This master thesis is a part of the PPilow project.

The project PPILOW has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N°816172

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FEEDING STRATEGY TO SUPPORT HEALTH IN ORGANIC LAYING HENS

The effect of fermented rapeseed meal with seaweed on *Ascaridia galli* in organic laying hens (*Gallus gallus domesticus*)

Master thesis in Agrobiology

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Feeding Strategy to Support Health in Organic Laying Hens

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Master Thesis (60 ECTS) by

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June 2021

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Preface

This master thesis has been written to fulfil the requirements for the Master degree in Agrobiology at the Faculty of Technical Sciences, Aarhus University, Denmark. The thesis is based on an animal experiment that lasted over 12 weeks and is the first of several collaborative research projects, which has the purpose of investigating feeding strategies to support health in organic laying hens.

I would like to express my gratitude to the best supervisors Ricarda Margarete Engberg, Rikke Brødsgaard Kjærup and Tina Sørensen Dalgaard, Department of Animal Science, Aarhus University, for including me in all the processes of the research work and for always being supportive, helpful, inspiring and encouraging, whenever I needed it. I am grateful to Rikke for spending a lot of time on helping me with the statistical analyses.

I would like to thank Lene Rosborg Dal for teaching me everything I have learned in the laboratory during my Master thesis and for your work with analysing samples with me. Also, thanks to Trine Poulsen, Inger Marie Jepsen and Hanne Purup, who contributed to the laboratory work.

Finally, I would like to express my love and appreciation to my children, Konrad and Johanna, and to my fiancé, Stefan Schultz Tiedemann, for being my rock and for always packing my lunch.

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List of abbreviations

A. galli	*Ascaridia galli*
A. nodosum	*Ascophyllum nodosum*
Art	Artificially infected
AUC	Area under curve
BALT	Bronchus-associated lymphoid tissue
BWG	Body weight gain
CALT	Conjunctiva-associated lymphoid tissue
ConA	Concanavalin A
DM	Dry matter
DP	Double-positive (CD4+CD8+)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immune sorbent assay
EPG	Eggs per gram faeces
FACS	Fluorescence-activated cell sorting
FI	Feed intake
GALT	Gastrointestinal-associated lymphoid tissue
H/L-ratio	Heterophils/lymphocytes ratio
IEL	Intraepithelial lymphocytes
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgY	Immunoglobulin Y
IL	Interleukin
I-NSP	Insoluble non-starch polysaccharides
ITC	Isothiocyanates

MALT	Mucosa-associated lymphoid tissue
MD	Meckel's Diverticulum
MHC	Major histocompatibility complex
Nat	Naturally infected
OD	Optical density
p.i.	Post infection
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PMA	Phorbol 12-myristate 13-acetate
r	Spearman correlation coefficient
RT	Room temperature
S. latissima	*Saccharina latissima*
S-NSP	Soluble non-starch polysaccharides
TCR1+	T cell receptor TCR1+ (expressed on $\gamma\delta$ T cells)

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Summary

Organic layers have higher risk for parasite infections due to access to outdoor areas. Infections with the nematode *Ascaridia galli* (*A. galli*) are highly prevalent in organic production systems. Dietary supplementation of feed components pre-fermented with lactic acid bacteria has recently attracted attention and is suggested to have positive effects on performance and gut health in poultry and pigs. The aim of this study was to investigate whether fermented rapeseed meal with seaweed had an effect on performance, short chain fatty acids and immunoprofiles of layers infected with *A. galli.* Sixty-four hens received either a feed supplemented with fermented rapeseed meal with seaweed (6%) or a non-supplemented control feed. The hens were infected with *A. galli* at 20 weeks of age by two different infection methods involving inoculation with 764 embryonated *A. galli* eggs orally or natural exposure to contaminated litter. Twelve weeks post infection, the experiment was terminated, and the number of intestinal adult worms were assessed. Supplementation of fermented rapeseed meal with seaweed did not affect performance, worm burden or immune competence as assessed by mitogen activation of peripheral blood mononuclear cells (PBMCs). However, compared to controls, supplemented hens had a lower humoral *A. galli* specific immune response. B and T cell populations were studied in Meckel's diverticulum (MD) and in the intestinal epithelium. Both fermented feed supplementation and artificial infection increased the frequency of activated CD4+CD8+ T cells (DP) indicating that the fermented feed may have immunomodulatory potential. In conclusion, the dietary inclusion of fermented rapeseed meal with seaweed did not show any notable health promoting effect, but more studies are needed to investigate a potential effect under production settings.

1. Introduction

In Denmark, organic egg production has increased through the last decade, which is mainly driven by increasing animal welfare concerns leading to an increased consumer demand for organic products. Parasite infections are very common in barn egg, free-range and organic egg production systems, where the hens have access to potentially contaminated litter or soil (Maurer et al., 2009, Heckendorn et al., 2009). The nematode, *Ascaridia galli (A. galli)*, is a dominant intestinal parasite in poultry, and of big concern in especially laying hens. The parasite is highly transmittable and spreads with the excreta. The hen ingests eggs containing the infective third larvae of *A. galli,* which hatch in the small intestine within 24 hours, later the larvae migrate to the intestinal mucosa, which may cause bleeding and involve increased risk of secondary infections (Skallerup et al., 2005). The adult worms excrete toxic metabolites and in severe infection cases, perforation of the intestinal wall can occur. *A. galli* infection may further present a risk factor for outbreaks of cannibalism. Experiments have shown that the number of *A. galli* eggs per gram faeces (EPG) correlates positively with cases of cannibalism (Heckendorn et al., 2009). Overall, the infection may lead to poor production results and a reduced feed conversion rate. Besides the negative effective on hen performance, *A. galli* may also affect food quality. Occasionally, *A. galli* enters the oviduct, either via the cloaca or by penetration (Akinyemi et al., 1980, Piergili Fioretti et al., 2005). There is a risk that worms are enclosed in the developing egg in the oviduct before shell formation, which has led to increased complaints from consumers finding worms in their table eggs.

Intestinal worm infections are treated with anthelmintic drugs, e.g., fenbendazol, flubendazol, which in practice are applied, when worm burden is estimated to be high, as indicated by the presence of high numbers of worm eggs in faecal samples. Currently, there is no withdrawal period for eggs in relation to the use of these drugs. However, EU regulation 2018/848 of the European Parliament and of the Council suggested to come into force in January 2022 (Regulation (EU) 2020/1693 of the European Parliament and of the Council of 11 November 2020), implies, that during the treatment period and 2 days after, eggs cannot be market as organic eggs. This will cause significant economic losses for the organic egg producers. Another aspect to consider in relation to the application of anthelmintic drugs is the development of resistance, which may complicate the control of parasite

infections. It is therefore highly relevant to investigate, whether new feed components or feed additives with anti-parasitic effects can reduce the incidence of worm infections in organic egg production.

Dietary supplementation of feed components pre-fermented with lactic acid bacteria in a solid-state fermentation process have recently attracted attention and are suggested to have positive effects on performance and gut health in poultry and pigs (Chiang et al., 2010, Satessa et al., 2020). In this context, fermented rapeseed meal or cake, which present locally grown protein sources rich in sulfur-containing amino acids and phosphorus (Chiang et al., 2010) may be of special interest in organic egg production, where the formulation of diets with balanced amino acid profile is a challenge. Fermentation is also an effective way to reduce anti-nutritional factors in the feed (Hu et al., 2016). Lactic acid fermentation has been suggested to positively influence immune functions in chickens (Sugiharto and Ranjitkar, 2019) and to effectively increase the level of lactic acid, lactic acid bacteria and organic acids in the feedstuff, which may have a positive effect on the intestinal microbiota (Engberg et al., 2009). Further, the fermented feedstuff lowers the pH in the gut, which creates a less favourable environment for acid sensitive pathogenic bacteria, i.e. Enterobacteriaceae including E. coli and Salmonella.

Brown macroalgae (seaweed), i.e. *Ascophyllum nodosum* (*A. nodosum*) and *Saccharina latissima* (*S. latissima*), provide a variety of components, e.g. alginate, laminarin and fucoidan, which are not found in terrestrial plants. These components are suggested to have prebiotic and antimicrobial effects. However, it is not known whether fermented rapeseed meal in combination with brown algae have an effect against intestinal helminths, such as *A. galli*.

2. Objective

The objective of this study was to investigate the impact of dietary inclusion of a lactic acid bacteria fermented rapeseed meal with seaweed (European protein EP 199, Fermentation Experts) on the infection pressure of *A. galli,* intestinal health and immune function of artificially (Art) and naturally (Nat) challenged laying hens.

3. Background

3.1 Organic egg production

The demand for organic food products is still increasing and organic eggs are no exception. In Denmark, the organic egg production makes up approx. 30% of the total amount of eggs produced in 2019, compared to 2009, where it only made up approx. 15% of the total amount of eggs produced (Figure 1 and 2). Data shows a significant decrease in cage egg production, which can be explained by the ban of the conventional cages in 2012. Farmers, producing cage eggs, were then forced to invest in new so-called enriched cages or to change to another production system. The enriched cages provided the animals with more space, nests, a scratching and dust bathing area and perches. However, consumer concerns regarding bird welfare, i.e. the limited possibility to express natural behaviour in cages, have resulted in the decision of most retailers in Denmark not to sell eggs derived from caged hens, which has driven the development to floor based egg production systems including barn egg, free-range and organic production (Figure 1). “Dansk Supermarked”, owning stores like Netto, Føtex, Salling and Bilka, stopped the sale of cage eggs in 2019, and “Coop” stopped the sale in 2020. Also, the current Minister of Food, Agriculture and Fisheries in Denmark, Rasmus Prehn, promotes phasing out the sale and production of cage eggs entirely (landbrugsavisen, 2020. URL: https://landbrugsavisen.dk/fødevareministeren-vil-arbejde-udfasning-af-buræg).

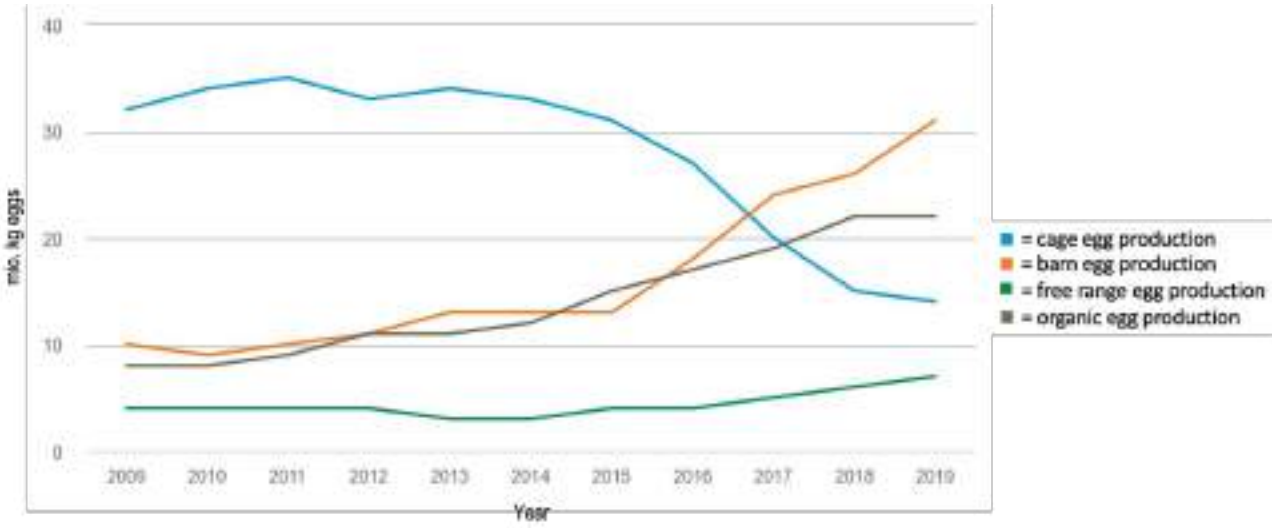

Figure 1: Egg production in Denmark 2009-2019 based on production system. Source: Danmarks Statistik (statistikbanken.dk)

