number of eggs}}$$

4.4.2. Immunocompetence samples

Blood samples were taken on 36 animals, 3 from each pen i.e. from 12 animals per treatment in total. The animals were chosen randomly, and the samples were taken at day 23 pf. Three milliliters were sampled from the jugular vein and was heparin stabilized in BD Vacutainer® collection tubes containing Sodium Heparin (BD bioscience Switzerland, cat. No. 367869). After securing part of the fresh blood sample for the flow cytometry analysis, the rest of the sample was centrifuged at 800 x g for 10 minutes at room temperature (RT). The plasma fraction was next collected and put into Eppendorf tubes and stored at -20 °C until further use.

From the same animals a piece of the intestine was taken at slaughter on experimental day 28 pf. This piece was cut proximal Meckel's Diverticulum, and a total of 6 cm intestine were cut. This was stored in PBS (Lonza 17-516F) and kept on ice for transport to the lab for assessment of local *A. galli* specific immunoglobulin titers.

4.4.3. *A. galli* infection measurements

Feces samples were taken 8x during the experiment. The samples were collected on days 0, 3, 7, 10, 14, 17, 21 and 24 pf. Samples were taken from each animal and pooled pr. pen. The following feces samples were also pooled pr. pen except for the last sampling. The pH in pooled feces was registered just after sampling.

At slaughter, digesta from caeca and ileum were collected for all 72 hens, and pH was measured for individual animals.

4.4.4. *A. galli* specific Ig from intestinal tissue

The intestinal tissues were used to assess intestinal B cell Ig production potential *in vitro*. Inspiration for the method was taken from Rees et al. (1989). First, the pieces of intestine were weighed and cut into smaller pieces. The tissue was put into tubes with 10 ml R10 (623 mL RMPI 1640, 70 mL 10 % FCS, 7 mL 1 % PenStrep). The tubes were kept at -20 °C until analysis. Prior to ELISA, the tubes were thawed and centrifuged at 2000 x g for 10 minutes. The supernatant was collected and analyzed by ELISA.

Components from three kits from Bethyl Laboratories (Chicken IgA ELISA quantitation set, E30-103; Chicken IgG ELISA quantitation set, E30-104; Chicken IgM ELISA quantitation set, E30-102), were used for the ELISA. First, a 96-well Maxisorp microtiter plate (Thermoscientific/Nunc, cat. No. 446612) was coated with antigen/antibody by adding coating buffer and incubated covered overnight at 4 °C. *A. galli* cruded extract (homemade) was used as coating antigen for wells for samples, and coating antibodies from the kits were used for the standard. The homemade *A. galli* cruded extract was made from worms that had been washed in 70% ethanol and stored in phosphate-buffered saline (OBS, pH 7.4) at -20 °C until further processing. The worms were shredded with scissors and mixed in PBS on a whirl mixer. Afterwards the extract was centrifuged at $1700 \times g$ for 10 min at 4 and after the supernatant was ready to use. Next day, the plate was washed three times with a washing buffer. Blocking (200 μ l) solution was added per well and the plate was covered and incubated for 38-45 minutes on a microtiter plate shaker at RT. Then the plate was washed 3 times with washing buffer. Afterwards, standard and samples were pipetted into the microtiter plate in duplicates and incubated for 2 hours on a microtiter plate shaker at RT. The samples were diluted 1:2 in dilution buffer. Sample with known Ig concentration were included in 8 dilutions as the standard curves. The first dilution was 500 ng/ml for IgA, 100 ng/ml for IgY and 250 ng/ml for IgM, followed by 2-fold dilutions, using dilution buffer. Then the plate was washed 3 times with washing buffer before adding 100 μ l diluted detection antibody from the kits per well and incubated for 1 hour on a microtiter plate shaker at RT. Then the plate was washed again 3 times with washing buffer, and 100 μ l of 3,3',5,5'-Tetramethylbenzidine liquid substrate (TMB) was added to each well, and the plate was covered with aluminum foil and incubated for 7½ minutes at RT. The reaction was stopped by adding 100 μ l 1 M H₂SO₄. The optical density was read at 450 nm with 650 nm as a reference on an ELISA-reader (Molecular Devices, California). The results were analyzed using the SoftMaxPro software (Molecular devices, California).

4.5.2. EPG and worm burden

The presence of eggs from *A. galli/Heterakis gallinarum* and *Capillaria spp.* in fecal samples was determined using the concentration McMaster technique with a sensitivity of 20 eggs per gram feces (EPG) as described by Permin et al. (1997) and Henriksen and Aagaard (1976). Briefly, feces were mixed with tap water and the mixture was stirred and rested for 30 minutes at RT. Afterwards, the mixture was filtered through two layers of gaze and 10 ml was collected in a test tube and centrifuged

at 300 x g for 7 minutes. The supernatant was removed and shortly before counting, flotation fluid (375 g glucose monohydrate and 250 g sodium chloride per liter distilled water) was added until a total volume of 4 ml. The sediment was resuspended and then transferred to both sides on a McMaster chamber in both sides and left for 3-5 minutes before the eggs were counted under a microscope. Both sides were counted, and the sides were called T1 and T2. The EPG was calculated as follows:

$$EPG \left(\frac{eggs}{g\ feces} \right) = (T1 + T2) \cdot 20$$

In addition, the total number of intestinal adult worms were counted in all animals when the experiment was terminated at day 28 and 29. The worms were kept in salt water until counted and sex determined later the same day.

4.5.3. Flow cytometric analysis

The blood samples were used for determining whole blood leucocyte counts by flow cytometry using a no-lyse no-wash protocol (Kjærup et al., 2014, Seliger et al., 2012) with adaptations. The blood was mixed well and diluted 100 x in RPMI (Roswell Park Memorial Institute 1640 Cell Culture Medium + GlutaMAXTM-1, GIBCO, cat. No. 61870-010). Antibody master mixes were prepared with monoclonal anti-CD antibodies in fluorescence-activated cell sorting (FACS) buffer (0.1 % BSA, 0.2 % Azide, 0.05 % horse serum in PBS). See Table 3 for the composition of the three antibody staining panels. In a microwell plate (Nunc A/S Denmark, cat. No. 262180), antibody master mix and diluted blood were mixed 1:1 by reverse pipetting. It was then incubated for 20 minutes in darkness at 4 °C. Before acquisition FACS buffer containing 1 % 123count e-beadsTM Counting beads (Thermofisher, cat. NO. 01-1234-42) and 4 mM EDTA were added. Forty microliters of the samples were then acquired on a BD FACSCelestaTM flow cytometer with a flow rate of 0.5 µl per second. The analyses of the acquired samples were performed in the FACS DIVA software, and the absolute number of cells was calculated according to 123count eBeadsTM manufactures instructions.

Table 3: Overview of the antibodies used for whole blood leucocyte counts. All the antibodies are Mouse Anti-Chicken.

Name	Target	Company	Cat. No.	Clone	Panel
CD41/61 FITC	Chicken integrin CD41/61 present on thrombocytes	BioRad	MCA2240GA	11C3	1 and 3

Kul-01 PE (Monocyte/Macrophage PE)	Mannose receptor C-type 1 present on monocytes/macrophage	Southern Biotech	8420-09	KUL01	1
CD45 PER-CP CY 55 ^a	Chicken CD45 present on leucocytes	BioRad	MCA2413GA	UM16-6	1 and 3
Bu-1 FITC	Chicken type I transmembrane glycosylated disulfide-linked homodimer on B cells	Southern Biotech	8395-02	AV20	2
TCR1 PE (TCR $\gamma\delta$ -PE)	Chicken T cell receptor present on TCR $\gamma\delta$ T cells	Southern Biotech	8230-09	TCR-1	2
CD8 α Cy5	CD8 as a heterodimer of CD8 α and CD8 β present on T cells	Southern Biotech	8405-15	3-298	2
CD4 pBlue (CD4-PACBLU)	MHC class II molecules present on T helper cells, monocytes, macrophages, and dendritic cells	Southern Biotech	8210-26	CT-4	2
CD8 α PE	CD8 as a heterodimer of CD8 α and CD8 β present on T cells	Southern Biotech	8405-09	3-298	3
CD3 A700	Complex for antigen-recognition signals into T cells	Southern Biotech	8200-27	CT-3	3
Bu1 pBlue ^a	Type I transmembrane glycosylated disulfide-linked homodimer present on B cells	Southern Biotech	8395-26	AV20	3

^a Fluorochrome conjugation by Lightning-Link[®] kits from Abcam

4.5.4. Plasma coloration

One hundred microliters of each plasma sample were added to the wells of a 96-well Nunclon Delta Surface plate (ThermoScientific, cat. No 167008). Optical density (OD) was measured at wavelengths 405, 450, 470, 490, 550, 590, and 650 nm on a spectrophotometer (Multiskan Sky, Fischer Scientific).

4.5.5. MCH haplotyping

Blood was used to determine the putative Major histocompatibility complex (MHC) haplotypes of the chickens. It was determined using the Lei025 microsatellite locus (McConnell et al., 1999) by PCR-based fragment analysis (Fulton et al., 2006) as described by (Dalgaard et al., 2005). Briefly, the PCR reaction was performed with the forward primer LEI258A (5'-CACGCAGCAGA AACTTGGTAAGG-2') and the reverse primer LEI258B (5'-AGCTGTGCTCAGTCCTCAGTGC-3'). The total reaction volume was 20 µl containing 0.5 µM of each primer, and 10 µl buffer Phusion® Blood Direct PCR Master mix (ThermoFisher, cat. No. F-175S). Blood was transferred to each tube containing PCR reaction mix by sticking a toothpick into the tube with blood and shortly sticking the toothpick with blood into the tube with PCR reaction mix. The reaction was performed at 98 °C for 5 min, 40 cycles of 98 °C for 1 minute, 62 °C for 5 seconds, and 72 °C for 15 seconds, and a final elongation at 72 °C for 1 minute.

All PCR products were visualized by 2 % Metaphor agarose gel electrophoresis and stained with GelRed™ Nucleic Acid Gel Stain, 10,000× in water from Biotium, Inc. (VWR-Bie & Berntsen A/S, Herlev, Denmark, cat. No. 41003). Four positive controls from animals with known MHC haplotypes and a GeneRuler 50 bp DNA ladder (Thermo Scientific, cat. No. SM0373) were loaded on the same gel.

4.5.6. Phagocytose assay

The phagocytic activity was expressed as the percentage of cells containing fluorescent beads (% Parent). Also, the mean fluorescence intensity (MFI) was recorded, and it reflected the mean number of ingested beads per phagocyte.

4.5.6.1. Whole blood assay

The heparin-stabilized blood was diluted 1:100 with RPMI and 50 µl of diluted blood was added per well in a round bottom 96-well plate. It was stained with CD45 percpcy55 (BioRad, cat. No. MCA2413GA) in RPMI as a master mix with 0.125 µl CD45 + 49.9 µl RPMI for each well before adding. Afterwards, the bacteria *S. Infantis* FITC (JEO1965 (S123443) diluted with RPMI were added. FITC labelled heat-killed *S. infantis*, as described by (Ulrich-Lynge et al., 2015). The mix in the wells were gently mixed by pipetting before incubation in 5 % CO₂ at 41 °C for 60 minutes. The

phagocytosis was stopped by adding paraformaldehyde (PFA) to a final concentration of 1 %. It was then mixed before incubation for 15 minutes at 4 °C. Flow cytometric analyses were conducted on a BD FACSCelesta™ flow cytometer and analyses of acquired samples were performed in the FACSDiva software.

4.5.6.2. Opsoniphagocytosis assay

The chicken macrophage-like cell line HD11 was used to determine the overall plasma opsonophagocytic potential (Opp) of the plasma from the chickens. For the growth of HD11 cells, 1.5×10^6 cells were cultured in T75 culture flasks with 15 mL of culture media (CM: RPMI medium with glutamine and 10% Fetal bovine serum (FBS), and antibiotics (100U/ml penicillin and 100ug/ml streptomycin (PEST)). The cells were passaged when 80-90 % confluence by removing CM and adding prewarmed Trypsin Versene (Lonza, cat. No. BE17-161F). They were incubated for 2 minutes at 41 °C followed by 2-4 min at RT, and then 5-10 ml CM without antibiotics was added. The cells were transferred to centrifuge tubes and spun down at 150 x g for 5 minutes. The supernatant was removed, and cells were resuspended in CM without antibiotics. Cells (90,000 cells/well) were seeded into 96-well flat bottom plates for bioassays, where the total volume was 100 µl/well. The cells were grown overnight at 41 °C and 5 % CO₂.

The assay for the Opp was performed as previously described by (Naghizadeh et al., 2018). Briefly, latex beads, carboxylate-modified polystyrene, fluorescent yellow-green (SIGMA, cat. No. L4655-1ML) were diluted 1:200 with RPMI. The plasma samples were vortexed before transferring 25 µl of plasma to each well in the opsonization plate (96-well plate with round bottom). Then 75 µl of diluted beads were transferred to each well with plasma so the total volume in each well was 100 µl. The beads and plasma were mixed by pipetting and incubated for 30 minutes at RT.

After incubation of the beads and plasma, the medium was removed from the HD11 cells, and 100 µl of cold RPMI was added to the cells. The cells were then placed on ice and 25 µl of the opsonized bead solutions were added to each well. This was mixed by pipetting and the plate was transferred to the incubator at 41 °C for 60 minutes. Afterward, the cells were washed 3 times with RT PBS. Then 150 µl RT PBS was added and the cells were harvested by adding EDTA to a final concentration of 2 mM in each well and incubating for 10 minutes at 41 °C. It was followed by vigorous pipetting up and down in circles for a minimum of 10 x. It was mixed 10 x again before transferring to a Microwell round bottom flow plate (NUNC, cat.no. 262180). PFA was added to a final concentration of 1 %,

followed by mixing and incubation for 15 minutes. Twenty-five microliters of sample were acquired as described in Section 4.5.6.1.

4.5.7. ELISA measurements of serum MBL

Measuring the concentration of Mannose-Binding Lectin (cMBL) in plasma was performed as described previously by Juul-Madsen et al. (2003) and Norup and Juul-Madsen (2007). First the 96 Maxisorp microtiter plate (Thermoscientific/Nunc, cat. No. 446612) was coated with antibody by adding coating buffer (5 µg MBL antibody (6B11) (Novus Biologicals, cat. No. NBP1-05085)/ml PBS). The plate was covered and incubated overnight at 4 °C. Next day the microtiter plate was brought to RT for 15 minutes and then washed 3 times using washing buffer (TBSTwash; 10mM Tris, 100mM NaCl, 0.05 (vol/vol) Tween 20, pH 7.6). Then 200 µl of blocking buffer (0.5 % (vol/vol) Tween 20 in TBS (10 mM Tris, 140 mM NaCl, pH 7.6) was added per well. The plate was covered and shaken for 30-45 minutes on a microtiter plate shaker at RT. Sample with known cMBL concentration were included in eight dilutions as the standard curve. The first dilution was 0.22 µg/ml in sample buffer (TBS with 10mM calciumchlorid₂, 0.05 % Tween 20) followed by 2-fold dilutions. Both high and low serum controls and the samples were diluted 1:610 in sample buffer. The plate was covered again and shaken for 2 hours on a microtiter plate shaker at RT. The plate was washed 3 times using washing buffer (10 mM Tris, 100 mM NaCl, 0.05% (vol/vol) Tween 20, pH 7.6) before adding 100 µl diluted biotinylated MBL antibody (6B11) (Novus Biologicals, cat. No. NBP1-05085) per well and the plate was covered again and shaken for 45 minutes on a microtiter plate shaker at RT. The plate was washed again 3 times using washing buffer before adding 100 µl diluted streptavidin (1:75.000) per well and the plate was covered and shaken for 30 minutes on a microtiter plate shaker at RT. The plate was then washed 3 times using washing buffer, 100 µl of TMB was added per well, and the plate was covered with aluminum foil and incubated at RT. The coloration was stopped by adding 100 µl 1 M H₂SO₄ when the color development was evaluated to be sufficient. The optical density was read at 450 nm with 650 nm as a reference on an ELISA-reader (Molecular Devices, California). The results were analyzed using the SoftMaxPro software (Molecular devices, California).

4.6. Statistical analyses

All data were analyzed for being normally distributed, and if not, the data were log-transformed and analyzed for normal distribution again. If the data were found to be normally distributed with homogeneous variance, ANOVA analysis was performed and statistically significant differences between treatments were compared by pairwise T-tests, and significance was declared at $p \leq 0.05$. If data were not normally distributed a Kruskal-Wallis one-way analysis of variance was used. Spearman correlation coefficient was used to determine the relationships between Ig/worm and female worm/EPG. Correlations greater than 0.6 were considered strong correlations, and correlations between 0.4 and 0.6 were considered moderate correlations. The statistical analyses were performed with RStudio (version 4.2.2, The R Foundation for Statistical Computing Platform) and the results were visualized using the ggplot 2 package (Wickham, 2009).

5. Results

5.1. Performance

Body weight, FI, FCR and egg production were measured during the experiment (Table 4).

Table 4: Performance data presented as mean \pm SEM. The daily BWL (g/day) and the daily FI were calculated as an average over the entire trial period from week 0-4.

Group	Control		Coriander/pumpkin		Propionic acid		p-value
	Mean	SEM	Mean	SEM	Mean	SEM	Treatment
Daily BWL (g/day)	5.28	1.23	3.81	0.84	6.44	1.83	0.91
Daily FI (g/day)	119	1.88	131	2.31	111	2.77	0.08
FI/egg (g/egg)	215	4.02	263	21.8	197	6.84	0.38
FCR (g egg weight/g feed)	0.60	0.01	0.72	0.05	0.54	0.02	0.32

The daily BWL, FI and FCR were calculated as an average over the entire trial period from week 0 to week 4. The mean daily BWL ranged from 3.81 for the layers fed with the coriander/pumpkin diet to 6.44 g/day for the layers fed with the propionic acid diet. No statistically significant differences were found between the treatments.

The mean daily feed intake (FI) ranged from 111 g/day for the layers fed with the propionic acid diet to 131 g/day for the layers fed with the coriander/pumpkin diet. Again, no significant difference between the treatments, but there was a tendency to a difference between the propionic acid diet and the coriander/pumpkin diet with a p-value on 0.072.

FI per egg produced was calculated as the total feed intake of the pen divided by the total number of eggs produced divided by the number of layers in the pen. The mean for the treatments ranged from 197 g/egg for the layers fed with the propionic acid diet to 263 g/egg for the layers fed with the control diet. Here no significant difference was observed.

The mean feed conversion ratio (FCR) ranged from 0.54 g egg/g feed for the layers fed with the propionic acid diet to 0.72 g egg/g feed for the layers fed with coriander/pumpkin diet. No significant difference between treatments was observed.

In addition, eggs from the hens were collected daily, and counted, and weighed every week. The egg production was calculated as means per pen per week (Figure 7).

The egg production in percentage decreased for all treatment during the first week. Afterwards it seemed to increase until week 2. From week 2 to 3 the egg production decreased again for layers fed with the propionic acid diet, increased further for layers fed with the coriander/pumpkin diet and remained stable for layers fed with the control diet. The egg production decreased for all treatments from week 3 to 4. There was no significant difference within any of the weeks for the three treatments.

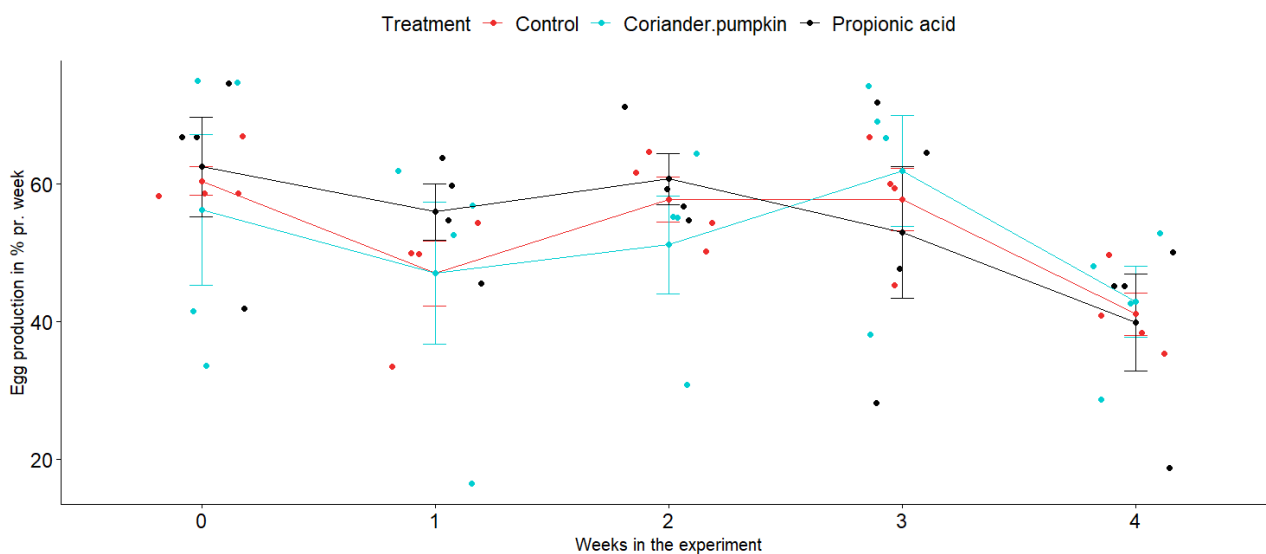


Figure 7: Egg production (%) for each treatment week 0-4. At week 0 the layers were 80 weeks old. The egg production (%) was calculated as a mean of each pen every week. Results are shown as means \pm SEM with the data for individual pens shown.

The egg weight was calculated as a mean per egg from the total collection of eggs every week from week 0-4 (Figure 8).

During the first week on the experimental diets the egg weight decreased a little for layers fed with the propionic acid diet, it remained stable for the layers fed with the control diet and it increased for layers fed with the coriander/pumpkin diet. Afterwards it increased a little for layers fed with the control diet and the propionic acid diet and stayed stable for layers fed with the coriander/pumpkin diet in week 2 (Figure 8A). In week 3 the egg weight increased a little for all three treatments. In the last week there was a decrease in egg weight for both layers fed with the control diet and the propionic acid diet and a further increase for layers fed with the coriander/pumpkin diet. At the beginning of the experiment layers fed with the propionic acid diet had a significantly higher egg weight than layers fed with the coriander/pumpkin diet ($p = 0.0406$) (Figure 8B). There was no significant difference between the three treatments overall.

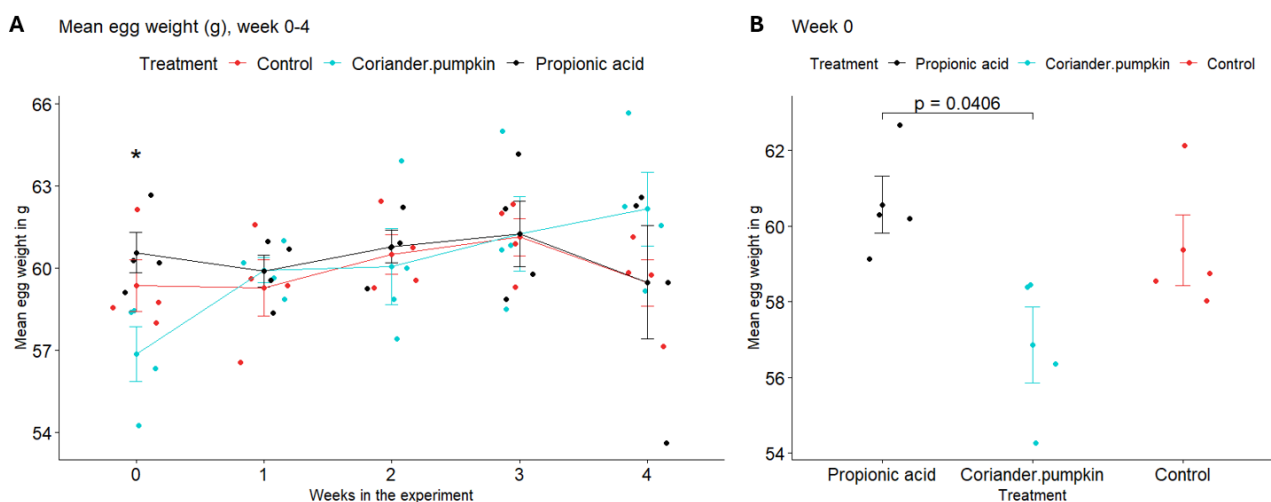


Figure 8: Egg weight during the experimental period. A) Mean egg weight (g) for each treatment over the 5 weeks. At week 0 the hens were 80 weeks old. Significant differences between groups are marked with *, $p < 0.05$. The egg weight (g) was calculated as a mean for the total collection of eggs from each pen every week. B) Mean egg weight (g) for each treatment at week 0 were there was a significant difference between propionic acid and coriander/pumpkin. The p -value is shown on the Figure. Results are shown as means \pm SEM with the data for individual pens shown.

5.2. Gut health

5.2.1 Fecal pH

Feces samples were collected 8 times during the experiment and pH was measured (Figure 9).

A significant difference in fecal pH between the three treatments was observed at day 21 where layers fed with the coriander/pumpkin diet had a higher pH compared to layers fed with the propionic acid diet (Figure 9A). The mean pH ranged from approximately 6.5 to 7.5 in the pooled fecal samples. The mean pH increased in the first 7 days except in feces from layers fed with the control diet where there was a decrease at day 7. Afterwards the pH was stable until day 17 where it decreased to day 21 most pronounced for the layers fed the propionic acid diet. The pH increased again till day 24 (Figure 9A). There was no significant difference at day 24, when the samples were measured individually (Figure 9B), but a large variation was observed within the groups.

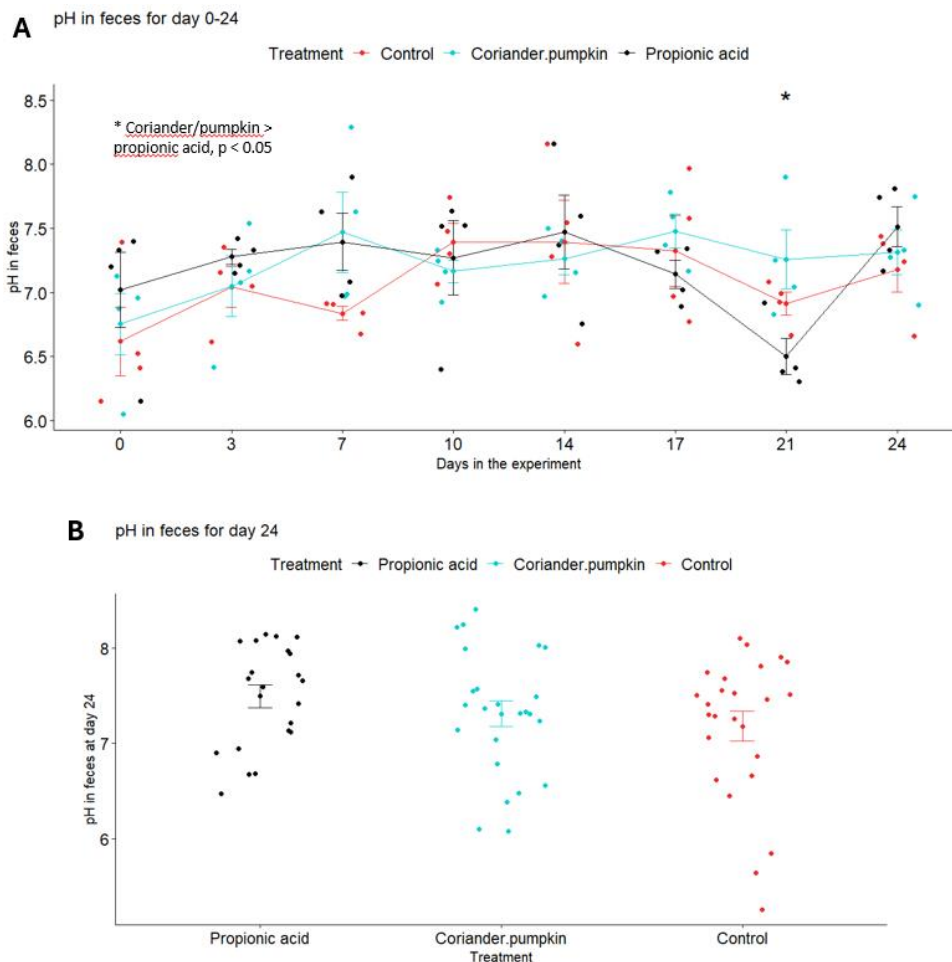


Figure 9: pH in fecal samples collected during the experiment. A) pH in feces at day 0, 3, 7, 10, 14, 17, 21 and 24 for each treatment. The feces samples were pooled per pen except for day 24 where a mean per pen was calculated. Results are shown as means \pm SEM with the data for individual pens shown. * = significant differences ($p < 0.05$). B) pH in feces at day 24 for individual animals for each treatment. Results are shown as means \pm SEM.