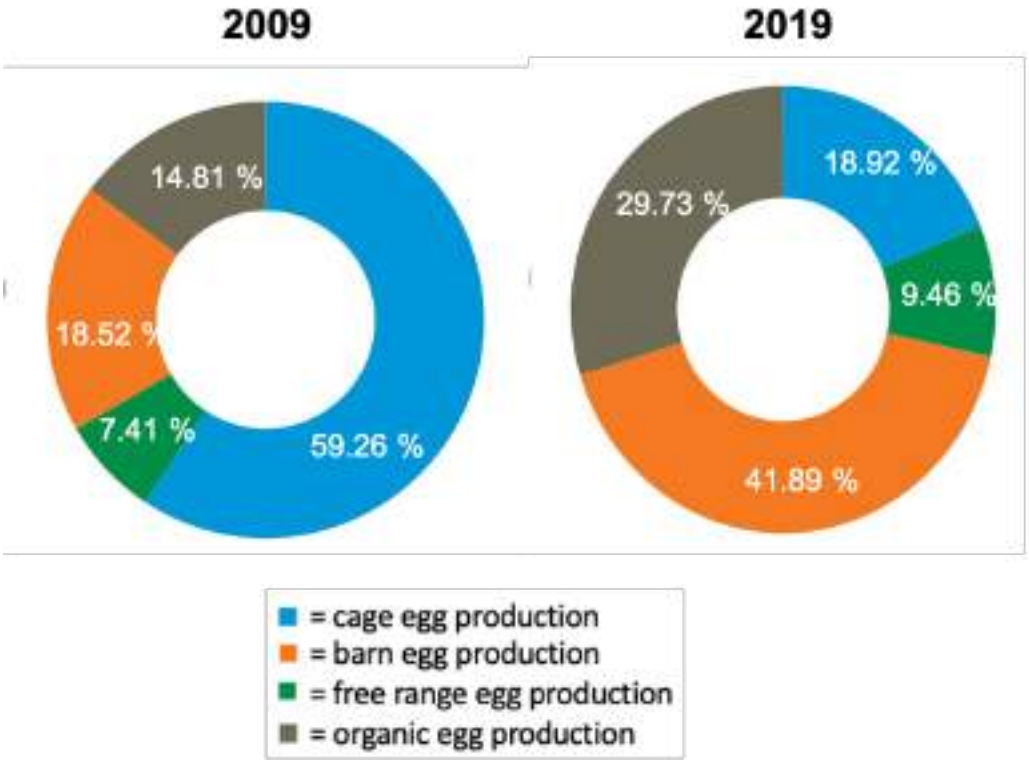

Figure 2: Egg production in Denmark 2009 and 2019. Source: Danmarks Statistik (statistikbanken.dk)

In organic egg production, flock sizes of 3000 hens/pen are allowed. There is a restriction of maximum six hens per m^2 in the indoor facilities and at least four m^2/hen in the outdoor area (Holdensen, 2020). Grass or other vegetation must cover the outdoor area, and at least once a year the hens are moved to another area to help vegetation to be re-established which also helps to alleviate parasite infections. *A. galli* infections occur in all floor systems, where layers have access to the litter (Jansson et al., 2010). With good management, nematode infections are less common in indoor system. Thorough cleaning, disinfection and burning of floors between new placements of hens are tools to limit infection transmission between flocks in indoor systems. In outdoor systems, like organic and free range, keeping a similar hygiene is impossible.

3.2 Gut health

Intestinal barrier function and a balanced intestinal microbiota are important features of gut health. The epithelium of the small intestine is responsible for the absorption of nutrients, but also presents the physical barrier protecting the host against invading pathogens. The gut microbiota competes with its host for available nutrients, but also supports intestinal integrity by preventing colonization of pathogenic bacteria (Pan and Yu, 2014). The composition and activity of the gut microbiota is affected by the composition of the feed and the substrate available in the intestine. Short chain fatty acids (SCFA) and lactic acid are the main bacterial metabolites produced from substrate present in the digestive tract. The concentrations of SCFA and lactic acid reflect intestinal microbial activity and give an indication of the quality and amount of fermentable substrate. If nutrient absorption is reduced, more substrate will be available for microbial fermentation.

3.3 Avian immune system

The avian immune system shares many similarities with that of mammalians, but also has some distinctive differences. The immune system of birds consists of lymphatic vessels, lymphoid organs and lymphoid tissue. In chickens, the primary lymphoid organs are thymus and Bursa Fabricii. In primary lymphoid organs, education and maturation of lymphocytes takes place. T cells are matured in the thymus, and B cells are matured in the Bursa Fabricii, which is only present in birds. In mammals, B cells mature in the bone marrow. Besides development and maturation, primary lymphoid organs also eliminate self-reactive B and T cells.

The secondary lymphoid organs and tissues are the spleen, bone marrow, lymph nodules, Harderian gland and mucosal associated lymphoid tissue (MALT). Birds lack structured peripheral lymph nodes but have lymph nodules associated with the walls of lymph vessels and in e.g. skin and lungs. Like mammals, chickens do have MALT, which consists of gastrointestinal-associated lymphoid tissue (GALT), bronchial-associated lymphoid tissue (BALT), and conjunctival-associated lymphoid tissue (CALT). GALT includes different lymphoid structures like MD, caecal tonsils, pyloric tonsils and Peyer's patches. In the secondary lymphoid organs activation of naïve lymphocytes takes place.

Like in mammals, the avian immune system consists of both an innate and an adaptive immune system, where both parts contain humoral and cellular immune components. The innate immune system is non-specific and provides the first line of defence against an infection. The adaptive immune system is more specific than the innate immune system and provides a more long-term defence against pathogens.

Cells of the innate immune system include phagocytes, basophils, natural killer cells and mast cells. Monocytes and neutrophils are the most common phagocytes in the mammalian immune system. Birds lack neutrophils but have heterophils, which have equivalent functions. The heterophils are involved in phagocytic activity and are the first cells to occur during inflammation (Genovese et al., 2013). Further, birds have a significant higher amount of basophils, which are also important for the early inflammation response. Avian thrombocytes deviate in morphology to mammalian thrombocytes, because they are nucleated, but they have similar functions. They play an important role in hemostasis and are some of the first cells to occur, when there is an arterial injury (Ferdous and Scott, 2015). However, avian thrombocytes have also proven to play a role in both the innate immunity, because of their expression of several toll-like receptors (TLRs) and pro-inflammatory cytokines as well as their phagocytic ability, but also creating a bridge to the adaptive immune system in terms of expressing CD40 ligand and transcripts of CD40, CD80 and MHC-II (Ferdous and Scott, 2015, St Paul et al., 2012). This gives the thrombocytes the potential to work as antigen presenting cells (APCs) and further produce cytokine IL-12, when activated by lipopolysaccharides (Ferdous et al., 2008).

Like in mammals, the cells present in the adaptive immune system are lymphocytes, which include T cells and B cells. T cells are involved in the cellular immune response, and B cells are involved in the humoral immune response. Avian T cells are divided into two subpopulations known as TCRαβ+ T cells and TCRγδ+ T cells (hereafter referred to as γδ T cells). The functions of αβ T cells are similar to the ones in mammals, including helper and cytotoxic functions, which is restricted by major histocompatibility complex (MHC) (Sharma et al., 1997). B cells are involved in immunoglobulin production. In birds, three classes of immunoglobulins have been identified: IgA, IgM and IgY, which is a homolog to mammalian IgG (Rose et al., 1974).

3.4 *Ascaridia galli*

3.4.1 Morphology

The roundworm, *A. galli*, belongs to the phylum Nematoda and is the largest parasite found in fowl (Figure 3). The male worm measures from 42 to 76 mm and the female are somewhat larger, measuring from 72 to 108 mm (Ramadan and Znada, 1992). The adult worms appear yellow to white and are semi-transparent. The anterior end contains three prominent trilobed lips and papillae are located on the lips and on the neck. The dorsal lip contains two papillae and the other two lips each contain one papilla (Ramadan and Znada, 1992). At the caudal end, the female worm appears to have a more symmetric pointed tail, whereas the tail of the male worm appears less symmetric (Figure 4).

Figure 3: Adult A. galli (MH own picture).

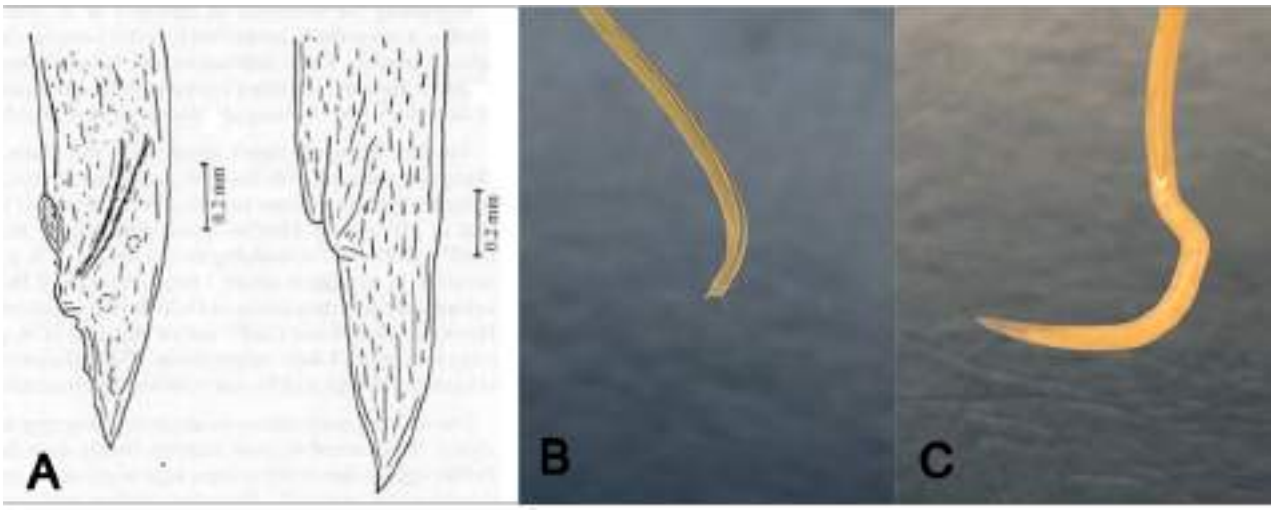

Figure 4: A) To the left: the posterior end of a 25 days old male. To the right: the posterior end of a 25 days old female (Ramadan and Znada, 1992). B) The posterior end of a male (MH own picture). C) The posterior end of a female (MH own picture).

3.4.2 Life cycle

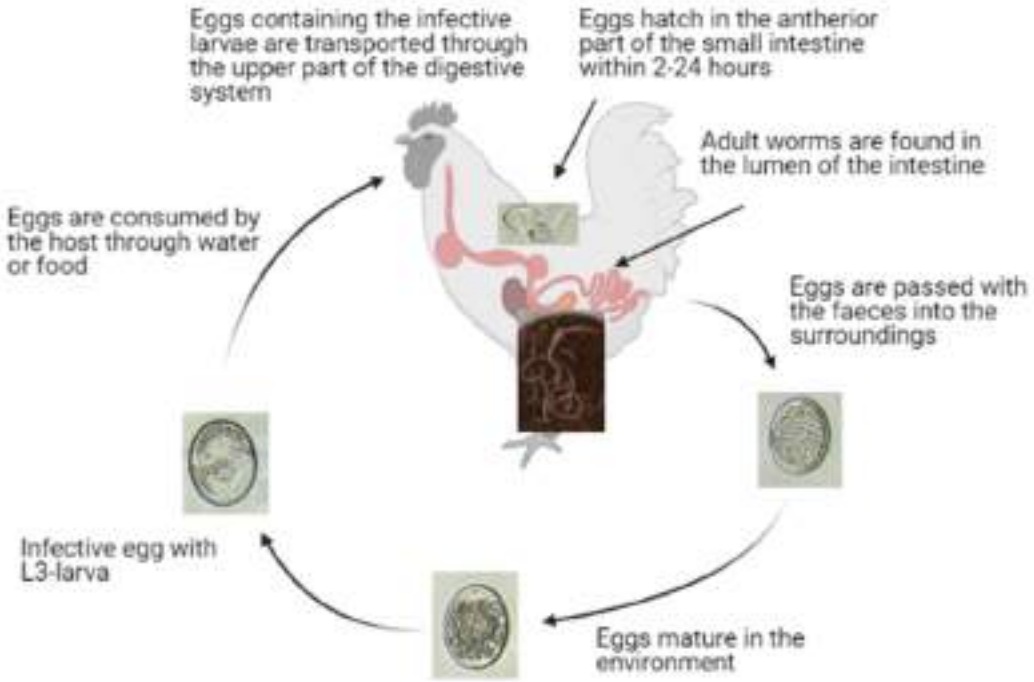

Figure 5: Life cycle of A. galli. Created in BioRender.com with own pictures.

Hens ingest the *A. galli* eggs containing the infective third stage larva from the soil or the litter (Figure 5). After 24 hours the larvae hatch in the intestinal lumen, either in the proventriculus or in the duodenum, and 1-9 days post infection (p.i.) the larvae will penetrate the mucosa causing haemorrhages (Luna-Olivares et al., 2012, Tugwell and Ackert, 1952). This is called the histotrophic phase, where the larva will grow and start maturing in the mucosa. In this phase, the larva is in direct contact with the host tissue. Usually, the larva returns to the lumen after 2-3 weeks for final maturation, but the histotrophic phase can last up to 7 weeks (Herd and McNaught, 1975). Adult larvae are primarily located in the anterior half of the jejunum (Ferdushy et al., 2012). After another 10-13 days, the larva has matured and is now an adult worm. The sexual formation occurs in the intestinal lumen and fertilized eggs are excreted with the faeces to the environment. An adult female worm can produce up to 5000 eggs/day. The eggs consist of three layers; first an inner permeable membrane, then a thick and highly resistant shell in the middle and finally an outer thin albuminous layer. These three layers make the eggs highly persistent to survive in the environment, which is very important, because it takes another 10-20 days for the L3-larva to develop within the eggs, which then become infective (Figure 6).

Figure 6: A. galli eggs in different developmental stages. A) Un-infective egg B) Infective L3-larva C) Infective L3-larva hatching (MH own pictures).

The time frame for larva maturation is affected by ambient humidity and temperature (Permin et al., 1997). When the eggs become infective, they can be ingested by the same or another hen and the *A. galli* life cycle is completed. This cycle makes the *A. galli* extremely transmittable and very persistent. Under ideal conditions, the eggs can stay infective for up to 2 years (Thapa et al., 2017, Cruthers et al.,

1974). The prepatent period, defined as the time from hatching of the third stage larva to final maturation, is 37 days on average, but can vary from 4-8 weeks (Ramadan and Znada, 1992, Hauck, 2017, Permin et al., 1998)

3.4.3 Pathogenesis

In general, *A. galli* has a negative impact on poultry health, welfare and productivity. Pathogenesis is influenced by age, sex, diet, stress level, size and genotype of the host, but also the age and dose of infective eggs affects the infection level (Gauly et al., 2005, Das et al., 2010). Small hens tend to have larger worm burden than larger hens (Das and Gauly, 2014).

Infection with *A. galli* in chickens might lead to poor feed efficiency, reduced growth, diarrhoea, damage of the intestinal epithelium, blockage of the intestinal lumen, increased incidences of cannibalism and increased mortality (Das et al., 2010, Danicke et al., 2009, Ramadan and Znada, 1992, Kilpinen et al., 2005, Gauly et al., 2007, Hinrichsen et al., 2016).

Damage of the intestinal mucosa further predisposes the chickens for secondary bacterial infections, like E. coli, Salmonella and Pasteurella. During the summer season the mortality rate on infected farms can increase by 100% (Hinrichsen et al., 2016).

Infection with *A. galli* also shows immunosuppressive effects by influencing antibody levels raised after vaccination against Newcastle Disease, both in relation to a faster decrease in antibody level after first vaccination, but the infected hens also lacked the boosting-effect from re-vaccination (Horning et al., 2003, Pleidrup et al., 2014).

3.4.4 Effects on the host immune regulation

In general, knowledge about the immune response directed against avian helminths is very limited. Maternal immunity does not protect poultry against *A. galli* infections (Rahimian et al., 2017).

In mammals, helminth infections induce a Th2 immune response (Degen et al., 2005, Marcos-Atxutegi et al., 2009, Schwarz et al., 2011). Cytokines, like IL-5, IL-4, IL-9, IL-13, are released during the Th2 immune response, activating a number of immune cells in mammals (Figure 7). IL-5 leads to eosinophilia, which is the primary defence against migrating larvae. Eosinophils are also a source of IL-

4 and help recruiting Th2 cells to the site of inflammation. IL-4 and IL-13 increase the motility in the smooth muscle cells, which helps parasite expulsion. IL-4 and IL-13 also increase permeability in the epithelial cells, mucus production in the goblet cells, IgE production of B cells, and secretion of inflammatory mediators by mast cells and basophils (Montaner et al., 2014).

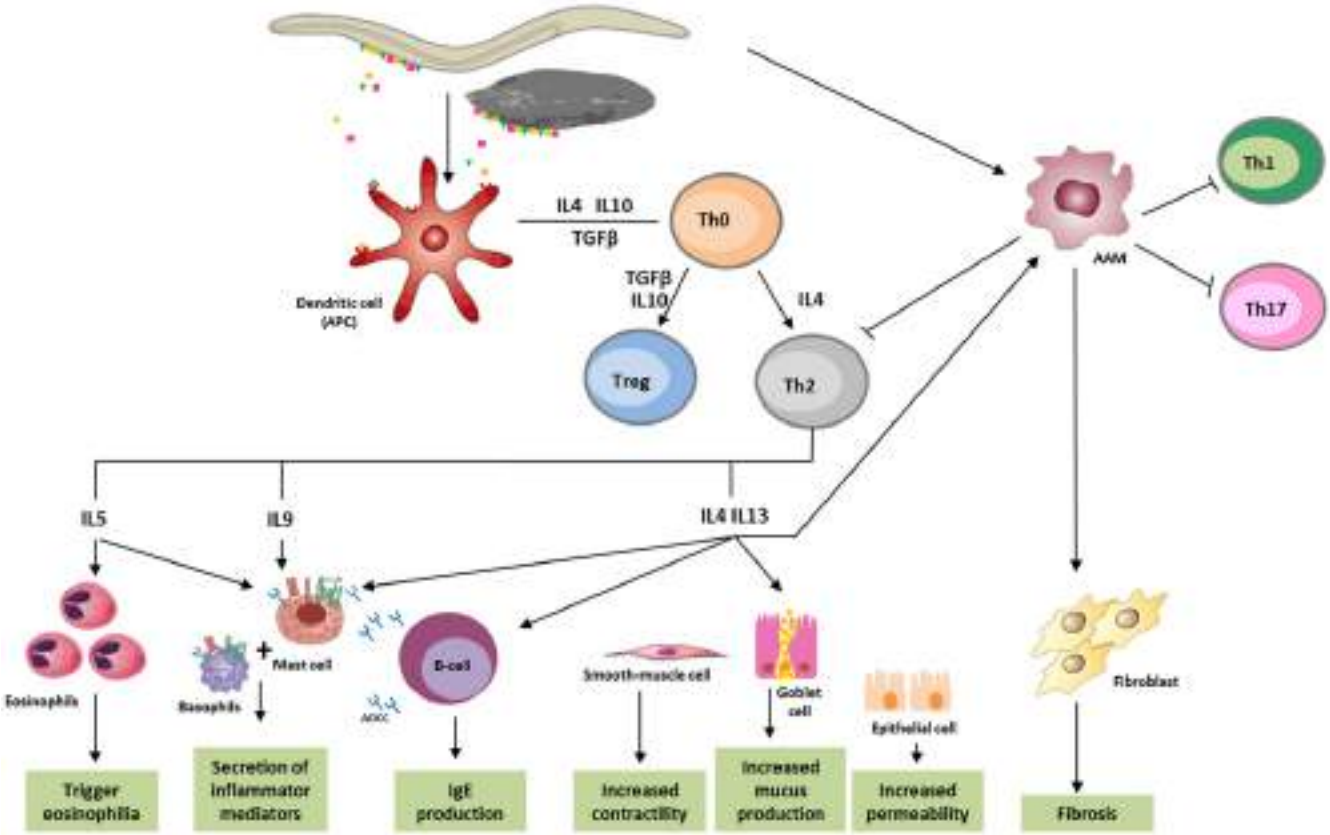

Figure 7: Th2 immune response against helminths in mammals (Montaner et al., 2014).

The avian immune system deviates slightly from the mammalian immune system. IL-5 is only found as a pseudo gene, and eosinophils are present in very low numbers compared to mammals. IgE has not been identified in birds, the IgG homolog, IgY, can act as IgE to some degree. However, IL-4 and IL-13 have been identified in the spleen and in ileum in response to *A. galli* infection (Degen et al., 2005). Poultry also produce and circulate *A. galli* specific antibodies in peripheral blood (Marcos-Atxutegi et al., 2009, Schwarz et al., 2011, Norup et al., 2013b). Dalgaard et al. (2015) found increased expression of IL-13 in the spleen of *A. galli* infected chickens in relation to the histotrophic phase (2 weeks p.i.), which is the major cytokine of the Th2 immune response and also known from the mammalian

immune response to helminths. However, they also found elevated expression of pro-inflammatory cytokines (IFN-alpha, IL-1-beta, IL-8, IL-12-beta and IL-18) and acute phase proteins (mannose-binding lectin and C-reactive protein) in infected chickens, when the juvenile worms re-entered the intestinal lumen (6 weeks p.i.).

Stehr et al. (2018) investigated the expulsion of *A. galli* in chickens and found that the chicken is able to eliminate the majority of the *A. galli* larvae before they mature to adult worms. The expulsion is strongly associated with the developmental stage of the larvae. The expulsion targets the early stages, which is the first day of infection, where the eggs must hatch in the duodenum. Some larvae spend the first day in the lumen, whereas others embed themselves in the tissue as the first step of the histotrophic phase. This is probably the most challenging part of the larva's life, because if hatching is unsuccessful within the retention time, the infective eggs are passively transported with the digesta through peristalsis and are excreted. Stehr et al. (2018) found that only 59% of the *A. galli* larvae survived the first day. Ferdushy et al. (2013) observed that only 20% of the larvae had survived on day 3 p.i., indicating that the expulsion rate is highly elevated in the first couple of days p.i., or it might simply be a sign of a low hatching rate of the *A. galli* eggs, which is impossible to distinguish. Stehr et al. (2018) suggests that the worm expulsion also targets the larvae embedded in the tissue and the larvae in the juvenile larva stages after re-entering the intestinal lumen after the histotrophic phase. This theory is based on the observation of a changed population structure, where only few of the larvae reached final maturity and became a mature adult worm. Unfortunately, expulsion or spontaneous self-cure only happens occasionally, and the chickens are quickly re-infected. The knowledge about what triggers this mechanism in chickens is very limited. In another experiment, faecal samplings from two different chicken lines were collected several times over 81 weeks to determine the fluctuations or variations over time in faecal *A. galli* egg count (Norup et al., 2013a). Both lines experienced a depletion of faecal egg count of *A. galli* during the experiment, but also a reinfection after the depletion, which indicates that spontaneous self-cure does happen during the hen's lifespan, but it does not make the hen resistant to a new infection (Norup et al., 2013a).

3.4.5 Treatment and prevention

Treating *A. galli* infection with anthelmintics, e.g. Flubendazole or Fenbendazol is possible and a very reliable way to control worm infections on the farm. Currently, it causes no withdrawal period for eggs, but a seven days withdrawal period for meat, which is relevant in broiler production. However, as mentioned before, the treatment of organic layers with anthelmintics will in the future cause a withdrawal period, where eggs cannot be marketed as organic, which presents a considerable economic burden for the organic producers.

Vaccination could be an effective way of preventing *A. galli.* However, the different life stages of the parasite make a vaccine development very difficult. The location of the nematode in the intestinal lumen, where immune effector cells have limited access, is a further obstacle.

Preventing *A. galli* infection in layers having access to the soil or litter remains difficult.

In indoor egg production systems, it may be somewhat easier to control hygiene, and nematode infections are less common (Jansson et al., 2010). Indoor barn systems can be disinfected between flocks, which can eliminate the transmission of *A. galli* to some extent between flocks. However, it is only effective if no *A. galli* eggs are left in both the barn, but also on boots, machines or any other equipment entering the barn. There are no developed methods to disinfect the field in organic systems (Tarbiat et al., 2015). Removing the top layer of the soil in the area of the pop holes between flocks is a way of reducing the prevalence in the field, but it is not very effective, because some vegetation must be maintained according to organic requirements. Pasture rotation is an option to reduce the contamination, but the worm eggs are highly resistant in the environment and can stay infective for as long as up to 2 years (Thapa et al., 2015).

Rules for strict biosecurity within the barn can reduce the transmission of *A. galli*. Measures involve thorough cleaning, disinfection and burning of floors between flocks. Maintaining a high hygiene with clean boots and clothes, clean machines and other equipment, providing the poultry clean feed and water, keeping control with rodents and wild birds and having limited access for visitors are also effective biosecurity measures in prevention of parasite infections.

Another way of reducing the prevalence of *A. galli* infection is selective breeding for parasite resistance. Genetic factors highly influence the establishment as well as the survival of the *A. galli*

(Schou et al., 2003). Different chicken lines have different resistance towards helminths. Gauly et al. (2002) found worm burden and EPG to be significantly higher in artificially infected Lohmann LSL than in artificially infected Lohmann Brown. The degree of worm burden had a high heritability. Across the two genotypes Lohmann Brown plus and Lohmann Brown classic, the heritability of worm burden was 0.56 ± 0.16 (Wongrak et al., 2015), which makes selection for this trait highly relevant. Another study discovered a heavier worm burden as well as a higher EPG in Danish Landrace chickens compared to hens of the Lohmann Brown breed (Permin and Ranvig, 2001). The genetic resistance is both related to the amount of harboured larvae, EPG, sex ratio and the size of the worms (Schou et al., 2003).