5.2.2. Ileal and cecal pH

Digesta from ileum and ceca was collected at slaughter day 28 and 29 for all animals and pH was measured (Figure 10).

The pH of the ileal and the cecal content was similar and the mean pH ranged from approximately 6.5-6.8 for both. There was no significant difference between the treatments for neither ileal nor cecal content, and again a large variation was observed within the groups.

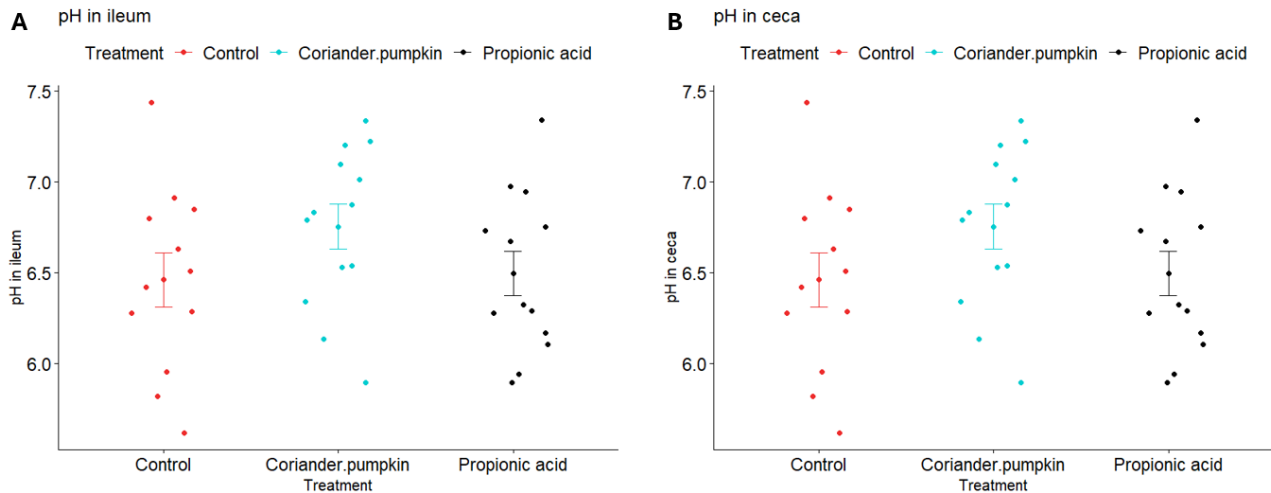


Figure 10: pH of digesta measured at day 28 or 29. A) pH of the digesta in ileum for each treatment. B) pH of the digesta in ceca for each treatment. Results are shown as means \pm SEM.

5.2.3. Intestinal integrity/plasma coloration

Plasma was collected one time at week 4 and plasma coloration was measured and used as a measure of intestinal integrity (Figure 11).

There was found significant difference between the control diet and the propionic acid diet on the OD for wavelength 405-490 ($p < 0.05$). For the wavelength 550 and 590 there was a tendency to a difference between the propionic acid diet and the control diet ($p = 0.055$ and $p = 0.075$, respectively).

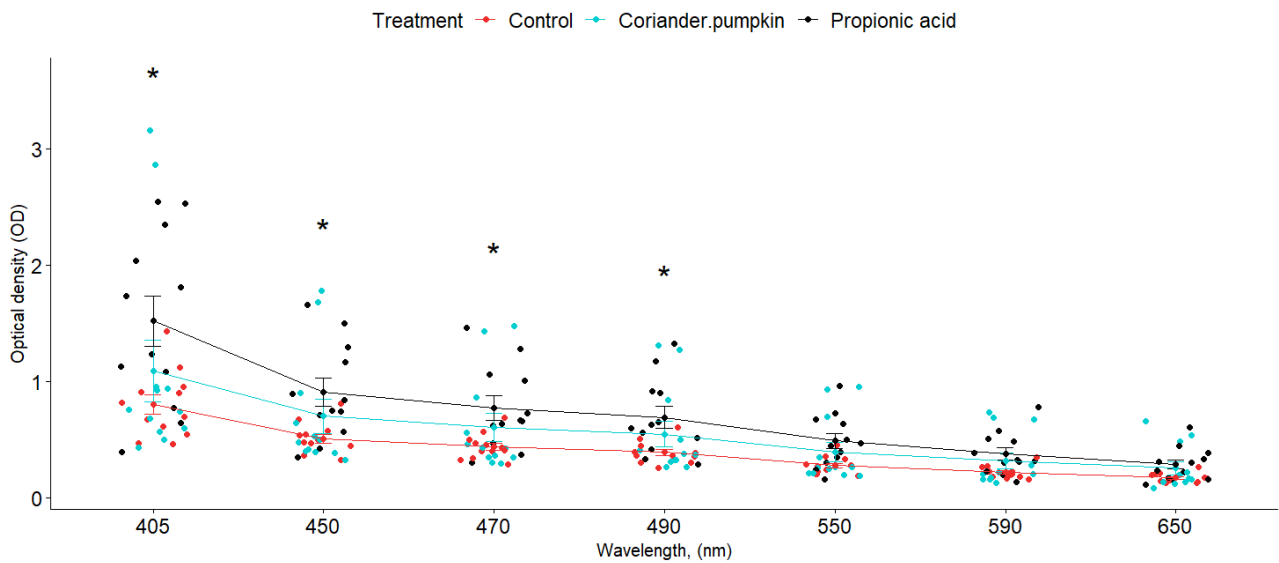


Figure 11: Plasma coloration shown as wavelength (nm) versus optical density (OD). Results are shown as means \pm SEM. Significant differences between groups are marked with *, $p < 0.05$.

5.3. *A. galli* burden

5.3.1. EPG for *A. galli* and *Capillaria spp.*

During the experiment the eggs in feces (EPG) of *A. galli/Heterakis gallinarum* and *Capillaria spp.* was counted 8 times (Figure 12). It is difficult to distinguish between eggs from *A. galli* and *Heterakis gallinarum*. However, it will from hereon be referred to as *A. galli* EPG.

A large variation was observed in EPG within each group most of the days both for *A. galli* and *Capillaria spp.* There is no clear tendency for either *A. galli* and *Capillaria spp.* since the EPG count showed large variation and fluctuations (Figure 12A and B). There was no significant difference between the treatments for any of the days for EPG from neither *A. galli* nor *Capillaria spp.* However, there was a tendency to a difference between propionic acid and coriander/pumpkin at day 3 for EPG of *Capillaria spp.* ($p = 0.085$) (Figure 12B). There was no significant difference at day 24 for EPG of both *A. galli* and *Capillaria spp.*, when EPG were counted for individual animals (Figure 12C and D).

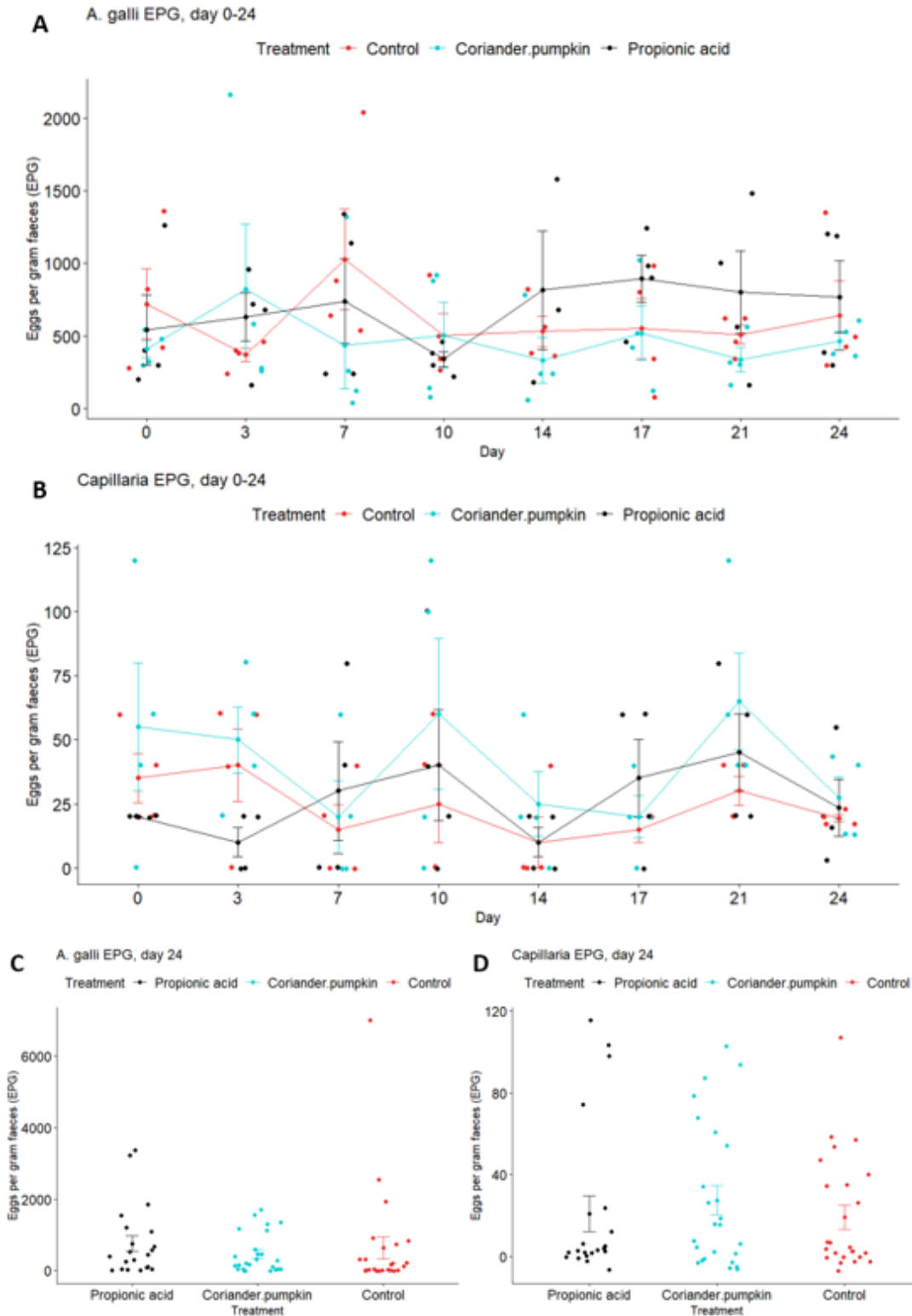


Figure 12: Worm eggs counted in fecal samples during the experiment. A) EPG of *A. galli* at day 0, 3, 7, 10, 14, 17, 21 and 24 for the different treatments. The feces samples were pooled per pen except for day 24 where a mean per pen was calculated. Results are shown as means \pm SEM with the data for individual pens shown. B) EPG of *Capillaria* spp. at day 0, 3, 7, 10, 14, 17, 21 and 24 for the different treatments. The feces samples were pooled per pen except for day 24 where a mean per pen was calculated. Results are shown as means \pm SEM with the data for individual pens shown. C) EPG of *A. galli* at day 24 for individual animals for the different treatments. Results are shown as means \pm SEM. D) EPG of *Capillaria* spp. at day 24 for individual animals for the different treatments. Results are shown as means \pm SEM.

The correlation was carried out between EPG count from *A. galli* at day 24 and female worm count at slaughter was calculated (Figure 13). The correlation between EPG from *A. galli* and female worm count is considered as a moderate positive correlation ($R = 0.4$), and it is significant ($p < 0.05$).