It has been shown that feed composition influences the outcome of worm infections. The presence of water soluble non-starch polysaccharides (S-NSP) increase the digesta viscosity (Danicke et al., 1999). Supplementation of S-NSP seems to create a gastrointestinal environment, which actually benefits the helminths (Das et al., 2012, Das et al., 2011). It was shown that the worm burden of *A. galli* and the prevalence of *Heterakis gallinarium* in caeca increased. Further, elevated EPG and a larger proportion of female worms were observed, when feeding S-NSP (Das et al., 2012, Das et al., 2011). Finally, the supplementation of structured feed, e.g. roughage to hens has shown to increase the worm burden of *A. galli* (Engberg, personal communication).

It is highly relevant to investigate different feeding strategies to reduce *A. galli* infection. These strategies may involve the dietary addition of herbs, plants or fermented feed.

In organic egg production, the identification of locally produced organic protein feedstuff is very important to address the requirement of 100% organic feedstuff from 2022 (Regulation (EU) 2021/181 of the European Parliament and of the Council of 15 February 2021), which has drawn increasing attention towards fermented rapeseed as an alternative protein source.

3.5 Fermented rapeseed meal with seaweed

Lactic acid fermentation of feed or feed components like protein has received increasing interest with respect to upcycling of less favourable protein sources thus increasing nutritional value and availability of the feed. Further, possible beneficial effects on the gut health have been suggested (Engberg et al., 2009).

3.5.1 Feed composition

In this trial, the effect of solid-state fermented rapeseed meal with seaweed (EP 199, Fermentation Experts) on the course of an *A. galli* infection was investigated. The experimental diet contained 6% of the product providing fermented rapeseed meal and 1% seaweed, which was added to the experimental diet mainly at the expense of rapeseed cake in the control diet. The composition of the experimental and control diet was otherwise quite similar (Table 2).

The rapeseed meal was produced after fat extraction and drying. Solid-state fermentation of rapeseed meal and seaweed was carried out in tanks after addition of *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Pediococcus acidilactici*. The applied seaweeds were *A. nodosum* and *S. latissima.* The seaweeds were dewatered before fermentation. The fermented feedstuff was dried to stop the fermentation process. The drying process kills the implemented microorganisms. After fermentation *Enterococcus faecium* was added to the feed, which allows the product a prebiotic declaration.

Rapeseed meal is not allowed for use in the organic production, but rapeseed cake is. When used for animal feed, rapeseed is crushed to remove oil and the first by-product is rapeseed cake. Rapeseed cake can further be solvent extracted and this second by-product is the rapeseed meal.

There are no publications available about the effects of EP 199 in poultry, but effects of the dietary feed supplement have been investigated in sows and piglets (Grela et al., 2019; Satessa et al., 2020). Grela et al. (2019) found that the addition of fermented rapeseed stimulated the immune system and reduced the incidences of E. coli diarrhea and mortality in piglets. Satessa et al. (2020) had similar conclusions and suggest that fermented rapeseed may be an interesting alternative to zinc oxide in weaners.

3.5.2 Lactic acid fermentation

Fermentation converts complex substrates into simpler compounds, which improves the nutritional value of the feed. Fermented feed also has higher concentrations of organic acids and a lower pH, which is beneficial for the gastrointestinal functions and health (Engberg et al., 2009). Lactic acid and lower pH inhibits the growth of acid-sensitive bacteria, such as *Salmonella*, *E. coli* and campylobacter

in the feed (Murry et al., 2004). Based on the fermentation technology used to produce the fermented feed, the feed is divided into two categories: solid-state fermented feed and fermented liquid feed, also known as submerged fermentation. Solid-state fermented feed can be used as fermented feed additives for a specific functional feature, for instance fermented medical plants to support the immune system, or it can be used to reduce anti-nutritional factors in protein or energy sources (Xu et al., 2020). Submerged fermentation is not relevant for poultry production, because liquid feed has undesirable effects on litter quality and keeping an appropriate feed hygiene is difficult (Engberg et al., 2009).

Solid state fermentation is an anaerobic process, where microorganisms are cultivated on a solid substrate, such as rice, grains and wheat bran. Fungi, e.g. *Aspergillus* spp., *Rhizopus* spp., and bacteria like *Lactobacillus* spp. and *Pediococcus* spp. are frequently used in solid state fermentation (Renge et al., 2012).

The feed in this experiment, is based on rapeseed meal as substrate, which is fermented by *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Pediococcus acidilactic*.

Fermented protein sources have shown to increase protein digestibility while decreasing the amount of anti-nutritive factors like trypsin. According to Xue et al. (2009), the change in nutritional values of rapeseed after fermentation included lower dry matter (DM) content, which is caused by the consumption of carbohydrates by the aerobic bacteria. There was an increase in crude protein, mainly due to the change in DM content. However, a considerable increase in rapeseed peptides was observed as well as an increase in the amount of lysine, methionine and cysteine. Xue et al. (2009) reported that fermented rapeseed contained far less isothiocyanates (ITC) compared to unfermented rapeseed. ITC are glucosinolates, which are undesirable substances in animal feed, because of their anti-nutritional properties. Reduced feed intake, reduced growth, goiter, irritation in the gastrointestinal tract, anaemia and hepatic lesions are possible consequences of high rations of glucosinolates fed to livestock (Bischoff, 2016). Glucosinolates are hydrolysed to polyphenols during the fermentation process. Xue et al. (2009) found the decline to be 85% in fermented rapeseed meal compared to rapeseed meal, and Aljuobori et al. (2014) reported, 38% less glucosinloates in canola meal after fermentation. However, glucosinolates have also shown some beneficial effects. They

regulate inflammation and stress-response, and works as an antioxidant as well as having antimicrobial properties, but because of the toxicity, the amount present in livestock feed should still be limited (Bischoff, 2016).

Use of fermented rapeseed meal as dietary feed supplement has mainly been tested on broilers and pigs, especially related to performance. Xu et al. (2012) found that fermented rapeseed meal could replace soybean meal with up to 10% without affecting the performance in broilers. When the replacement was increased to 15%, the performance was affected with reduced bodyweight gain (BWG) and reduced feed conversion. Broilers offered diets where 10% or 15% of the soybean meal was replaced by fermented rapeseed meal, had a higher IgY content in serum and reduced urea N in serum. Chiang et al. (2010) compared fermented rapeseed meal with rapeseed meal fed to broilers and found increased performance, better nutrient digestibility, increased number of lactobacilli in colon and caeca and an improved intestinal morphology. Hu et al. (2016) confirmed the improved gut morphology in terms of increased villus height and further observed improved antioxidative status functions in terms of increased antioxidant capacity. Further, the latter authors report a decrease in phytate concentration after fermenting the rapeseed meal. Phytate is the storage form of phosphorus in plants, and phosphorus is less bio-available in this form. Phytate is considered an anti-nutritional factor in poultry, because of the very low phosphorus availability in poultry. Fermentation decreases the amount of phytate phosphorus and thereby improves the bioavailability of the phosphorus. The increased digestibility of phosphorus as well as a lower demand for inorganic phosphorus as a feed supplement leads to reduced phosphorus excretion in chickens (Hirabayashi et al., 1998).

3.5.3 Bioactive peptides

Bioactive peptides are organic substances, which consist of amino acids and linked together by either amide bonds or peptide bonds. Usually, they are encrypted in the structure of the precursor protein, but protein can be degraded to bioactive peptides by microbial fermentation or an enzymatic process (Sánchez and Vázquez, 2017). The composition and sequence of the amino acids determine which properties the bioactive peptides have. Bioactive peptides can for instance have antimicrobial, antioxidative, immunomodulatory or antihypertensive activity (Sánchez and Vázquez, 2017). Xue et al.

(2009) suggest that bioactive peptides from fermented rapeseed meal may improve the immune function in a tumor-bearing murine model. They reported increased macrophage phagocytosis activity and a better potential to recruit antigen-specific T cells as well as modulating the T cell mediated memory response. In general, they found increased antioxidative effects in terms of reduced free radical formation and reduced oxidative stress.

3.5.4 Effects of seaweed

Seaweed is an interesting feed additive, because of its potential health improving effects. Algae contain beneficial minerals and organic acids, for instance alginate, fucoidan and laminarin (Choi et al., 2018). Alginate converts to alginate oligomers and stimulates the cytokine secretion in the immune cells. Fucoidan has antioxidant and anticancer effects. Choi et al. (2018) also found that the performance of layers was enhanced by dietary supplementation of brown algae (0.5%) and had both significant higher egg production rate and a greater egg mass. Overland et al. (2019) investigated the health improving effects of the seaweed, *A. nodosum*, and found that the effects were primarily linked to fucoidan and laminarin in pigs. Extracts from *A. nodosum* was shown to increase growth performance of weaned piglets and reduce coliform bacteria in finishing pigs (Overland et al., 2019). Feeding pigs with 10% intact *A. nodosum* in the diet was furthermore shown to significantly decrease the prevalence of *E. coli* and anaerobic bacterial growth in the small intestine and stomach in newly weaned pigs (Dierick et al., 2009).

4. Materials and Methods

4.1 Animal material

192 Bowans brown pullets were purchased from a commercial breeder (TOPÆG Aps., Nybrovej 19, 8800 Viborg, Denmark). Each hen was tagged with a foot ring providing an individual number. At arrival 2 weeks pre infection, the hens were 18 weeks old. All hens received worm treatment via the diet with Flubendazol (Flubenol Vet) during the first 7 days to make sure none of the hens had worm infection at the beginning of the *in vivo* experiment. The pens were not cleaned or emptied after deworming to create two different infection groups, naturally infected with *A. galli* (Nat) from the litter or artificially infected (Art) with *A. galli* eggs inoculated. During the experiment 3 hens were taken out of the experiment due to poor health condition not related to the infection.

4.2 Study design

The study design consisted of 4 treatments and 8 repetitions/treatment (Table 1). The hens were distributed randomly in 32 pens with 6 hens/pen. Nat and Art hens were distributed in two different houses, and the two diets were randomly distributed within each house. Each pen was equipped with a latter and two nest boxes. Wood shavings were used as litter material. The temperature in the facilities was 21 degrees, and the light-dark cycle was 14 hours of light and 10 hours of dark.

Table 1: Study design overview

Treatment #	Feed stuff	Infection	Number of pens	Number of hens per pen
1	B - 6% fermented rapeseed meal with seaweed	Nat	8	6
2	B - 6% fermented rapeseed meal with seaweed	Art	8	6
3	A - Control	Nat	8	6
4	A - Control	Art	8	6
Total			32	192

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. 2017-15-0201-01211).

4.3 Infection material

Harvesting of *A. galli* eggs was performed in June 2020 at the Institute of Animal Science, Aarhus University, 62 days pre the infection trial. Ten naturally infected organic hens were received from a local farmer and adult worms were harvested from the gastrointestinal tract of the hens. The eggs of the worms were extracted under a microscope, and the eggs were embryonated in 0.2 M H_2SO_4 at room temperature for 30 days (Permin et al., 1997). Afterwards they were stored at 4°C until inoculation.

Five days before inoculation, the number of embryonated eggs were counted as infective eggs per ml. On the day of inoculation, a suspension of 0.2 M H_2SO_4 with an amount of 764 embryonated eggs was given orally. The Nat group was inoculated with 0.2 M H_2SO_4 without *A. galli* eggs to eliminate any effect of the suspension medium or the handling during inoculation.

4.4 Feed

The treatment group was fed diet B and the control group was fed diet A. Both diets were very similar regarding their nutritive values. In diet B, the rapeseed meal product EP 199 was added at a concentration of 6% mainly at the expense of rape seed cake in diet A. The compositions of the experimental diets used during the entire experiment are shown in Table 2. Water and feed were provided *ad libitum*.

Table 2: Feed composition of diet A and diet B. Diet A contains more rapeseed cake, than diet B to compensate for the amount of fermented rapeseed meal.

Diet	**A**	**B**
Nutrients	**Control diet**	**Fermented rapeseed meal with seaweed, 6%**
Wheat	43.93	42.95
Oat	8.00	8.00
Triticale	4.00	4.00
Sunflower cake	8.00	6.00
Soybeans (toasted)	9.00	9.00
Fish meal (standard)	3.00	3.00
Calcium carbonate	4.00	4.00
Oyster shells	4.25	4.25
Rapeseed oil	0.20	0.80
Rapeseed cake (9%)	6.52	2.93
Soybean cake (toasted)	8.00	8.00
Monocalcium phosphate	0.30	0.27
Vitamin/Mineral DLG kylling slut	0.40	0.40
Salt	0.18	0.18
Sodium bicarbonate	0,18	0,18
Choline chloride	0,04	0,04
Fermented rapeseed meal with seaweed	-	6,00
Total	**100**	**100**

4.5 Samples

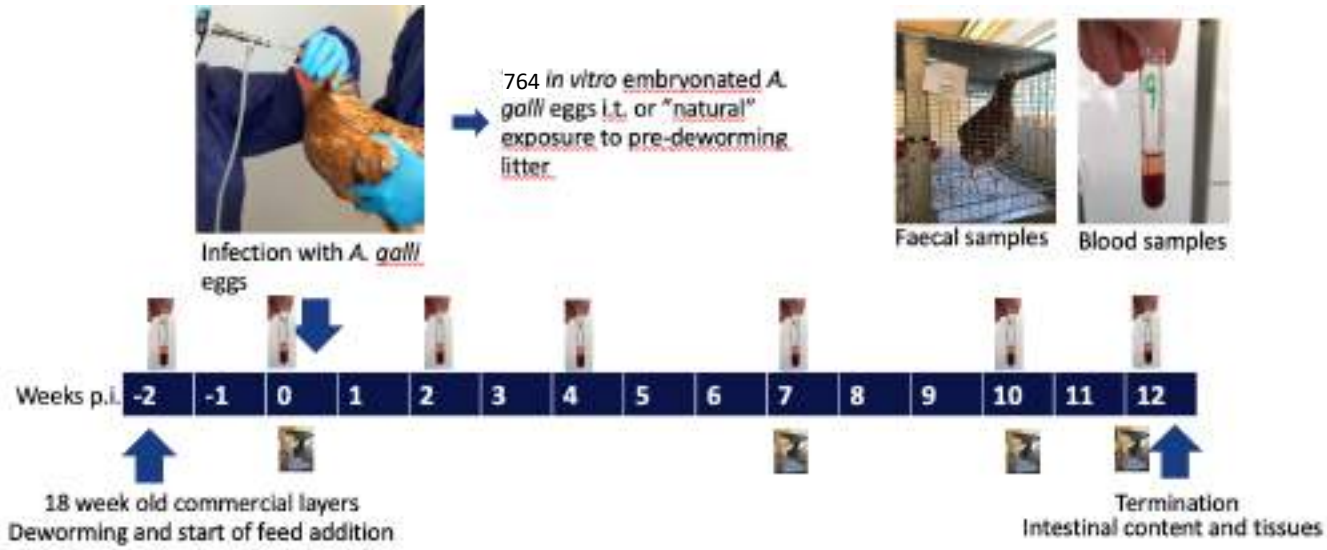

Figure 8: Sample overview for the 12 weeks of the experiment. Picture of hen in sampling cage represent faecal samples and picture of collection tube represents blood samples (MH own pictures).

4.5.1 Body weight and feed consumption

Body weight was registered at arrival (week -2 post infection (p.i.)) and on the days of slaughtering (week 12 p.i.) for each individual animal. 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 naturally infected birds were killed after 99 days, and for artificially infected birds after 100 days.

$$Daily\ BWG\ (g/day) = \frac{BW_{Week\ 12\ p.i.}(g) - BW_{Week-2\ p.i.}\ (g)}{Trial\ duration\ (days)}$$

Feed consumption was registered for each pen for the total period of 12 weeks. Daily feed intake (FI) was calculated as a mean for each pen:

$$Daily\ FI = \frac{Feed\ given\ (g) - left\ overs\ at\ the\ end\ of\ trial\ (g)}{number\ of\ hens\ in\ the\ pen}$$

4.5.2 Egg production

Eggs were collected from each pen every day and counted 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:

$$Egg\ production\ (\%) = (Total\ number\ of\ eggs/Total\ number\ of\ hens\ in\ the\ pen/7) * 100$$

4.5.3 Blood

Blood samples (Ethylenediaminetetraacetic acid (EDTA) stabilized blood for whole blood and non-stabilized blood for serum) were collected from the jugular vein of 2 hens per pen (a total of 64 samples) at weeks -2, 0, 2, 4, 7, 10 and 12 p.i. The same hens were used for blood collection at each time point, except for heparin stabilized blood collected from the jugular vein at 11 weeks p.i. for mitogen stimulation. Only one hen per pen was used for this. The EDTA stabilized blood was used for flow cytometry to enumerate different blood cell subsets. Serum was used for an enzyme-linked immune sorbent assay (ELISA) to determine *A. galli* specific IgY in blood and for serum coloration. Blood samples in serum tubes were placed at room temperature (RT) for minimum 2 hours after sampling and stored at 4 °C over night. The samples were placed at RT for 10 minutes before centrifuged at 1850 x *g* for 10 minutes at 20 °C. Serum was transferred to Eppendorf tubes and stored at -20 °C until further use.

4.5.4 Faeces

Faeces samples (4 per pen, a total of 128) were collected at week 0, 7, 10 and 12 p.i. The same four hens were used for sampling at each time point. Samples from each pen were pooled and pH in faeces was registered. Samples were furthermore used for DM analysis and count of worm eggs per gram faeces (EPG).

4.5.5 Intestinal content

Samples of content from caeca and ileum were collected when the hens were slaughtered 12 weeks p.i. The 4 hens per pen used for collection of faeces were also used for sampling of gut content. The

samples from these 4 hens per pen were pooled, pH was measured, and short-chain fatty acid (SCFA) and lactic acid concentrations were measured.

4.5.6 Intestinal tissue

At termination of the experiment, Meckel's Diverticulum (MD) and a total of 6 cm intestine at the site of MD (3 cm of jejunum and 3 cm of ileum) was cut from 32 hens (1 bird from each pen) to phenotype intra epithelial lymphocytes as well as to measure total IgM, IgY and IgA production from cultured intestinal tissue. The tissue was flushed with phosphate-buffered saline (PBS), residual fat tissue was removed, and the intestine tissue was transported in PBS (BioWhittaker® PBS without calcium and magnesium, Lonza, cat. no. 17-516F) containing 1% antibiotics (penicillin and streptomycin; cat. no. 17-602, Lonza) on ice to the lab.

4.6 Methods

4.6.1 A. galli specific IgY

A. galli specific IgY titres were determined using an ELISA method previously described (Norup et al., 2013a) in serum samples from 64 animals (2 from each pen). Briefly, *A. galli* crude extract was made of adult worms that were washed in 70% ethanol and shredded with scissors in PBS, mixed on a whirl mixer and centrifuged at 1700 x *g* for 10 minutes at 4 °C. The supernatant was filtered and centrifuged at 12,000 x *g* for 10 minutes at 4 °C, and the final supernatant was the ready for use *A. galli* crude extract. The *A. galli* crude extract was used as the coating antigen at a protein concentration of 5 µg/ml in coating buffer (0.15 M Na_2CO_3 and 0.35 M $NaHCO_3$). The coating antigen was applied to flat-bottomed 96-well microtiter plates and PBS with 0.5% bovine serum albumin (BSA, Roche, cat. no. 10735086001) was used for blocking, while PBS with 0.1% BSA was used as diluent and wash buffer. After incubation, washing, blocking, and a second step of washing, the serum samples and controls were added and incubated. A dilution series of a highly positive sample was used as standard, and the highest titre was set at the relative value 2. The standard curve is relative and does not give an actual concentration of *A. galli* specific IgY, but it gives the opportunity to compare the titres between sample animals. A high titre and a negative sample were included on all plates in

order to correct for inter-plate variation. Horseradish peroxidase conjugated polyclonal goat anti chicken IgG (IgY)-Fc antibodies (Bio-Rad, cat. no. AAI29P) was used as detection antibody and 3,5,3',5'-tetramethylbenzidine (TMB, Invitrogen, cat. no. SB02) was used for visualisation of antibody binding. The TMB reaction was stopped with 1 M H_2SO_4, and the spectrometric values was measured at A_{450} with A_{650} as a reference wavelength on an ELISA reader (Multiskan FC, ThermoFisher). For each sample, the titre was determined based on the arbitrary standard curve.

4.6.2 Dry matter

Cups used for faeces samples were weighed before sampling. Pooled faeces samples from each pen were weighed as "wet" after sampling (including weight of cup) and weighed again after freeze-drying. Dry matter (DM) was calculated as:

$$DM\% = \frac{Dry\ weight - cup\ weight}{Wet\ weight - cup\ weight} * 100$$

4.6.3 EPG and parasite burden

EPG as an indication of the parasite infection pressure (Permin et al., 1997) were determined in faeces pools at each faecal sampling time point. The presence of *A. galli* eggs in faecal samples were determined using a modified McMaster counting technique with a sensitivity of 20 EPG (Henriksen and Aagaard, 1976, Permin et al., 1997). Briefly, 2-3.5 g faeces were mixed with 14 ml tap water per gram faeces and filtered through two layers of gaze. 10 ml of the mixed sample was collected in a test tube and centrifuged at 300 x g for 5 minutes. The supernatant was removed, and flotation fluid (375 g glucose monohydrate and 250 g sodium chloride per litre distilled water) was added to the sediment, until a total volume of 4 ml was obtained. The samples were then transferred to a McMaster chamber and left for 3-5 minutes before eggs were counted under a microscope. The chamber had two sides called T1 and T2. EPG was calculated as followed:

$$EPG\ (\frac{eggs}{g} faeces) = (T1 + T2) * 20$$

Adult worms present in the intestine were counted for each hen (189 in total), when the hens were slaughtered at the termination of the experiment. Further, the worms were sex determined to calculate the sex ratio between male and female worms. The sex ratio was determined for the two hens per pen also used for blood sampling (in total 64 hens).

4.6.4 Intestinal integrity/barrier function

Serum coloration was performed in Nunclon Delta Surface 96-well plates. 100 µl serum was added to each well, and optical density (OD) was measured at wavelengths 405, 450, 470, 490, 550, 590 and 650 nm on a spectrophotometer (Multiskan Sky, Fischer Scientific) for weeks 0, 2, 4, 7, 10 and 12 p.i.

4.6.5 Mitogen stimulation

Mitogen stimulation was performed as described by Naghizadeh et al. (2021) with a few modifications. Briefly, 2 ml heparinized blood samples were taken at 11 weeks p.i. from 32 animals (1 hen from each pen). Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll density gradient centrifugation as previously described (Dalgaard et al., 2010). The PBMC concentration was adjusted to 1×10^7 cells/ml with R5 medium (RPMI 1640 (cat. no. BE12-115F/U1, Lonza) with ultra-glutamine supplemented with 5% foetal bovine serum (FBS, Gibco, cat. no. cat. no. 10082147) and 1% antibiotics (penicillin and streptomycin; Lonza, cat. no. 17-602))). The PBMCs (1×10^6 cells/well) were stimulated in 96-well plates (Nunclon Delta Surface plates, Thermoscientifi, cat. No. 163320) with two different plant lectins, Concanavalin A (ConA, Sigma, cat. no. C5275) and phorbol 12-myristat 13-acetate (PMA, Sigma, cat. no. 79346). The samples were incubated in 5 minutes on a shaker at room temperature and afterwards incubated in an incubator with 5% CO_2 and 41 °C to create an environment equivalent to the chicken body environment. The cells stimulated with PMA was incubated for 48 hours and cells stimulated with ConA plate was incubated for 72 hours. As negative controls for both stimulations, cells with medium were included.

After incubation, cells were detached with 2 mM EDTA, pelleted by centrifugation at 600 x *g* for 5 minutes before flow cytometric analysis.

4.6.6 Isolation of intraepithelial cells

Intestinal tissue from 64 animals was used for *in vitro* production of immunoglobulins and immunophenotyping by flow cytometry.

For cell immunophenotyping by flow cytometry the intestinal samples (equivalent weights) were cut longitudinally. Intra epithelial lymphocytes (IEL) was isolated from jejunum/ileum or MD. Procedure was as followed; The intestinal tissue pieces and MD was incubated in PBS containing 2 mM dithiothreitol (PBS/DTT) for 5 min. at 37°C in shake incubator (150 rpm). Afterwards, the samples were shaken before filtered twice through a metal sieve for intestine and a 100-µm cell strainer for the MD. The supernatant was discarded, and tissue was put in PBS with 2 mM EDTA. The pieces were then cut into smaller pieces before incubation for 30 minutes at 37°C in a shake incubator (150 rpm). The suspensions were poured through a 70 µm cell strainer several times until the supernatant was clear, and then the supernatant was centrifuged (300 x *g* for 10 min.) and washed once with PBS. The pellet was resuspended in R10 medium RMPI 1640, 10% FCS and 1% antibiotics (penicillin and streptomycin). The concentration of cells was adjusted to $2x10^7$, and $1x10^6$ was used for flow cytometric analysis.