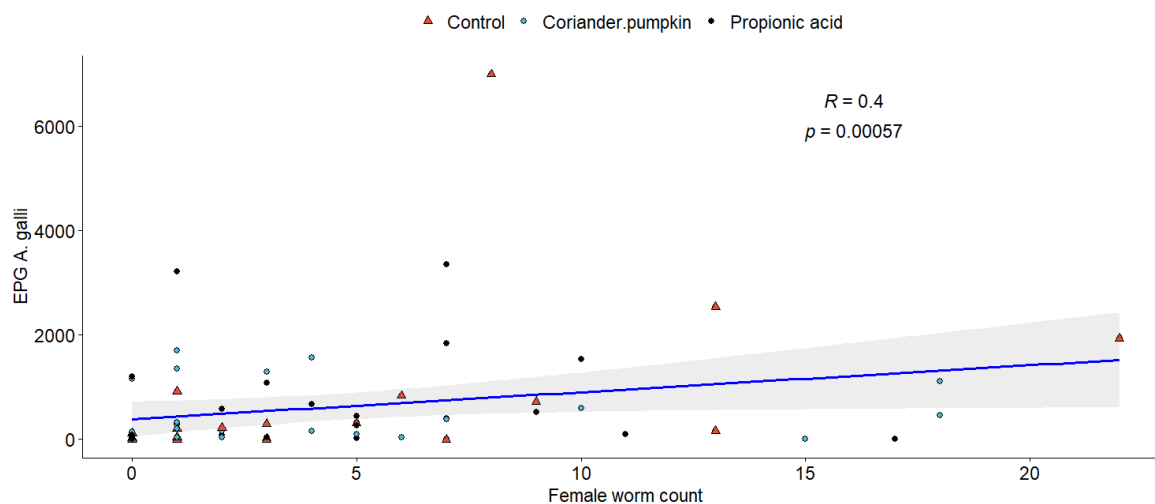


Figure 13: *A. galli* EPG in feces for day 24 and the correlation to female worm count. It is shown with spearman correlation coefficient (r) and the p -value.

5.3.2. *A. galli* specific IgY, IgA and IgM production potential

A. galli specific IgY, IgA and IgM production potential of intestinal B cells were determined from intestinal cultures from 36 hens at week 4 (Figure 14). The amount of IgY and IgA are based on a standard curve but for IgM the values were below the standard curve, so the absorbance was used for statistics.

There was no significant difference between the treatments on the amount of IgY and IgA (Figure 14A and B). A significant difference was found for IgM between the control treatment and the coriander/pumpkin treatment ($p = 0.00873$) with control having the highest absorbance, and between the control treatment and the propionic acid treatment ($p = 0.0103$) with control having the highest absorbance (Figure 14C).

No significant correlation was found between IgY and worm count and IgM and worm count (Figure 14D and F). But there was a significant correlation between IgA and worm count ($p = 0.0041$) and it was a moderate positive correlation ($R = 0.47$) (Figure 14E).

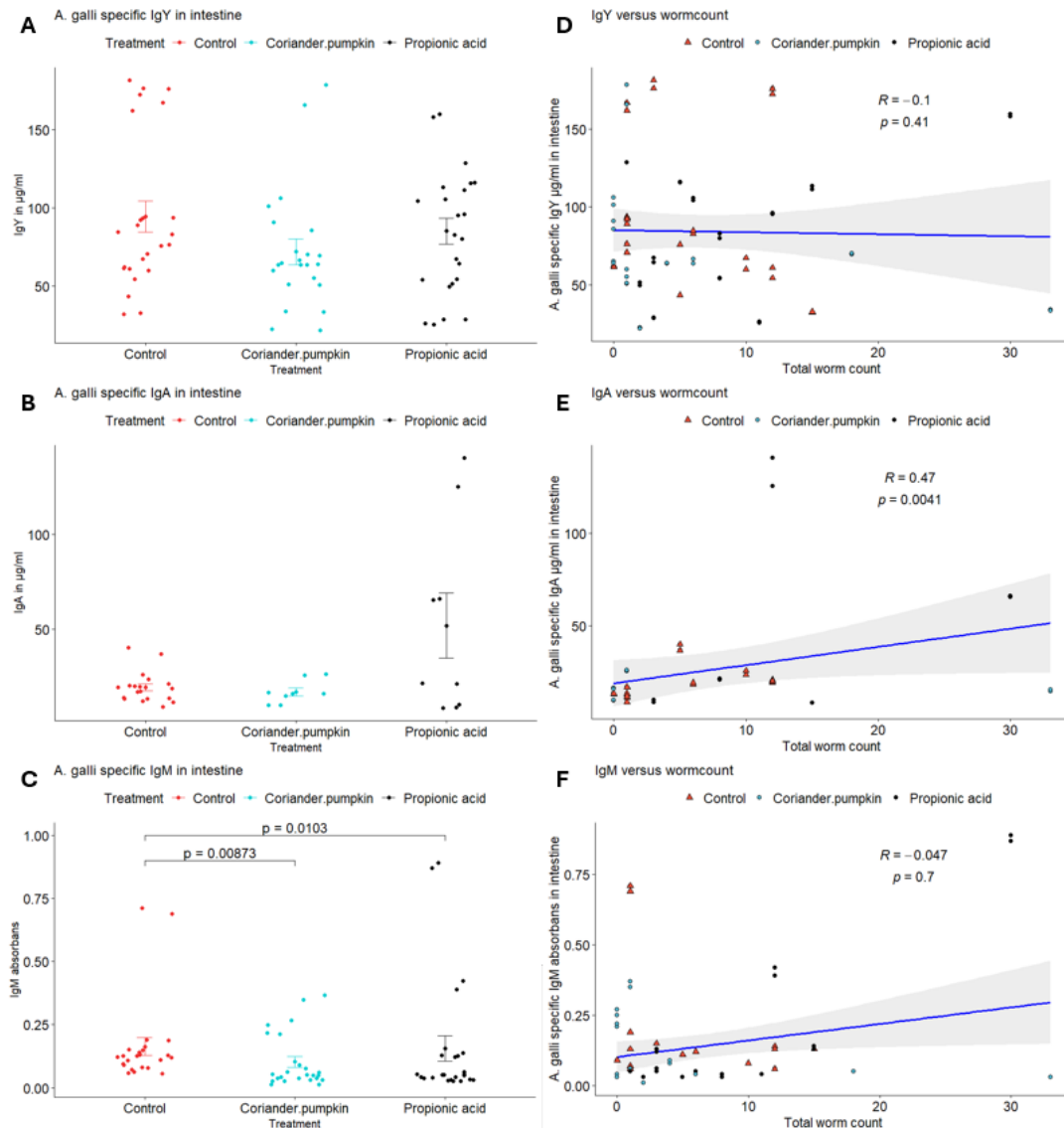


Figure 14: Potential of intestinal intraepithelial lymphocyte to produce IgY, IgA and IgM. A-C) *A. galli* specific IgY, IgA and IgM in serum. Results are shown as means \pm SEM with individual measurements. D-F) Correlation between Ig and worm count shown with the spearman correlation coefficient (r) and the p -value. The titers are shown for IgA and IgY while the absorbance is shown for IgM.

5.3.3. Worm burden and co-infections

The worm burden was counted at slaughter at the end of the experiment for the three treatments (n = 72 animals) (Figure 15).

For the mean of the worm burden there was no significant difference between the three treatments and a large variation was observed within the treatments (Figure 15A). There was no significant difference for the number of male and female *A. galli* (Figure 15B and C). For the proportion of female *A. galli* of worm count in percent was seen a tendency ($p = 0.1$) to coriander/pumpkin treatments (81.3 %) being higher than the two others (control: 63.8 % and propionic acid: 65.4 %).

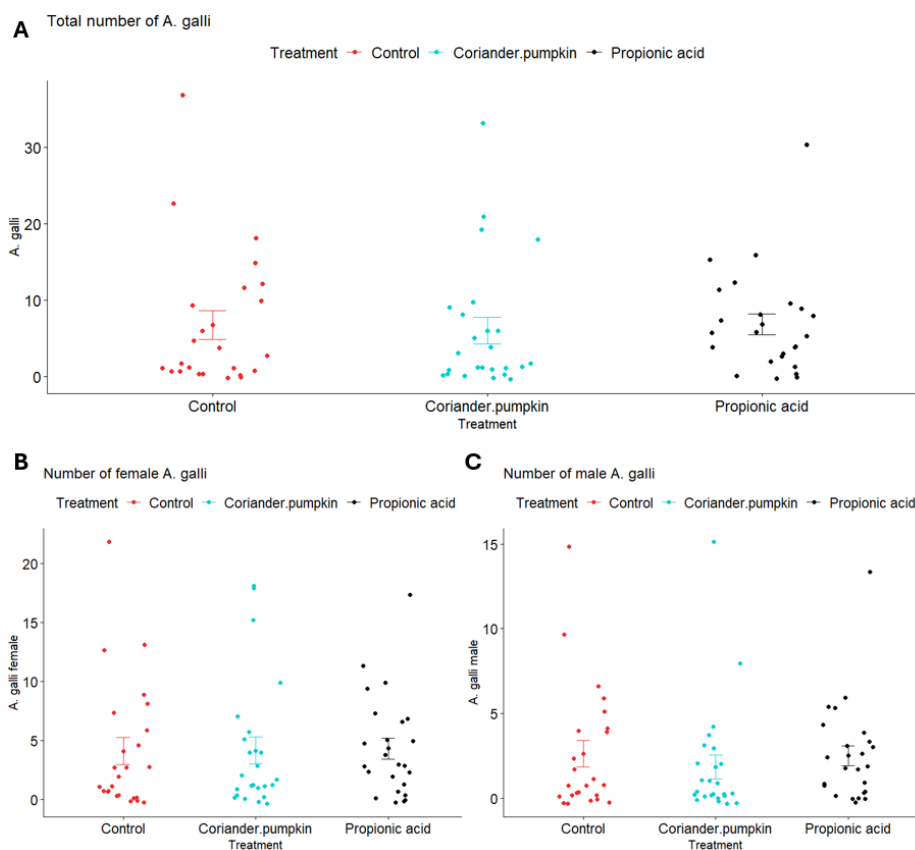


Figure 15: *A. galli* worm count at week 4. A) total worm count, B) female worm count, C) male worm count. All are shown as means \pm SEM with individual worm burdens.

At slaughter co-infection with other parasites was observed. Tapeworms were found in a few hens, but they were not counted. *Heterakis spp.* in caeca were also observed in a few hens, but these were not counted either. However, the worms were observed in an equal amount of birds from each treatment.

5.4. Immunocompetence

5.4.1. Leukocyte counts in peripheral blood

For quantifying blood cell subsets in peripheral blood an absolute count flow cytometric analysis was used (Figure 16 and Figure 17).

No significant differences between treatments for any type of cells counted were observed. But there was a tendency to a difference in B cells where the coriander/pumpkin treatment had a higher concentration than the control treatment ($p = 0.078$) (Figure 16D).

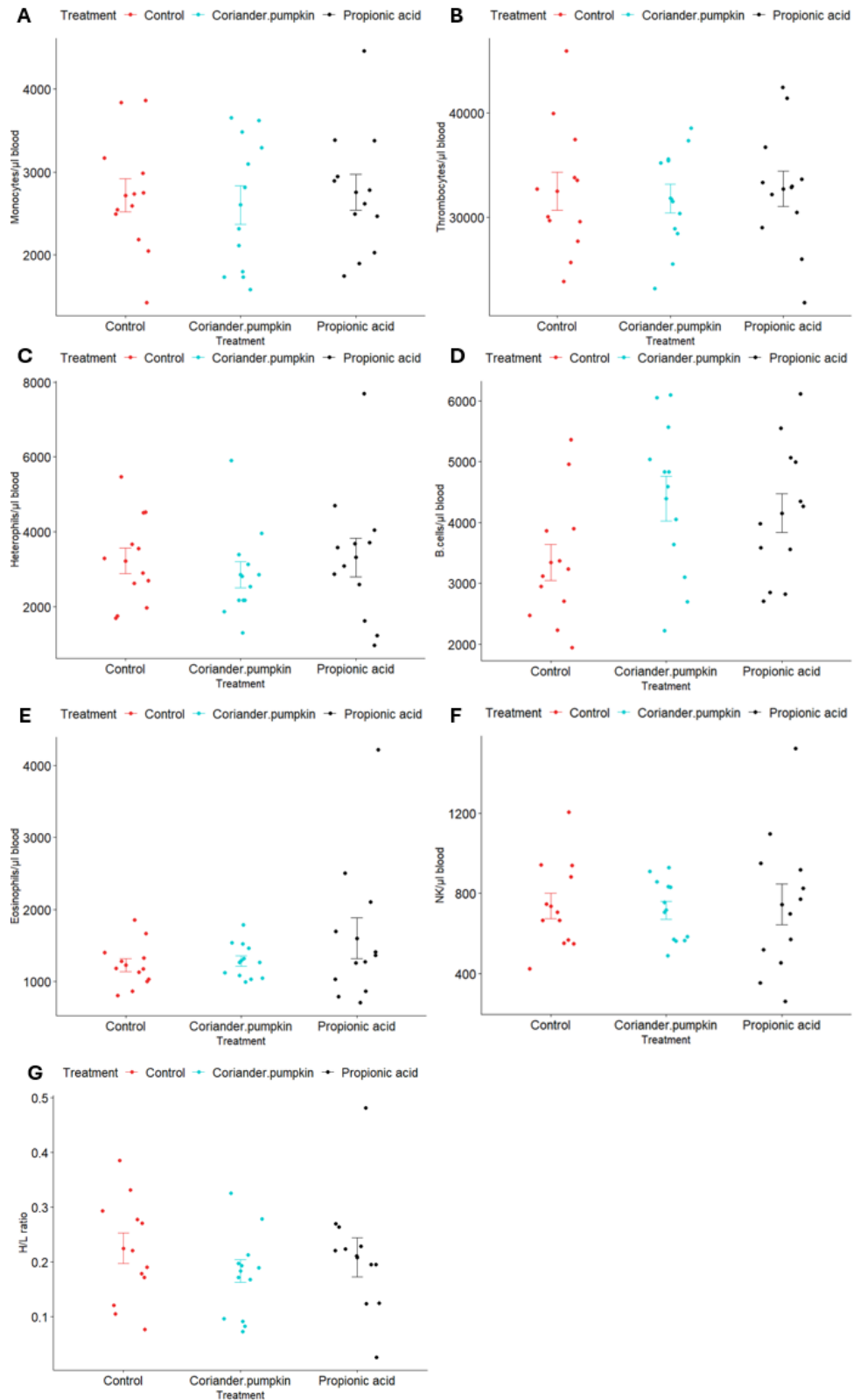


Figure 16: Blood cell subsets in peripheral blood in week 4. Flow cytometry gating of blood cell subsets were performed as shown in Appendix A. A) Absolute counts of monocytes. B) Absolute counts of thrombocytes. C) Heterophils. D) Absolute counts of B cells. E) Absolute counts of eosinophils. F) Absolute counts of natural killer cells. G) Heterophil-lymphocyte (H/L) ratio. Results are shown as means \pm SEM with individual counts indicated.