4.6.7 Total IgY, IgM and IgA

For antibody production the pieces of intestine (equivalent weights) were rinsed with R10 medium (RMPI 1640, 10% FCS and 1% antibiotics (penicillin and streptomycin)) before chopped into smaller pieces. Equal amounts of tissue were placed in two T25 tissue culture flasks containing 10 ml R10 medium). One T25 flask was stored at -20°C (served as control (0 h) and the other was incubated overnight at 41°C and 5% CO2. After incubation the supernatant was stored at -20°C until further analysis. The unthawed supernatants were centrifuged at 2,000 x *g* and supernatants used for the ELISA analysis. Analyses of the total IgY, IgM and IgA concentration in the intestine was performed by sandwich ELISA using commercial kits (Bethyl Laboratories Inc, cat. no E33-103 (Chicken IgA ELISA Kit), E33-104 (Chicken IgG ELISA kit) and E33-102 (Chicken IgM ELISA kit). Instructions from manufacturer were followed.

4.6.8 Flow cytometric analyses

a) Whole blood leucocyte counts

Enumeration of different cell subsets in peripheral blood were determined using a no-lyse no-wash flow cytometric method as previously described (Kjaerup et al., 2014a, Seliger et al., 2012) with a few modifications. Fifty microliters of EDTA stabilized blood were diluted 25 times with Fluorescence-activated cell sorting (FACS) buffer (0.2% BSA, 0.2% sodium azide, 0.05% normal horse serum in PBS). Fifty microliters of this were then mixed with 50 µl of antibody master mix (Panel 1&2, Table 3) in 96-well U-bottom plates. Staining was done for 20 minutes in darkness at 4°C and immediately before acquisition 123count eBeads™ Counting Beads (Thermofisher, cat. no. 01-1234-42), diluted 1:10 in FACS buffer and 4 mM EDTA were added. Forty microliters of each sample were acquired in the flow cytometer. Absolute number of cells was calculated according to 123count eBeads™ manufactures instructions.

b) Mitogen stimulated PBMC

Mitogen-stimulated cells, pelleted by centrifugation, were resuspended in 100 µl of antibody master mix (Panel 3&4, Table 3), mixed and incubated for 15 minutes in darkness at RT. After incubation, 150 µl FACS buffer was added, and cells were centrifuged at 600 x *g* for 5 minutes. Wash was repeated twice with 200 µl FACS buffer. Before acquisition cells were resuspended in 200 µl FACS buffer. 100 µl of each sample was acquired.

c) IEL and MD

Fifty microliter of cells isolated from tissue was mixed with 50 µl of antibody master mix (Panel 5, Table 3) in 96-well U-bottom plates and incubated for 15 minutes in darkness at RT. After incubation, the plate was washed with FACS buffer and incubated for 15 minutes with 100 µl of 1:100 diluted secondary antibodies at RT. The cells were washed and resuspended in 200 µl FACS buffer. Forty microliter of each sample was acquired.

All flow cytometric analyses were conducted on a BD FACSCelesta™ flow cytometer with a flow rate of 0.5 µl per second and analyses of acquired samples were performed in the FACS Diva software.

Table 3: Monoclonal antibodies used for immunolabelling, and combinations (panels) used in the different experimental sets.

				Panels				
Abbreviation	**Clone**	**Specificity**	**Fluorochrome**	**1**	**2**	**3[f]**	**4[f]**	**5[g]**
CD41/61-FITC[b]	11C3	Chicken integrin CD41/61	Fluorescein	X				
Kul-01 PE[a]	KUL01	Chicken monocyte/macrophage	R-phycoerythrin	X				
CD45-PerCP/ Cy5.5[bd]	UM16-6	Chicken CD45	Peridinin chlorophyll-cyanine 5.5	X				
Bu-1-FITC[a]	AV20	Chicken Bu-1	Fluorescein		X		X	
TCR1-PE[a]	TCR-1	Chicken TCR$\gamma\delta$	R-phycoerythrin		X			
CD8a-Cy5[a]	3-298	Chicken CD8α	Cyanine 5		X	X		
CD4-pBlue[a]	CT-4	Chicken CD4	Pacific Blue™		X	X		X
MHC-II-PE[a]	2G11	Chicken MHC class II β-chain	R-phycoerythrin			X	X	
CD25-PerCP/Cy5.5[bd]	AV142	Chicken IL-2Rα (CD25)	Peridinin chlorophyll-cyanine 5.5			X	X	X
TCR1-FITC[a]	TCR-1	Chicken TCR$\gamma\delta$	Fluorescein			X		X
CD57-APC[c]	NK-1	CD57	Allophycocyanin				X	
Bu-1-PE[a]	AV20	Chicken Bu-1	R-phycoerythrin					X
Kul-01-A647[a]	KUL01	Chicken monocyte/macrophage	Alexa fluor® 647					X
CD8a-APC/Cy7[d]	3-298	Chicken CD8α	Allophycocyanin-cyanin 7					X
CD45-BV650[bc]	UM16-6/R19-15	Chicken CD45	BD Horizon™ BV650					X

X = used in panel
[a] Purchased from Southern Biotech (www.southernbiotech.com).
[b] Purchased from BIO-RAD AbD Serotec (www.abdserotec.com).
[c] Purchased from BD Biosciences (www.bdbiosciences.com).
[d] Fluorochrome conjugation by Lightning-Link® kits from Abcam (www.abcam.com).
[e] Use of secondary antibody
[f] Panel combined with LIVE/DEAD™ fixable Near-IR dead cell stain kit (Invitrogen, cat. no. L10119) for dead cell exclusion.
[g] Panel combined with BD Horizon™ Fixable Viability Stain 510 (BD Biosciences, cat. no. 564406) for dead cell exclusion.

Single-stained compensation controls and fluorescence minus one (FMO) negative controls were included in all experiments. Titrations of all antibodies on the different cell types were performed to determine optimal labelling conditions prior to the experiments.

4.6.9 Short chain fatty acids and lactic acid

The concentrations of SCFA and lactic acid in digesta samples were measured as previously described by Canibe et al. (2007). Briefly, 10-fold diluted intestinal digesta (10 g) with a 0.028 M sodium hydroxide solution were extracted using 0.5 mL of concentrated HCl and 2 mL of diethyl ether, centrifuged (1,000 x *g* for 10 minutes at 4°C), and derivatized with 10 µL of the N-methyl–N-t-butyldimethylsilyl-trifluoroacetamide (Sigma-Aldrich Denmark A/S) after incubation at 80°C for 20 min, and at room temperature for 48 h. Quantification of SCFA, and lactic acid , was performed on a Hewlett Packard gas chromatograph (Model 6890, Hewlett Packard, Agilent Technologies, Naerum, Denmark) configured with flame-ionization detector and a capillary column. All samples were analysed using 2-ethylbutyric acid (Sigma-Aldrich Denmark A/S, Vallensbæk Strand, Denmark) as internal standard.

4.7 Statistical analyses

For normal distributed data, statistical analyses were performed using a general linear model's procedures in R followed by pairwise T tests to assess statistically significant differences between groups, treatments or feed. Kruskal-Wallis one-way analysis of variance was employed on non-normal distributed data and glm with negative binomial distribution was employed for worm count. Correlations between variables were assessed by Spearman correlation coefficient (r), and p-values are reported. Correlations greater than |0.6| were considered as strong correlations, whereas correlations between |0.3| and |0.6| were considered as weak correlations. For all the statistical analyses, a nominal p-value ≤ 0.05 was considered statistically significant in all cases. All the statistical analyses were performed in RStudio (Team, 2016) and all the results were visualized using the ggplot2 package (Wickham, 2009) or the ggpubr package for R (Kassambara, 2017).

5. Results

5.1 Feed analysis

Both the fermented product EP199 and the final diet after cold pelleting were analysed for the presence of probiotic bacteria on plate count agar plates (non-selective medium for aerobic bacteria count) and Slanetz agar (selective for *Enterococci)*. The number of bacteria on plate count agar plates were 10^3 bacteria/g in the diet and the fermented product. Enterococci numbers were below the detection limit (10^3 bacteria/g). Hence, the feed supplement did not provide the claimed concentration of probiotic bacteria (10^6 *Enterococcus faecium* /g).

5.2 Performance

5.2.1 Body weight, feed intake and egg production

Different performance data, related to body weight, FI and egg production, was measured during the experiment (Table 4).

The daily BWG and the daily FI was measured as an average over the entire trial period from week -2 p.i. to week 12 p.i. The mean daily BWG between each group ranged from 4.17 g/day in naturally infected chickens given the control diet to 4.60 g/day in artificially infected chickens given the control diet. No significant differences were observed between the groups for the BWG.

The mean daily FI ranged from 116 g/day in the naturally infected chickens given the diet supplemented with EP199 diet to a mean of 126 g/day in artificially infected chickens given the same supplement.

Feed intake per egg produced was calculated as the total FI of the pen divided by the total number of eggs produced divided by the number of hens in the pen. The mean between the groups ranged from 143 g feed/egg in artificially infected chickens given the control diet to 152 g feed/egg in the artificially infected chickens given diet B. No significant differences between the four treatment groups were observed.

Table 4: Performance data presented as mean ± SEM. The daily BWG (g/day) and the daily FI was measured as an average over the entire trial period from week -2 p.i. to week 12 p.i.

Group	**A - Art**		**A - Nat**		**B - Art**		**B - Nat**		**p-values**		
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Infection**	**Feed**	**Infection*Feed**
Daily BWG (g/day)	4.60	0.18	4.17	0.22	4.51	0.19	4.49	0.18	0.227	0.545	0.394
Daily FI (g/day)	121	1.97	125	5.35	126	3.78	116	1.36	0.466	0.531	0.177
FI/egg (g/egg)	143	2.58	148	5.43	152	7.56	147	8.58	0.954	0.610	0.823

Eggs from each pen were collected daily, and egg production was calculated as means per pen per week (Figure 9).

The egg production in percentage increased during weeks 0-3 weeks p.i. followed by a relatively stable egg production at 4-12 weeks p.i. (Figure 9A). The artificially infected hens had a significant higher egg production rate than naturally infected hens at week 1 p.i. (p=0.041) (Figure 9B) and at week 2 p.i. (p=0.042) (Figure 9C). There was no significant difference between the two diets.