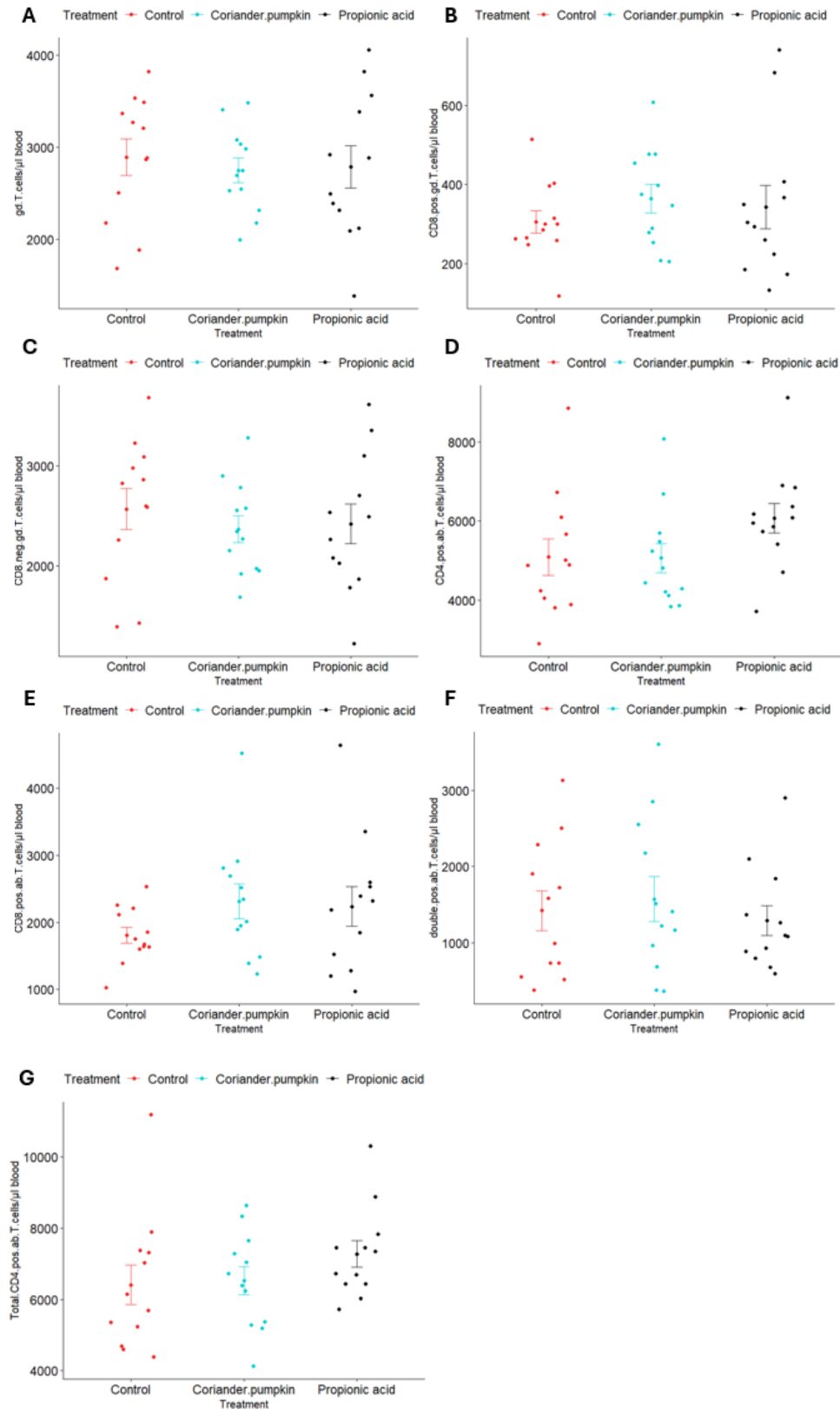


Figure 17: Blood T cell subsets in peripheral blood in week 4. Flow cytometry gating of blood cell subsets were performed as shown in Appendix A. A) Absolute counts of TCRγδT cells. B) Absolute counts of CD8+ TCRγδT cells. C) Absolute counts of CD8- TCRγδT cells. D) Absolute counts of CD4+ ab T cells. E) Absolute counts of CD8+ TCRab T cells. F) Absolute counts of double pos. ab T cells (CD8+CD4+). G) Absolute counts of CD4+ ab T cells. Results are shown as means ± SEM with individual counts indicated.

5.4.2. Phagocytosis assay

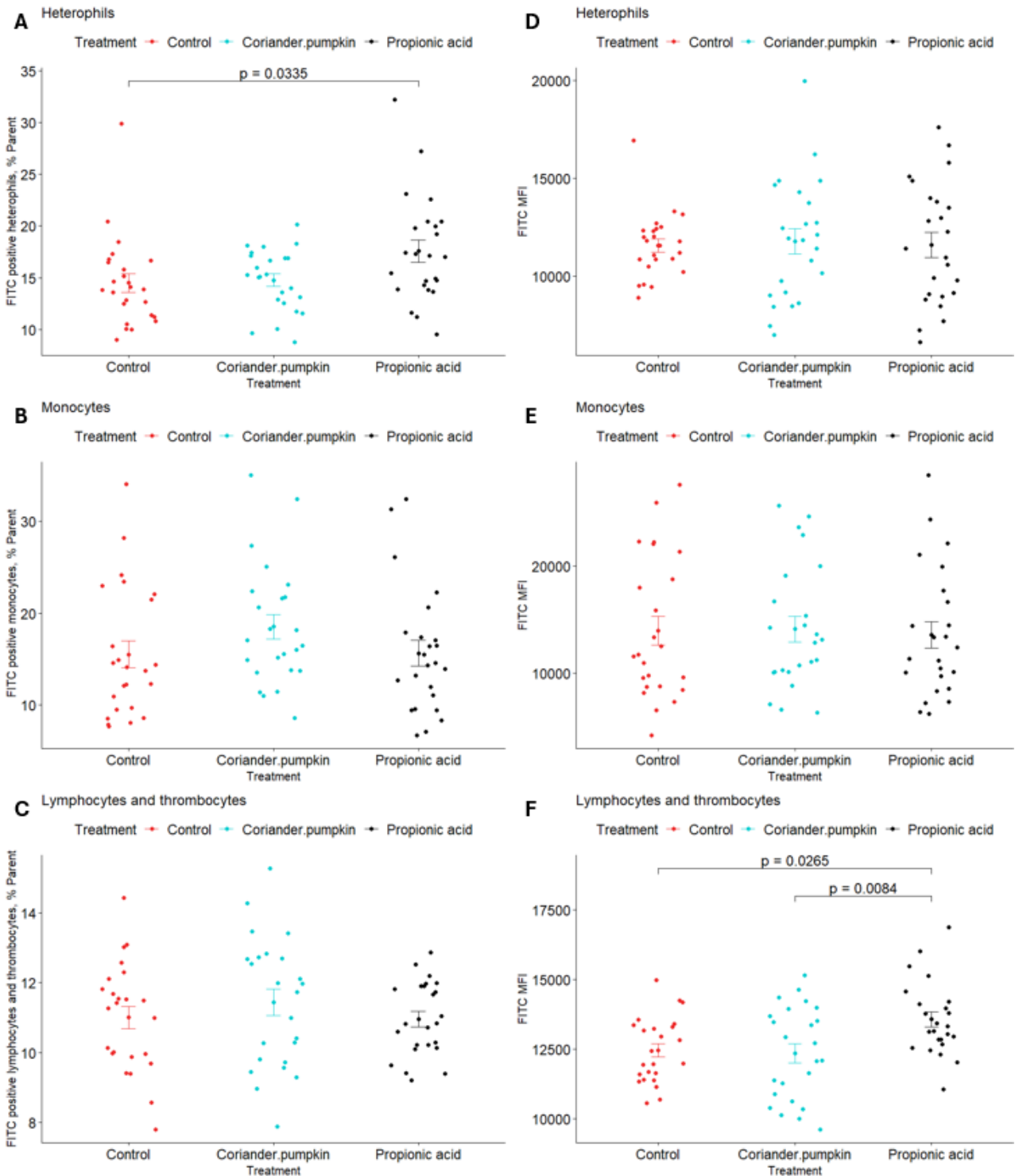


Figure 18: The phagocytosis ability of leucocytes in peripheral blood from week 4 for the three treatments. Flow cytometry gating of leucocyte subsets were performed as shown in Appendix B. A) Heterophils that have phagocytosed beads as % of the parent population (total heterophils). B) Monocyte that have phagocytosed beads as % of the parent population (total monocytes). C) Lymphocyte and thrombocyte that have phagocytosed beads as % of the parent population (total lymphocytes and thrombocytes). D) Heterophils that have phagocytosed beads as mean fluorescence intensity. E) Monocytes that have phagocytosed beads as mean fluorescence intensity. F) Lymphocytes and thrombocytes that have phagocytosed beads as mean fluorescence intensity. Results are shown as \pm SEM with individual measurements indicated.

The phagocytosis ability of leucocytes in peripheral blood plasma was measured using flow cytometry (Figure 18).

For heterophils the propionic acid treatment was significantly higher than the control treatment ($p = 0.0335$) for the results as cells that have phagocytes beads as % parent population but there was no significant difference for the results in the phagocytic activity, as measured by mean fluorescence intensity (MFI), reflecting the mean number of ingested beads per cell (Figure 18A and D). There was no significant difference between the phagocytose ability between treatments for monocytes for either % of parent population or MFI (Figure 18B and E). For the lymphocytes and thrombocytes was found no significant difference between treatment for % of parent population but for the MFI the propionic acid treatment was significantly higher than the control ($p = 0.0265$) and the coriander/pumpkin treatment ($p = 0.0084$) (Figure 18C and F).

5.4.3. Plasma opsonization potential

Determination of opsonization potential was performed by measuring HD11 cell's ability to phagocytose latex beads pre-opsonized with plasma from hens from the three treatments (Figure 19).

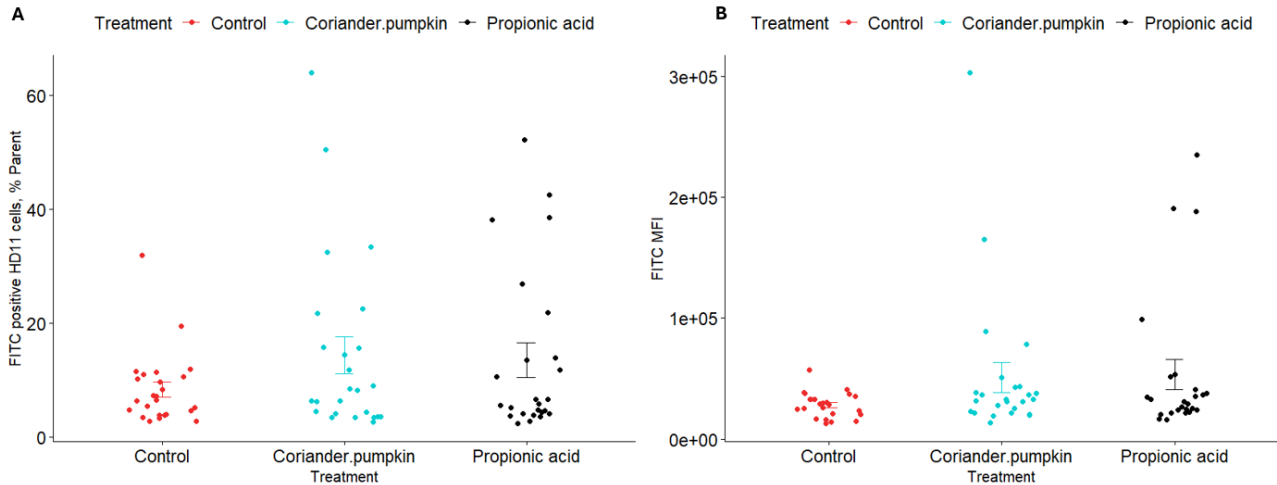


Figure 19: Opsonophagocytosis potential of plasma from week 4 for the tree treatments. Gating HD11 cells were performed as shown in Appendix C. A) HD11 that have phagocytosed beads as % of the parent population (total HD11 cells). B) HD11 that have phagocytosed beads as mean fluorescence intensity. Results are shown as mean \pm SEM with individual measurements indicated.

The opsonization potential of the HD11 cells did not differ significantly between the tree treatments. There was observed a large variation between plasma from different individuals, especially for the treatments coriander/pumpkin and propionic acid.

5.4.4. cMBL concentration

Quantification of cMBL concentrations in plasma was done with an ELISA analysis (Figure 20). No significant differences between cMBL concentration for the different treatments were found.