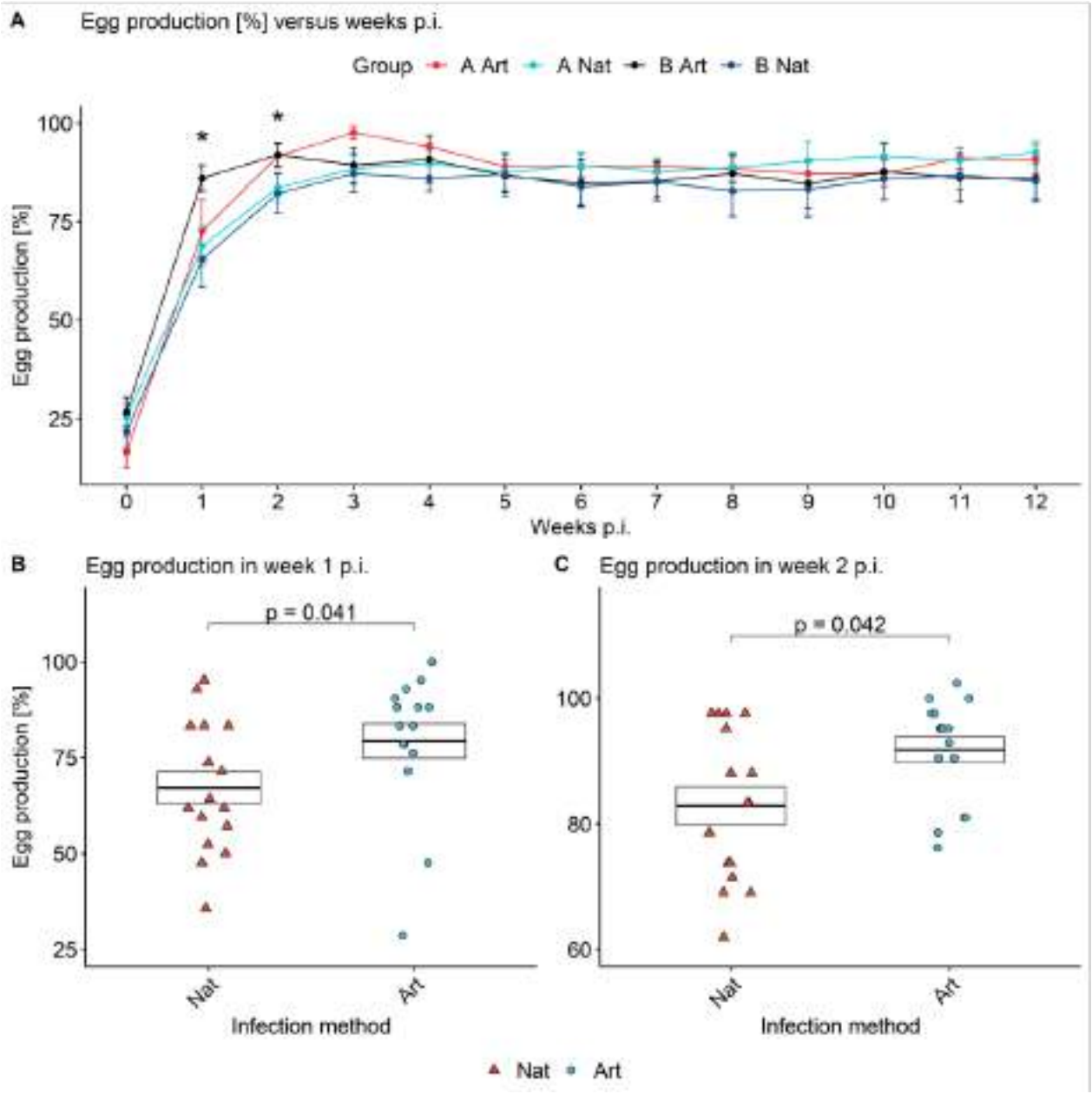

*Figure 9: A) Egg production (%) for each treatment group over the 12 weeks p.i. At week 0 p.i., the hens were 20 weeks old. Significant differences between groups are marked with *, p<0.05. The egg production (%) is calculated as a mean of each pen every week. B) Egg production (%) at week 1 p.i. (hens are 21 weeks of age) in relation to infection method. C) Egg production (%) at week 2 p.i. (hens are 22 weeks of age) in relation to infection method. Results in B) and C) are shown as mean ± SEM. P-values are indicated.*

5.3 Gut health

5.3.1 Faecal pH and dry matter content

Faeces was collected four times during the experimental period and pH and DM was measured (Figure 10).

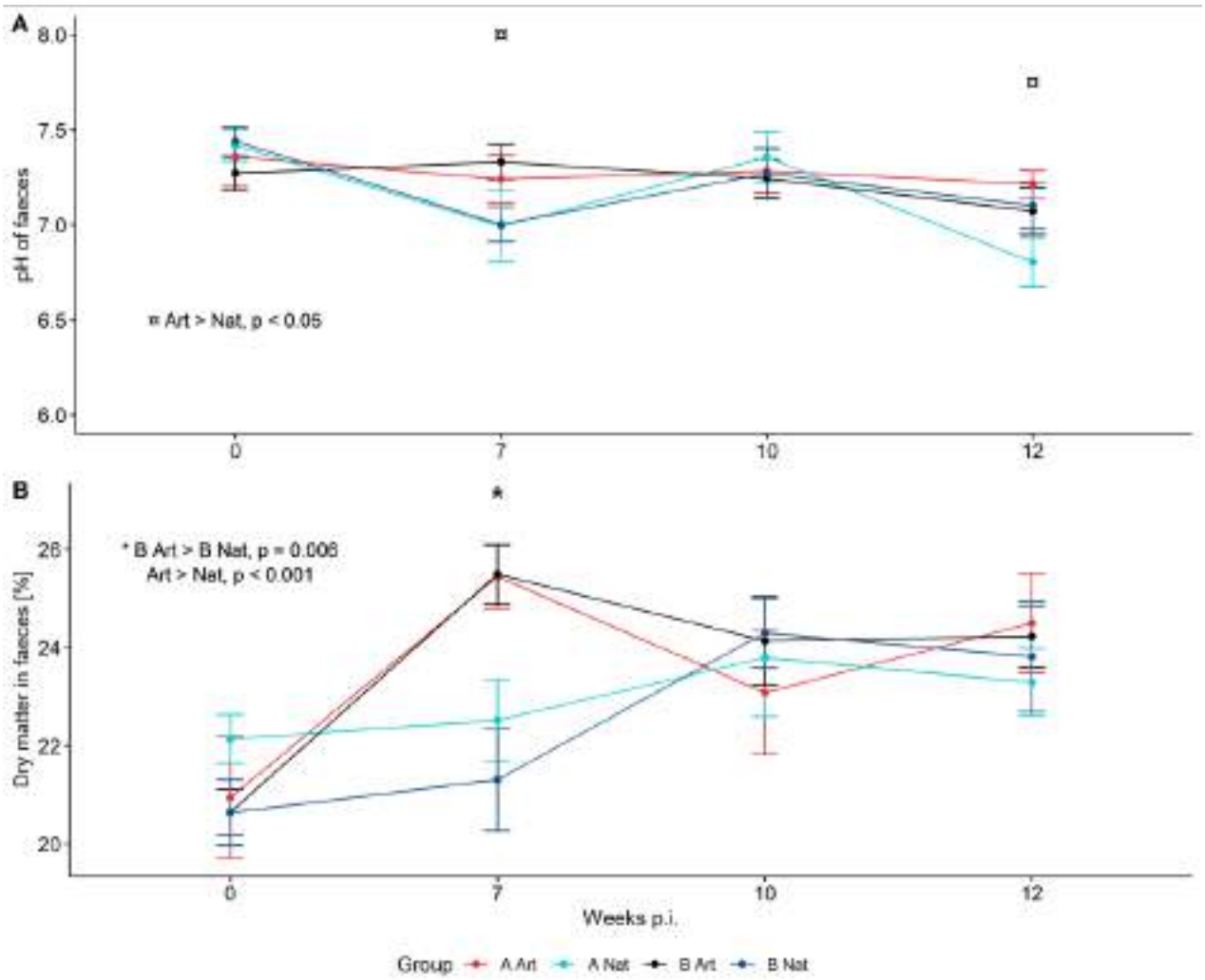

*Figure 10: A) pH of faeces at week 0, 7, 10, 12 p.i. * = significant differences (p<0.05). B) DM content (%) of faeces at week 0, 7, 10 and 12 p.i. Four samples of faeces per pen were pooled, and results are shown as means ± SEM.*

There was a significant difference in faecal pH between infection methods at weeks 7 and 12 p.i. ($p<0.05$), where artificially infected hens had a higher faecal pH compared to naturally infected hens

(Figure 10A). The artificially infected hens also had a higher DM content of faeces 7 weeks p.i. compared to the naturally infected hens ($p<0.001$) (Figure 10B). No significant differences in pH or DM were observed between the two diets ($p>0.05$).

5.3.2 Ileal and caecal pH, short chain fatty acids and lactic acid

A large difference between ileum and caeca was found with respect to the composition of organic acids (Table 5). In ileal content, lactic acid was the dominant, followed by acetic acid, formic acid and succinic acid, which was found only in very low concentrations. In the caeca, acetic acid was found in highest concentrations. Further propionic acid and butyric acid were highly abundant in caecal content, formic acid was only detected in caecal content of very few birds. Branched chained SCFA, i.e. Isobutyric and isovaleric acid, as well as succinic acid were measured in smaller amounts. In contrast to ileal content, no lactic acid was measured in caecal content.

The pH of ileal content was approximately one unit higher compared to that of caecal content (5.87 *vs.* 6.83, respectively). A significant effect of the mode of infection or the diet on ileal and caecal pH was not detected ($P>0.05$).

Naturally infected hens had slightly lower concentration of acetic acid ($p=0.05$) and slightly higher concentrations of succinic acid in ileal contents (0.66 ± 0.06 mmol *vs.* 0.48 ± 0.06, respectively, $p= 0.04$) (Table 6). No effect of feed on the concentration of SCFA and lactic acid was found in the ileum ($p>0.05$).

Table 5: Digesta pH and content of short chain fatty acids and lactic acid (mmol/kg digesta).

	Treatment groups								p-value		
	A - Art	SEM	A - Nat	SEM	B – Art	SEM	B - Nat	SEM	Infection	Feed	Infection * Feed
Ileum											
Formic acid	1.63	0.25	1.48	0.36	2.18	0.31	1.54	0.34	0.21	0.35	0.39
Acetic acid	3.84	0.30	3.14	0.27	3.72	0.30	3.21	0.38	0.05	0.94	0.29
DL-Lactic acid	13.67	1.59	18.87	3.17	15.91	2.26	19.14	2.85	0.21	0.47	0.53
Succinic acid	0.47	0.08	0.64	0.11	0.48	0.09	0.68	0.08	0.04	0.08	0.79
pH	6.73	0.12	6.87	0.07	6.86	0.11	6.85	0.07	0.48	0.58	0.71
Caeca											
Acetic acid	70.63	2.46	71.39	3.68	79.12	2.43	80.49	5.38	0.79	0.02	0.15
Propionic acid	30.01[b]	1.37	30.11[b]	1.05	32.68[ab]	1.63	37.29[a]	2.05	0.20	0.01	0.01
Isobutyric acid	1.57	0.15	1.13	0.14	1.38	0.10	1.18	0.15	0.03	0.64	0.13
n-Butyric acid	13.96[b]	1.05	15.41[ab]	0.75	16.03[ab]	1.16	18.58[a]	0.91	0.07	0.02	0.02
Isovaleric acid	0.86	0.09	0.61	0.08	0.80	0.08	0.65	0.09	0.03	0.81	0.20
n-Valeric acid	1.82	0.14	1.59	0.13	1.87	0.08	1.78	0.16	0.21	0.37	0.45
Succinic acid	0.91	0.14	0.71	0.12	0.76	0.13	0.61	0.05	0.14	0.32	0.36
pH	5.88	0.08	6.05	0.14	5.67	0.05	5.89	0.12	0.13	0.07	0.12

[ab] Different letters within a row indicates statistically significant difference

In caecal content, significantly higher concentrations (mmol /kg digesta) of acetic acid (p=0.02) and propionic acid (p=0.01) were found where found in hens receiving the feed supplemented with the fermented product (79.81 ± 2.86 mmol *vs*. 71.01 ± 2.14 mmol and 30.06 ± 0.83 mmol *vs.* 34.99 ± 1.40 mmol, respectively). Likewise, the concentrations of butyrate were higher in caecal contents of hens fed the supplement (14.69 ± 0.65 mmol/kg *vs*. 17.31 ± 0.79 mmol/kg, p=0.02). There, was a significant interaction (p=0.02) between mode of infection and feed, naturally infected hens fed the supplemented diet had the highest concentrations of propionic and butyric acid in caecal contents. Artificially infected hens had higher concentrations of isobutyric acid (1.47 ± 0.09 mmol *vs.* 1.17 ± 0.10 mmol, p=0.03) and isovaleric acid (0.83 ± 0.06 mmol *vs*. 0.63 ± 0.06 mmol, p=0.03) than naturally infected hens in the contents of the caeca (Table 5).

5.3.3 Intestinal integrity/barrier function

Serum was collected six times during the experiment and OD was used as a measure of serum coloration (Figure 12).

Hens receiving the control diet (diet A), showed a higher OD at week 2 p.i. for artificially infected hens compared to naturally infected hens at wavelengths 450, 470 and 550 nm (Figure 11B). If only looking at artificially infected hens at week 2 p.i., there was also an effect between the different diets; the hens fed diet A had a higher OD than the hens fed diet B at wavelengths 405, 450, 470 and 550 nm (Figure 11B). At week 4 p.i. the hens fed diet A had a higher OD than the hens fed diet B at 405 nm (Figure 11C). At week 7 p.i., artificially infected hens had a higher OD than the naturally infected hens at wavelength 490 nm (Figure 11D).