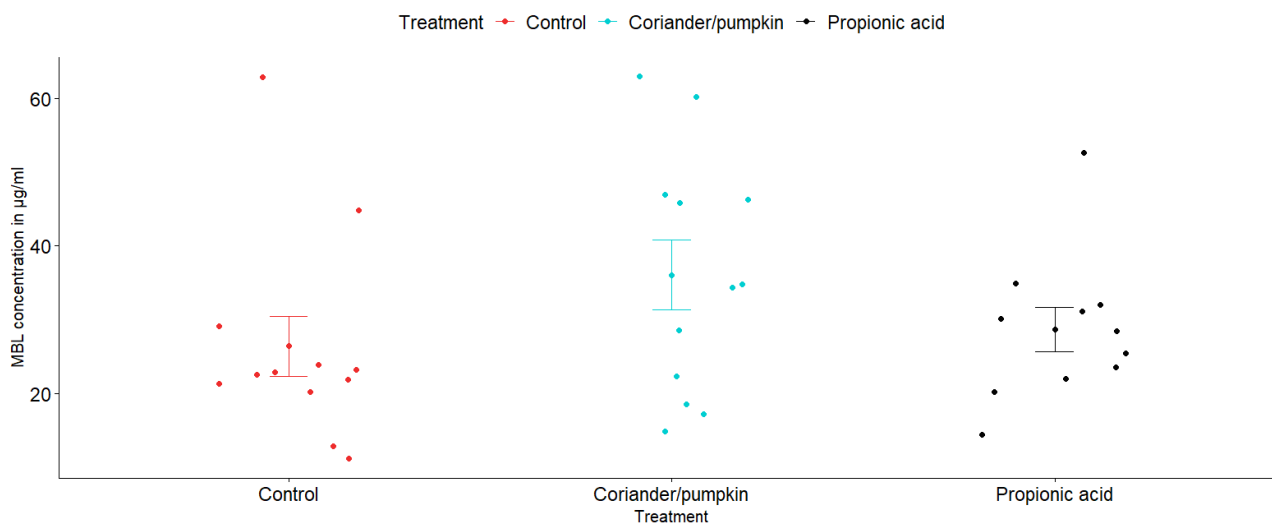


Figure 20: Concentration of Mannose-Binding Lectin (MBL) in plasma from week 4 for the three treatments. Results are shown as means \pm SEM with individual means of replicate measurements indicated.

5.4.5. MHC haplotypes

The Lei0258 microsatellite fragment length method was used to determine the MHC haplotype of the layers in the experiment (Table 5 and Figure 21). It was observed that there are only three different MHC types: one homozygous (357/357) and two heterozygous (261/357 and 443/357) in the commercial hens (Figure 21). The percentage of the different band sizes for each treatment is shown in Table 6.

Table 5: Overview of the controls used for MHC haplotype determination.

Lei0258 microsatellite size	B haplotype	Chicken Line	Source
261	B2	32	AU inbred line
357	B131	131	AU inbred line
443	B6	36	AU inbred line
552	B19.1	131	AU inbred line

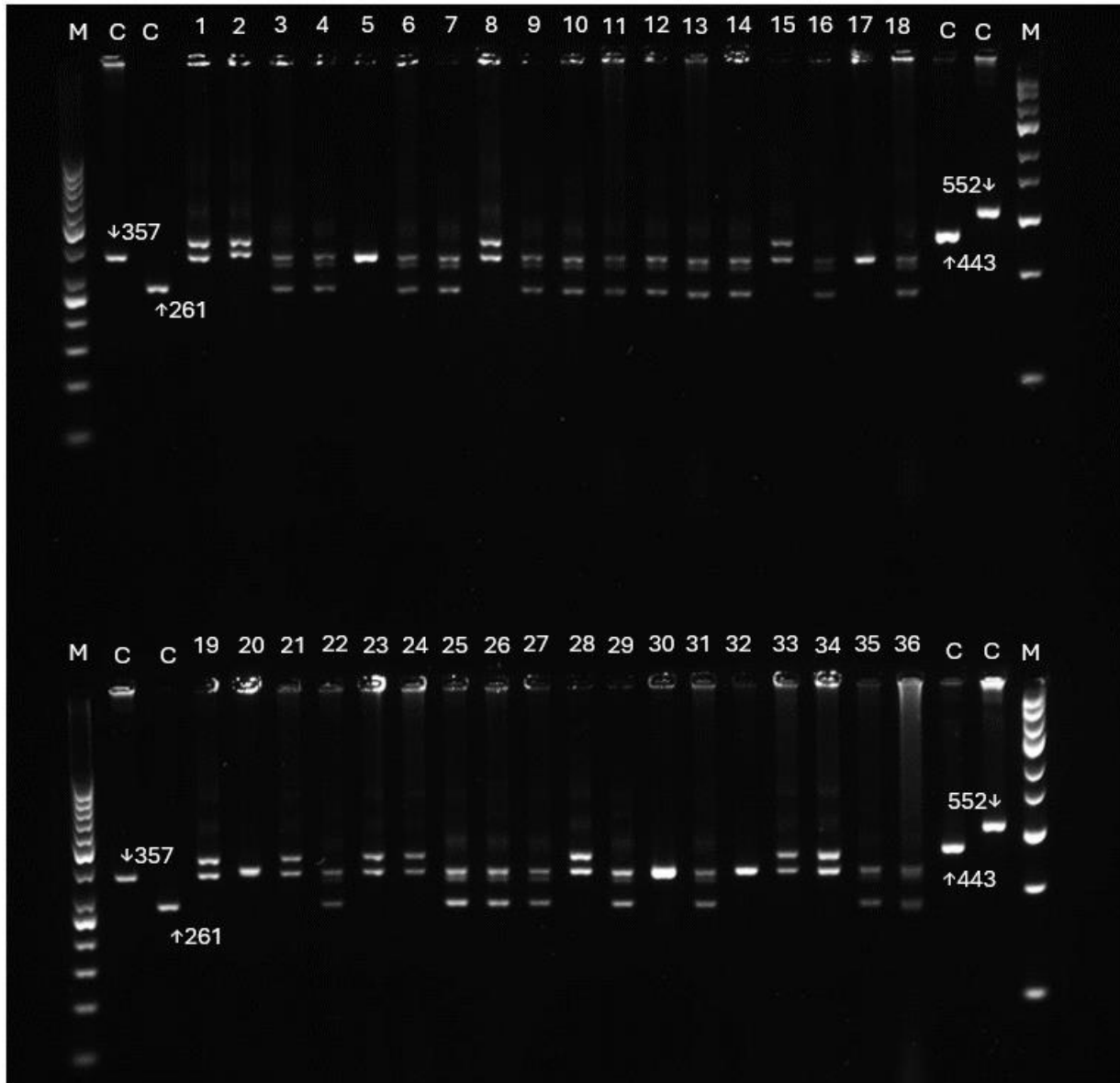


Figure 21: PCR amplification patterns from MHC haplotyping of 36 layers. M is the DNA size marker (50bp), and the numbers above the lanes are lab numbers. The predicted sizes of the bands of the four controls are shown next to the band from the controls (C).

Table 6: The different band sizes found in the hens in the experiment. It is shown as percentage for the different treatments.

Band size		Percent of layers		
		Control	Coriander/pumpkin	Propionic acid
Allele 1	Allele 2			
357	357	16.67	8.33	16.67
443	357	50.00	25.00	16.67
261	357	33.33	66.67	66.67

The combination of the three haplotypes was not entirely identical in the three treatment groups (Table 6). The homozygous (357/357) hens were less represented in the coriander/pumpkin treatment, but equal for the two other treatments. The heterozygous (443/357) hens were overrepresented in the control group with fewer hens with that haplotype in the coriander/pumpkin treatment group and even fewer in the for propionic acid treatment group. There were fewer hens heterozygous (261/357) in the control group compared to the two other treatments which had equal number of hens with this haplotype.

6. Discussion

6.1. Performance

To my knowledge, no other studies have used the same treatments as the current study (propionic acid treated grains and the combination of pumpkin seed oil and essential coriander seed oil). Hence, the results will be compared with studies using either pumpkin (*Cucurbita pepo*) seed oil, coriander seeds/powder or organic acids.

The daily BWL, the FI/egg and the FCR did not show a significant difference between the treatments (Table 4). However, higher values for the coriander/pumpkin on FI/egg and FCR and a lower value for BWL compared to the other treatments were observed. The difference between coriander/pumpkin and control on BW is in consistence with another study on pumpkin seed oil (Çelik et al., 2011). The observed BWG in the latter study instead of BWL is likely due to the age difference of the hens where the hens in the current study was 80 weeks old when starting the treatments and the other study used 30 weeks old hens. The study from Çelik et al. (2011) also showed no significant difference for the FCR for pumpkin compared to the control, but had lower values for both. The difference is caused by the hens laying more eggs than the hens in the current study and therefore the total egg weight is higher in the study by Çelik et al. (2011).

The mean daily FI showed a tendency to a difference between the treatments propionic acid and coriander/pumpkin ($p = 0.072$) (Table 4). This could be because the hens preferred one taste over the others since the FI of the birds receiving the propionic acid treated grain also had a lower numerical mean daily FI than the control diet. The greater mean daily FI for the hens with the coriander/pumpkin diet also influenced the higher values of FI/egg and FCR and lower BWL compared to the other treatments. The FI levels for the different treatments is in consistence with other studies with birds

fed pumpkin seed oil and organic acids (Çelik et al., 2011, Soltan, 2008). Another study on coriander seeds showed lower FI than in the current study (Habiyah et al., 2016). Hence, it could be the addition of pumpkin seed oil in the current study, that makes the layers eat more of the coriander/pumpkin diet.

The egg production in percentage did not show a significant difference between the treatments (Figure 7). For all treatments, a decrease in egg production during the first week was observed. This is probably due to the relocation of the chickens and the change of feed since the egg production increased for all treatments again afterwards. At the end of the experiment, a decrease in egg production again was seen for all treatments. This could be explained by the age of the hens since they were 80 weeks old at the beginning of the experiment. Other studies have also found no significant difference between treatments of pumpkin seed oil and coriander seed powder compared to control (Çelik et al., 2011, Habiyah et al., 2016). Other studies on the effect of organic acid mixtures including propionic acid have found an increase in egg production compared to the control for the hens at 20-38 weeks of age (Yesilbag and Colpan, 2006) and 70 weeks old hens (Soltan, 2008, Rahman et al., 2008). This is different from the current study, but it could be because the latter studies worked with a mixture of organic acids as feed additives and not grains treated with pure propionic acid.

The mean egg weight showed a significant difference at week 0 between the propionic acid diet and the coriander/pumpkin diet ($p = 0.0406$) (Figure 8B). At this point, the hens had not started on the experimental diets, and therefore it cannot be the reason. Afterwards there were no significant differences between the treatments. This is in consistence with other studies where no differences were found in egg weight from birds fed control diets or diets complemented with pumpkin oil, organic acids or coriander seed powder (Çelik et al., 2011, Yesilbag and Colpan, 2006, Soltan, 2008, Habiyah et al., 2016).

The performance data from the current study comes with some uncertainty since some eggs were laid on the floor instead of in the nest boxes. This resulted in some eggs breaking or being eaten by the hens. This made the egg counting difficult and may give an underestimated egg production rate. Also feed spillage was observed in the pens which potentially gives an overestimated feed intake.

6.2. Gut health

The parameters investigated to characterize gut health of the hens was pH of feces and ileal and cecal contents, and the barrier status of the gut epithelium as measured by serum coloration. The plasma coloration represents the ability of nutrient absorption of the gut by measuring uptake of e.g. carotenoids. The test showed a significant difference between the control diet and the propionic acid diet for the short wavelengths (405-490 nm) and a tendency in the following wavelengths (550-590 nm) with the propionic acid having a higher OD than the control diet (Figure 11). Studies with plasma coloration of plasma from birds infected with *A. galli* have never been published but it has for plasma from broilers with coccidiosis. The latter study show that chickens with damaged intestinal epithelium caused by coccidiosis had lower OD of serum compared to uninfected chickens (Hamzic et al., 2015). From that, it seems like the hens fed with the propionic acid diet had a higher ability of nutrient absorption than the ones fed the control diet and thereby a less damaged intestine. This could indicate a lower worm burden, but this was not the case since there was no significant difference in worm burden between the treatments (Figure 12). However, as mentioned previously, the intestine is mostly damaged during the histotrophic phase where the migrating larvae penetrate the intestinal mucosa leading to intestinal damage, and plasma was not taken at this timepoint in this study.

6.3. *A. galli* burden

EPG of *A. galli* and *Capillaria spp.* was counted as measure of the nematode burden during the experiment but since it only refers to the female worms the EPG it does not necessarily correlate to the total worm burden. A large variation within the treatments was observed for both nematodes and there were no significant differences for any of the days (Figure 12). A tendency was observed at day 3 for *Capillaria spp.* where coriander/pumpkin had a higher EPG than the propionic acid (Figure 12B). Pumpkin seeds has earlier shown that it is moderately effective at reducing EPG from *A. galli*, *H. gallinarum* and *Raillietina spp.* which is contrary the EPG of *A. galli* observed in the current study (Acorda et al., 2019). Another study on pumpkin seeds showed no effect of treatment on *A. galli* EPG like in the current study (Rodenbücher et al., 2023).

A correlation between EPG and *A. galli* female worm count at slaughter showed a significant moderate positive correlation (Figure 13). Others have also found a significant strong correlation between *A. galli* egg count and total worm burden (Sharma et al., 2018b, Thapa et al., 2015, Train and Hansen, 1968, Gauly et al., 2005).

For further determination of immune parameters which may correlate with the *A. galli* burden the concentrations of intestinal *A. galli* specific IgY, IgA and the absorbance of IgM was measured (Figure 14). The Ig production potential of intestinal B cells showed no significant difference between treatments for *A. galli* specific IgY or IgA (Figure 14A and B). For IgA another study has found higher levels of total IgA in the control (100-200 µg/ml) than in the current study (Levkut et al., 2022). In a study by Daş et al. (2021), it was observed that nematode infections with *A. galli* and *H. gallinarum* had no effect on the total immunoglobulin concentrations for IgY, IgM or IgA. Also, higher immunoglobulin values than in the current study was observed, but also higher values for IgY compared to the two others like in the current study (7.49 mg/ml IgY, 0.76 mg/ml IgM and 0.33 mg/ml IgA). The reason for the different values for Ig compared to the current study is likely because the other studies measured Ig's in blood samples and the current study used intestinal samples. The difference of concentration of different antibodies within a chicken is normal since total IgY normally is found in higher concentrations than total IgA and IgM in serum (Rose and Orlans, 1981, Kowalczyk et al., 1985). For IgM significant differences between the control treatment and the coriander/pumpkin diet as well as the control treatment and the propionic acid treatment were observed where the control had the highest absorbance compared to both other treatments (Figure 14C).

A correlation between the intestinal *A. galli* specific antibodies and worm count showed a significant correlation for IgA which was a moderate positive correlation (Figure 14E). Earlier studies have established that specific serum antibody levels seldom correlate with worm burden in late stages of the infection when adult worms are present. Instead it has been shown that it is the larvae stage of *A. galli* that induces a strong serum antibody response in the host, rather than the mature worms (Daş et al., 2018). This is likely because the larvae penetrate the intestinal wall and thereby causes damage that will initiate the immune response whereas the adult worms live in the intestinal lumen.

The worm burden at slaughter showed no significant difference between the three treatments (Figure 15) but for the proportion of *A. galli* females there was a tendency to coriander/pumpkin having more females than the two other groups ($p = 0.1$). In a study using pumpkin seed, the treatment was effective against *A. galli*, where a significant decrease in *A. galli* worm load was observed and it was almost as effective as treatment with mebendazole (Acorda et al., 2019). They suggest that the presence of cucurbitin is responsible for the anthelmintic effect of the seeds. Pumpkin seed ethanolic extract have also shown an effect on reducing *A. galli* worm burden (Abdel Aziz et al., 2018). However, another study on pumpkin seeds failed to show an effect of treatment on worm burden like

observed in the current study (Rodenbücher et al., 2023). The difference may be because of differences in the amount of additive used, with 2000 mg/kg pumpkin seed ethanolic extract compared to 10 g pumpkin seed per hen per day. Furthermore, cucurbitin may be more concentrated in the ethanolic extract of pumpkin seeds. Promising results have been found in vitro on propionic acid treatment where it showed nematocidal effects on larvae of *S. papillosum*, *M. capillaris*, and *H. contortus* (Boyko and Brygadyrenko, 2022). Unfortunately, this was not observed in this study in vivo on *A. galli*.

6.4. Immunocompetence

The parameters absolute counts of peripheral blood cell subsets, phagocytosis ability of leucocytes in blood, plasma opsonization potential, cMBL concentrations and MHC haplotypes were all measured to investigate the effects of the feed on the immune system and the general immunocompetence of the hens.

The cellular immunity against *A. galli* is characterized by T helper cells that secrete cytokines and activation of macrophages, eosinophils, basophils and mast cells and an increase in heterophils (Sharpe et al., 2018, Luna-Olivares et al., 2012). In case of detection of pathogens heterophils are the first cells in action (Genovese et al., 2013, Chuammitri et al., 2009). The leucocyte counts in peripheral blood did not show a significant difference between treatments for any type of cells (Figure 16 and 17). But a tendency was found for B cells where coriander/pumpkin treatment had a higher concentration than the control treatment ($p = 0.078$) (Figure 16D). Earlier studies have shown an increased amount of T helper cells in *A. galli* infected hens in both blood and intestine and infected hens had significantly more T helper cells in blood than controls (Ruhnke et al., 2017, Schwarz et al., 2011). Ruhnke et al. (2017) did not find a significant difference between infected and controls on the number of monocytes and eosinophils, which could indicate that the number of those cells are not affected by infection of *A. galli*. An immune-histochemical analysis of T lymphocytes has demonstrated a significant increase in all T cell subtypes in the jejunal lamina propria of *A. galli*-infected chickens (Schwarz et al., 2011, Norup et al., 2013). This was most significant in the beginning of an infection. In cellular immunity T helper cells and TCR $\gamma\delta$ T cells have been found to form a protective band during *A. galli* infection (Shohana et al., 2023).

The H/L-ratio is a parameter for measuring long-term stress in domestic fowls (Gross and Siegel, 1983, Hofmann et al., 2020). The control animals that were not exposed to stress showed a H/L ratio

around 0.4 (Gross and Siegel, 1983) which may indicate that the hens in the current study did not suffer from long-term stress with values around 0.2.

Blood parameters like chicken mannose-binding lectin (MBL) of the chicken can be used as an indicator for diseases diagnosis since it is considered an acute phase protein. The cMBL concentrations in plasma was not significant different between the treatments (Figure 20). The concentrations are similar to earlier findings (Laursen et al., 1995). The latter study tested the mean level of cMBL in 14 different chicken lines, both layers and broilers and found an average on 6 µg/ml blood. But this can vary greatly in healthy chickens from 0.4 µg/ml blood to 37.8 µg/ml blood (Laursen et al., 1995). Studies on the effect of cMBL on other chicken diseases showed that cMBL plays a role in the innate immunity against Infectious Bronchitis Virus (IBV) and *Pasteurella multocida* (Juul-Madsen et al., 2007, Schou et al., 2010a). The cMBL performs an acute phase response, is able to activate complement, and inhibits the propagation of the virus in the trachea (Juul-Madsen et al., 2007).

The MHC haplotype determination showed only three different types: one homozygous (357/357) and two heterozygous (261/357 and 443/357). The combination of the three haplotypes was not entirely identical in the three treatment groups (Table 6). This is relevant since studies have shown that different haplotypes are more or less susceptible towards *A. galli*-infection (Schou et al., 2003, Kaufmann et al., 2011, Permin and Ranvig, 2001, Gaulty et al., 2007, Norup et al., 2013). Lohmann Brown laying hens was considered more resistant to parasites than other breeds (Gaulty et al., 2007, Permin and Ranvig, 2001). Permin and Ranvig (2001) found differences in worm burden and egg excretion between two layer types and Kaufmann et al. (2011) found significant differences between two layer types on the establishment rate of several helminth species. Another study have found a low level of variation between hybrids but owe it to the small number of flocks per hybrid (Thapa et al., 2015). This indicate that it is possible to select hens based on genetics for parasite resistance and this can be important in the battle against *A. galli*.

7. Conclusion

The aim of the study was to evaluate if the helminth burden and immunocompetence in organic layers are affected by the feeding the chickens propionic acid treated grain or grains with pumpkin seed oil in combination with essential coriander seed oil.

The present study showed no evidence of anthelmintic effects of pumpkin seed oil combined with essential coriander seed oil or grains treated with propionic acid. Health, welfare, and production parameters were unaffected by the treatments indicating that the treatments are tolerated by the hens, but further studies are needed for long term effects. The coriander/pumpkin treatment increased the daily FI but no improvement on performance was found.

From the plasma coloration it seemed that the hens fed with the propionic acid diet had a higher ability of nutrient absorption than the ones fed the control diet and thereby a less damaged intestine. But no difference in worm burden was found between the treatments which relates to the EPG where no difference was found either.

The absolute counts of blood cell subsets were not affected by the treatment but a tendency to a higher B cell concentration in coriander/pumpkin compared to the control was found. However, the phagocytosis ability of leucocytes showed a significant higher activity for heterophils, lymphocytes, and thrombocytes from the propionic acid diet. In contrast no difference was found for the plasma opsonization potential that affects the efficiency of phagocytosis.

In summary, anthelmintic effects found in *in vitro* experiments with propionic acid, pumpkin oil and coriander oil were not proven *in vivo* in the current experiment. Further research with other allocation methods, other concentration or treatment over longer periods could therefore be of interest. It could also be interesting to further investigate the connection of genetics and resistance towards helminths to reduce the incidence of infections as alternative to drug treatment.

8. Literature

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Appendix A: Gating strategy for flow cytometric analysis (Absolute count)

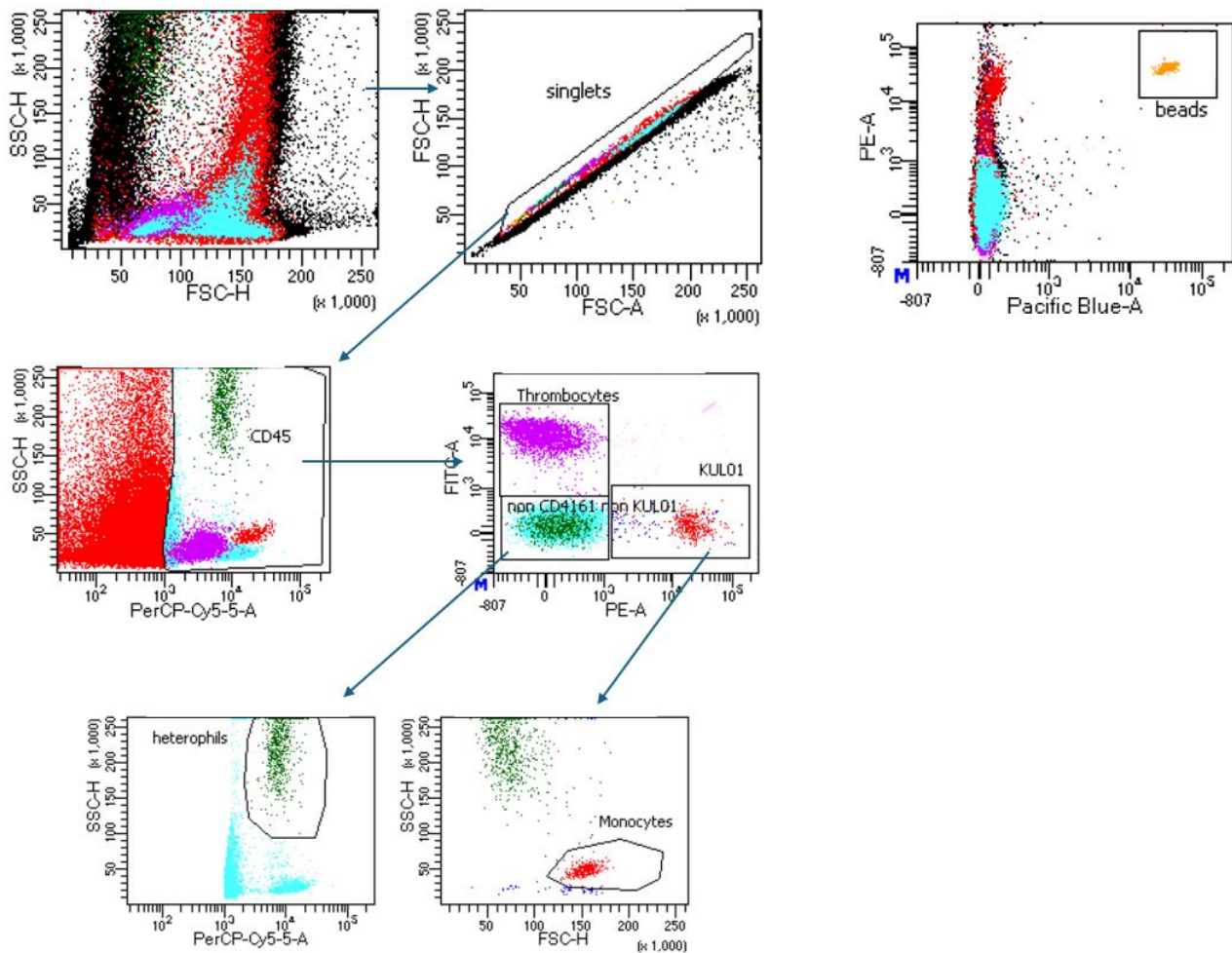


Figure 22: Gating strategy for flow cytometric analysis of absolute counts of blood cell subsets panel 1. The strategy for Figure 16 panel 1 was CD45⁺ cells, CD45⁺CD41/61⁺ cells (thrombocytes) / CD45⁺KUL01⁺ cells / CD45⁺CD41/61⁻KUL01⁻ heterophils and CD45⁺KUL01⁺ FSC-H monocytes. PE versus Pacific Blue was used for gating of 123count eBeads™ counting beads. Staining of whole blood from a representative animal is shown as an example.

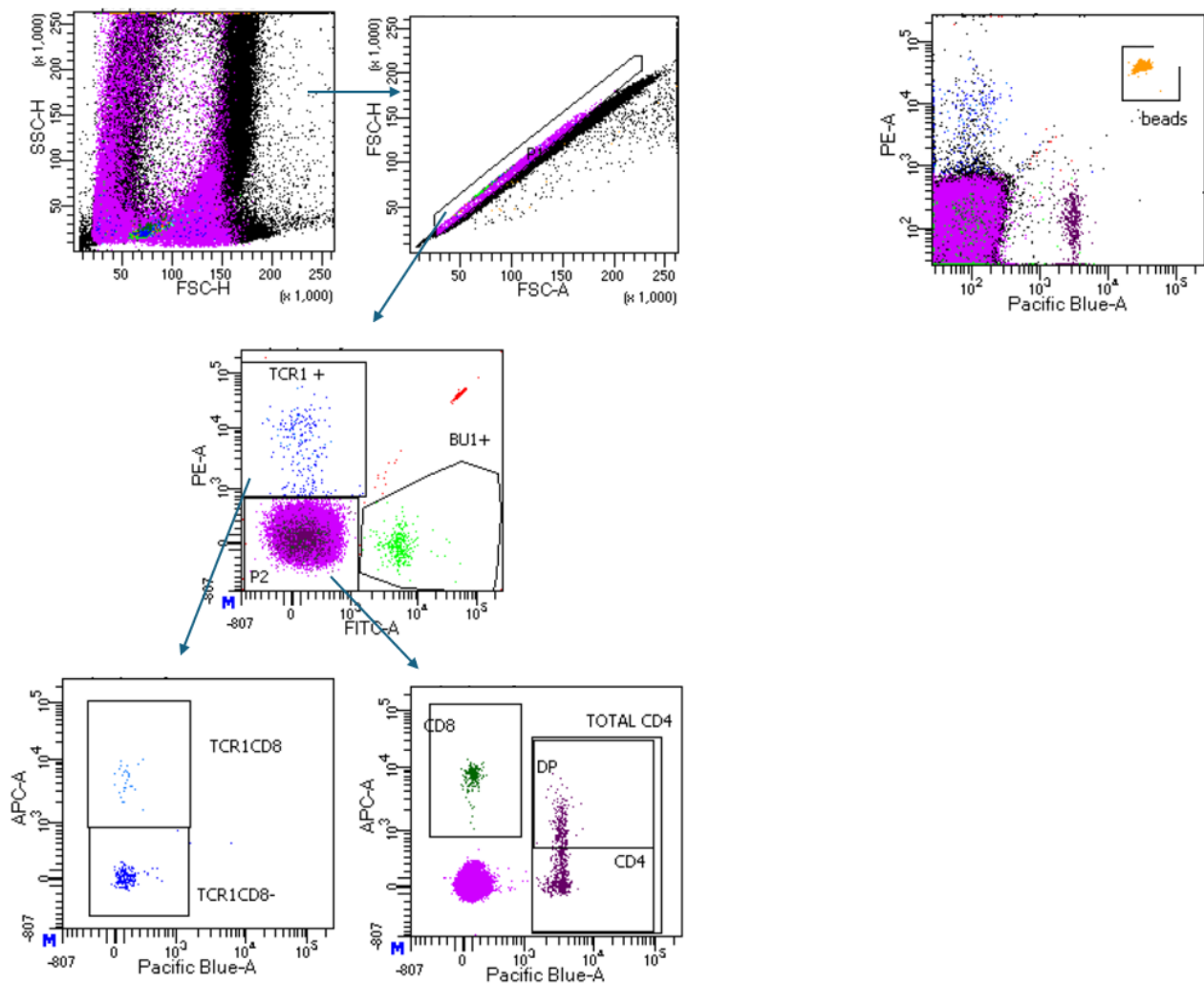


Figure 23: Gating strategy for flow cytometric analysis of absolute counts of blood cell subsets panel 2. The strategy for figure 16 and 17 panel 2 was BU1+ B cells, TCR1+ T cells, TCR1+CD8+ T cells, TCR1+CD8- T cells, TCR1-BU1- cells (P2), CD8+ T cells, Total CD4+ T cells, CD8+CD4+ T cells. PE versus Pacific Blue was used for gating of 123count eBeads™ counting beads. Staining of whole blood from a representative animal is shown as an example.

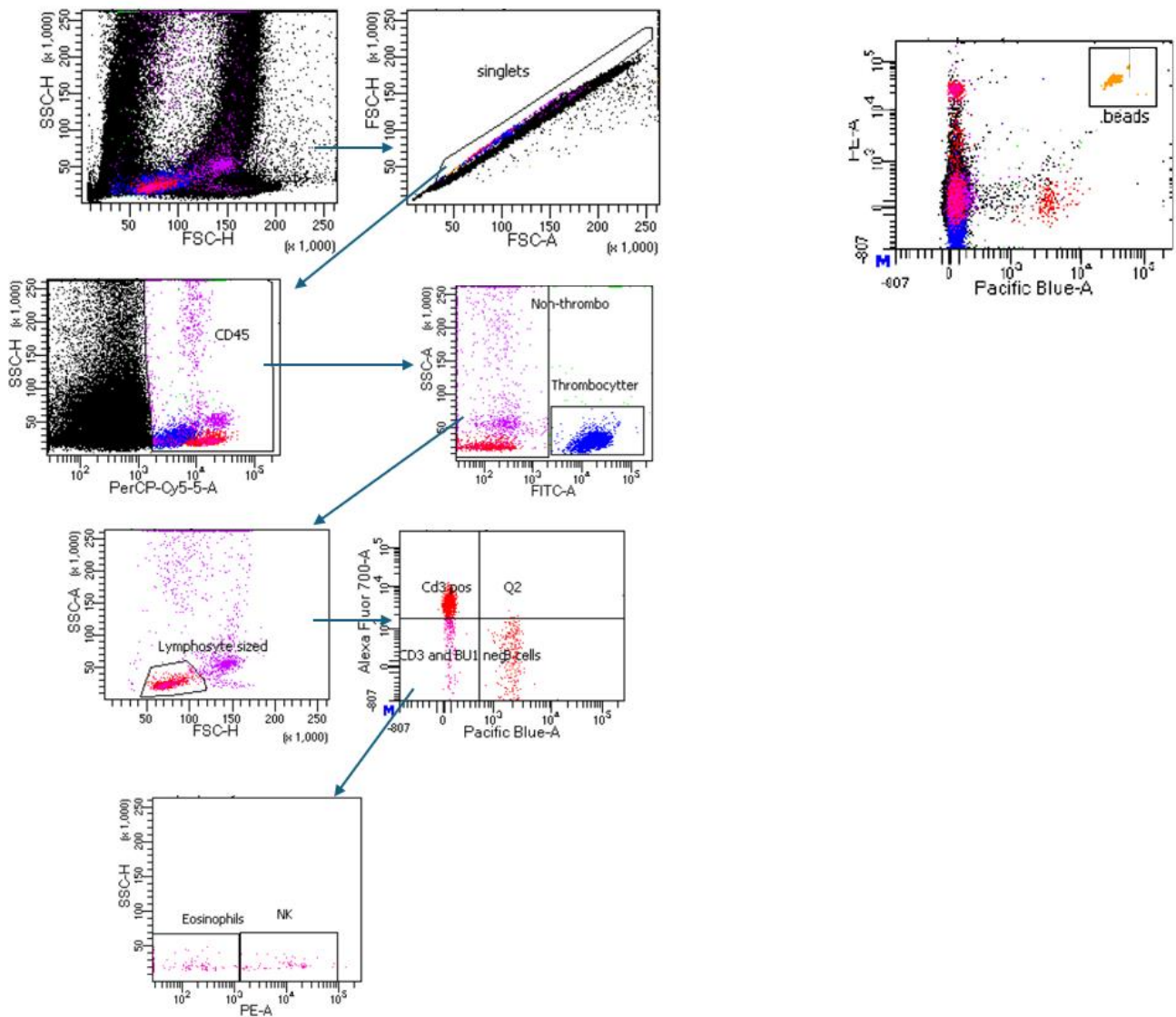


Figure 24: Gating strategy for flow cytometric analysis of absolute counts of blood cell subsets panel 3. The strategy for Figure 16 and 17 panel 3 was CD45+ cells, CD41/61 +(thrombocytes), non-CD41/61+ cells, SSC-A-FSC-H lymphocyte size, CD3+ cells, BU1+ cells, CD3-Bu1- cells, CD8+ cells (NK), Cd8- cells (eosinophils). PE versus Pacific Blue was used for gating of 123count eBeads™ counting beads. Staining of whole blood from a representative animal is shown as an example.

Appendix B: Gating strategy for flow cytometric analysis (Phagocytose assay)

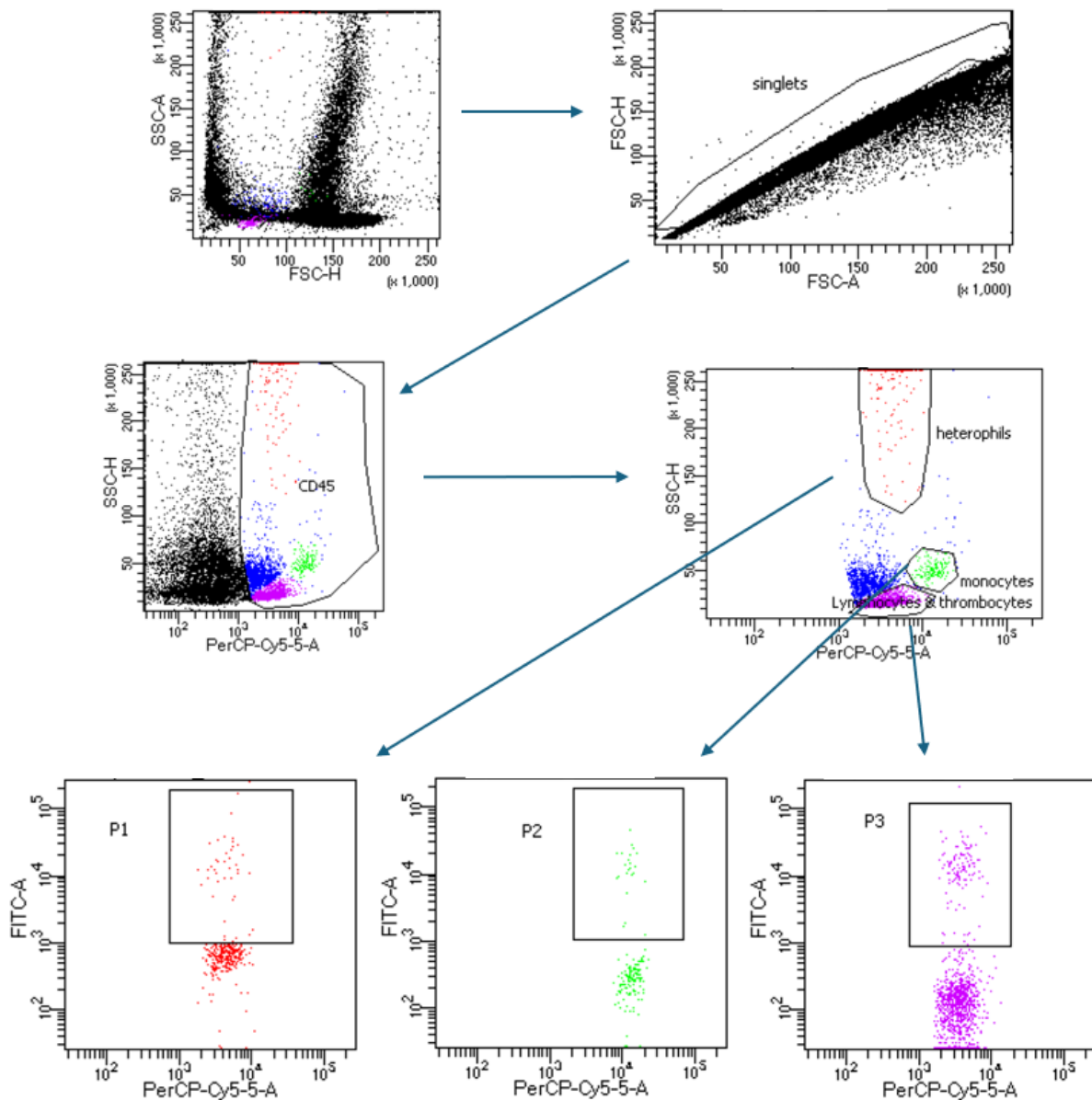


Figure 25: Gating strategy for flow cytometric analysis of phagocytose assay on whole blood. The gating strategy for Figure 18 was CD45- cells, SSC-H heterophils, SSC-H monocytes, SSC-H lymphocytes & monocytes, and Salmonella FITC+ cels (P1, P2 and P3). Staining of whole blood from a representative animal is shown as an example.

Appendix C: Gating strategy for flow cytometric analysis (Opsonization potential)

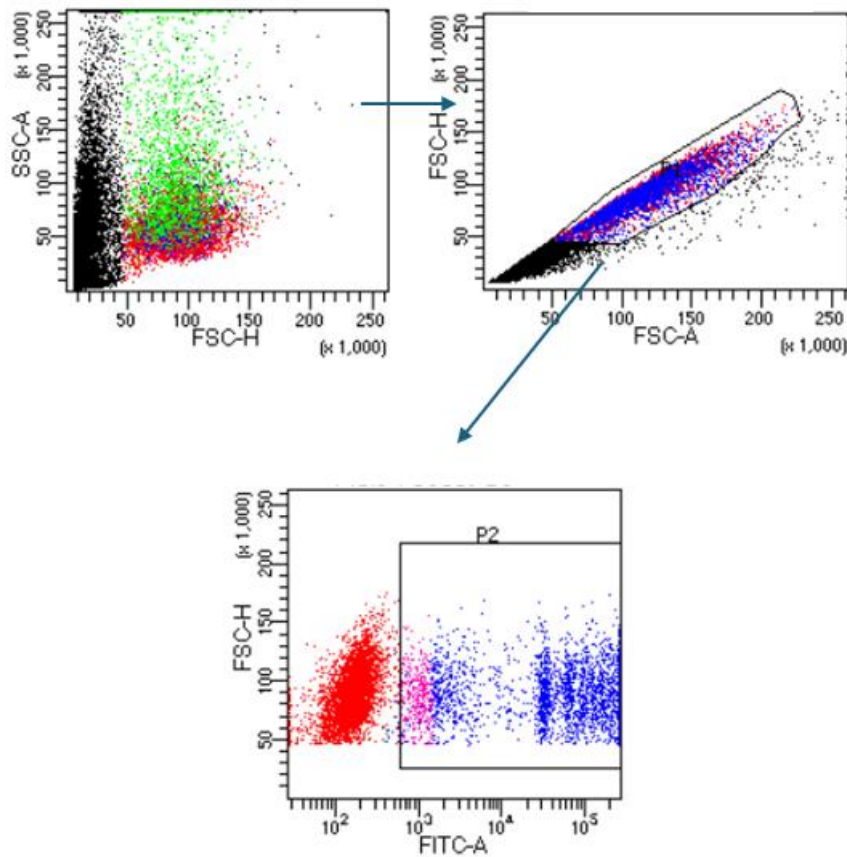


Figure 26: Gating strategy for flow cytometric analysis of opsonization potential with HD11 cells. The gating strategy for Figure 19 was single cell gating (FSC-H vs FSC-A) followed by FITC+ cells. Results from opsonization potential with plasma from a representative animal is shown as an example.