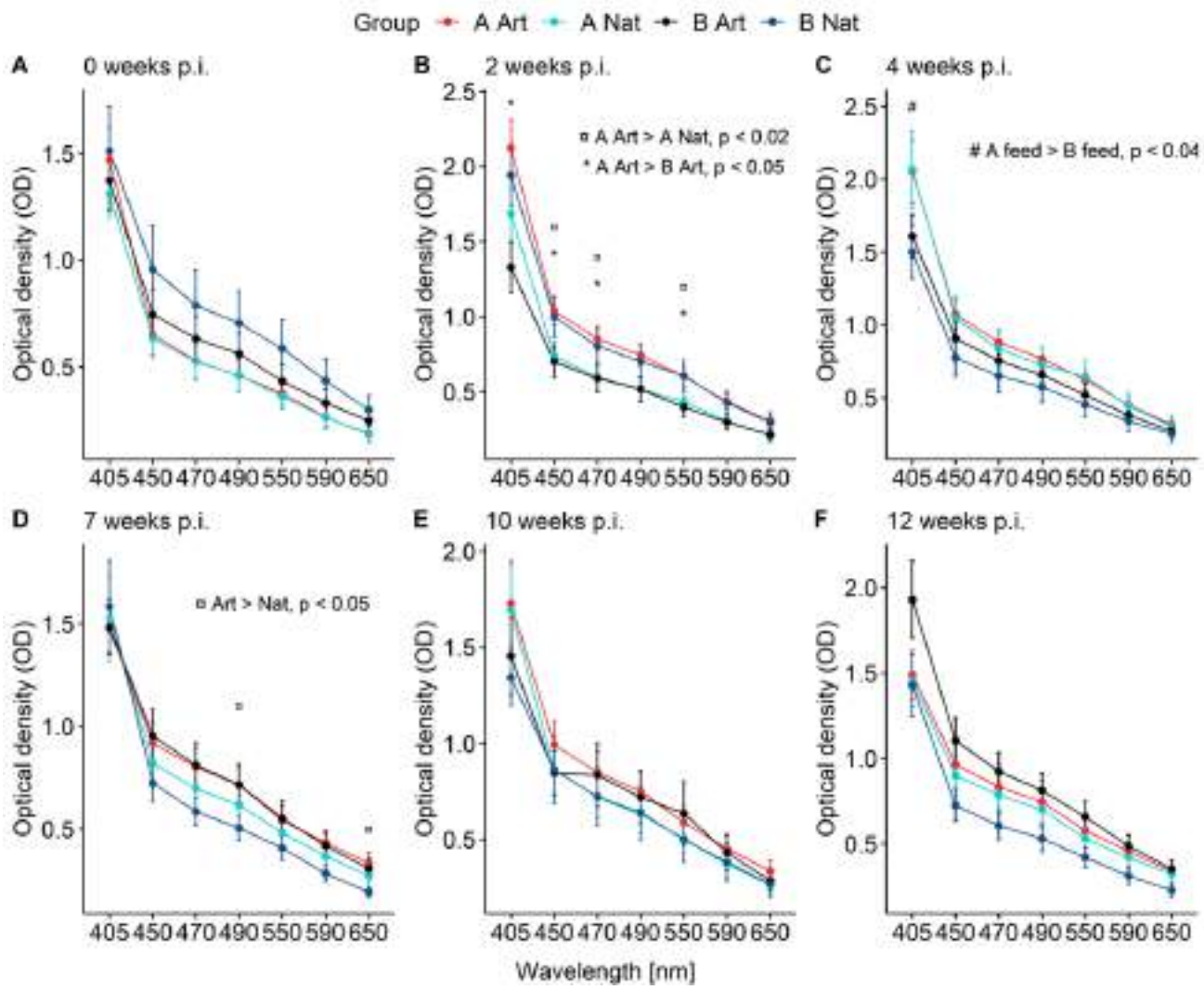

Figure 11: Serum coloration, wavelength (nm) versus optical density (OD). A-E represents different weeks p.i. Results are shown as means ± SEM, n=64 chickens (2 hens per pen).

5.4 *A. galli* burden

5.4.1 EPG

During the experiment the burden of *A. galli* infection was assessed four times by counting *A. galli* eggs in faeces (EPG).

Table 6: EPG for the four groups in the four different sampling weeks. Results presented as mean ± SEM, and p-values are indicated.

Group	A - Art		A - Nat		B - Art		B - Nat		p-values		
EPG	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Infection**	**Feed**	**Infection*Feed**
Week 0 p.i.	0	0	0	0	0	0	0	0	-	-	-
Week 7 p.i.	452.5	296.6	362.5	83.9	412.5	145.8	207.5	49.1	0.713	0.908	0.519
Week 10 p.i.	405	162.4	205	31.3	505	112.6	410	167.7	0.253	0.225	0.413
Week 12 p.i.	420	73.1	262.5	50.8	445	172.1	295	75.4	0.133	0.740	0.500

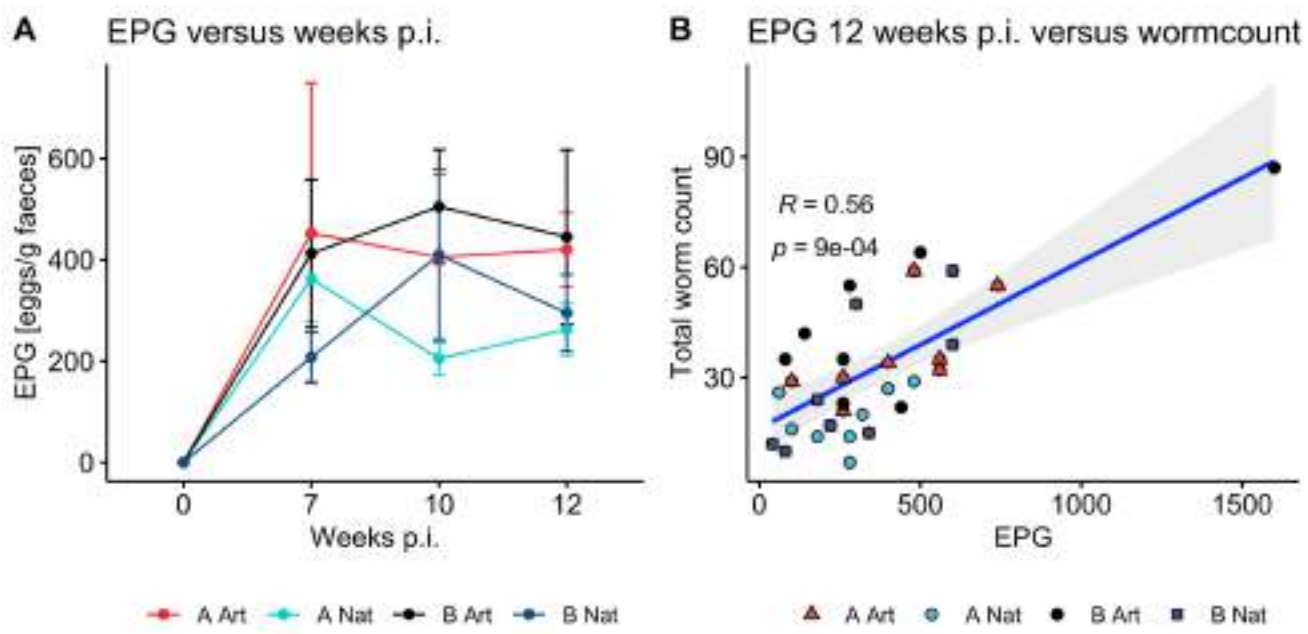

Figure 12 A) A. galli EPG at weeks 0, 7, 10, 12 p.i. for the four different treatment groups. Results presented as mean ± SEM. B) Correlation between EPG and the total worm count per pen at week 12 p.i. presented with spearman correlation coefficient (r) and p-value.

In general, there was a large variation in EPG within each group (Table 6). There was no significant difference between the treatment groups in any of the weeks, but it appears that the different diets have different kinetics over the trial period. Feed A appears to peak at week 7 p.i., whereas feed B peaks at week 10 p.i. (Figure 12A).

EPG was calculated based on a pooled sample from four animals in each pen and the total worm burden (see later, Figure 14) in this case is only related to the four animals used to determine EPG from each pen (Figure 13B). The correlation between EPG and total worm burden at slaughter at week 12 p.i. (Figure 12B) is a moderate positive correlation ($r=0.56$) and it is found to be significant ($p<0.05$).

5.4.2 A. galli specific IgY

A. galli specific IgY was determined in serum from 64 hens at weeks 0, 2, 4, 7, 10 and 12 p.i. (Figure 13A). There was no significant correlation between the *A. galli* specific IgY serum titre and the worm burden observed in the same 64 hens at week 12 p.i. (Figure 13B).

The artificially infected hens had a significantly higher serum titre of *A. galli* specific IgY compared to naturally infected hens ($p=0.003$) (Figure 13C). Diet A, the control diet, had significantly higher *A. galli* specific IgY serum titre at week 10 and 12 p.i. compared to diet B ($p=0.002$ and $p=0.025$, respectively) (Figure 13D and 13E).

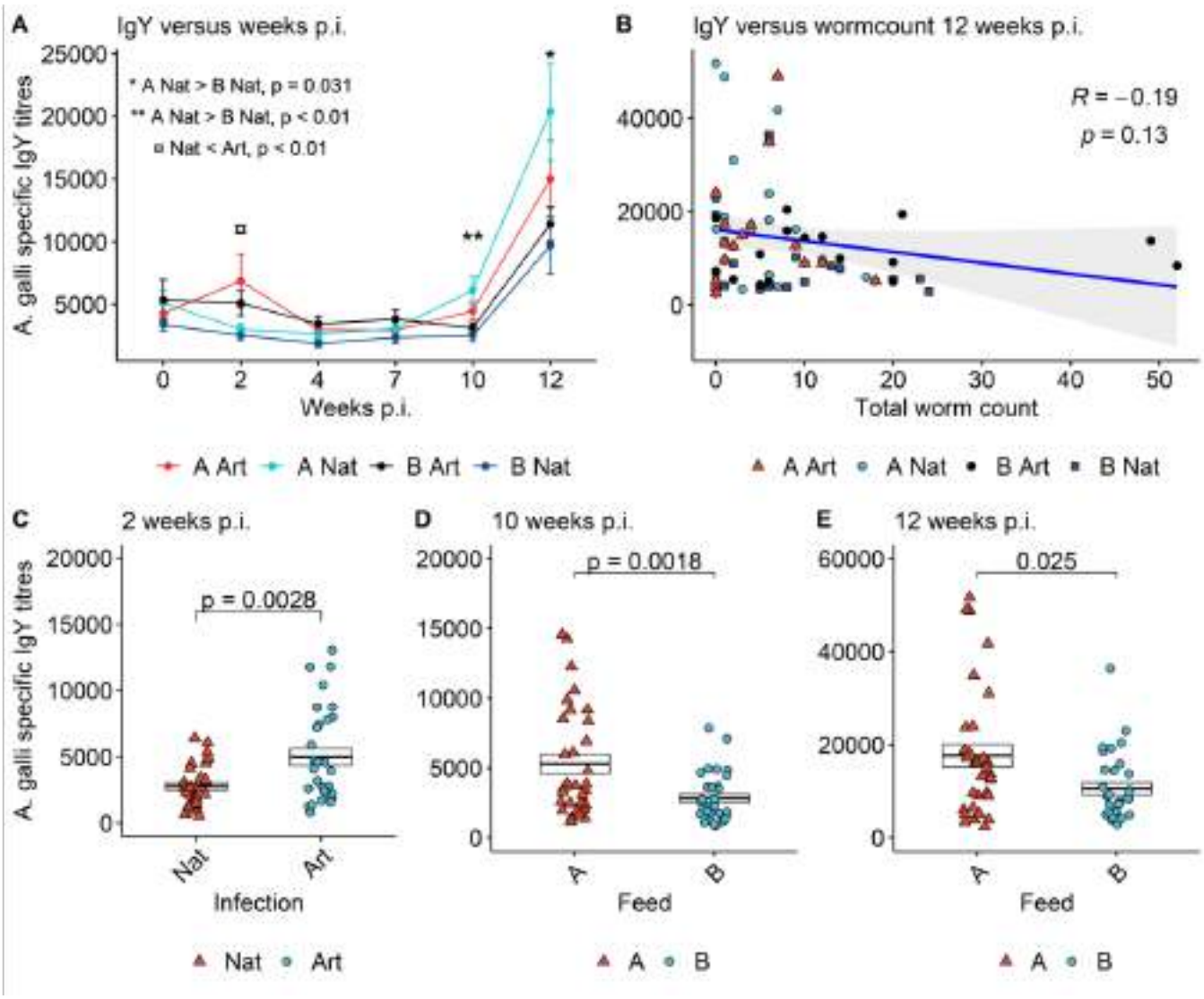

*Figure 13: A. galli specific IgY titre in serum A) Titre at week 0, 2, 4, 7, 10 and 12 p.i. Results are shown as means ± SEM. Significant differences is marked with symbols, *, **, ¤. Explanation of symbols are shown on graph. B) Correlation between titre and worm burden at week 12 p.i. presented with spearman correlation coefficient (r) and p-value. C) Titre in relation to natural and artificial infection at week 2 pi. D) Titre in relation to the two diets at week 10 p.i. E) Titre in relation to the two diets at week 12 p.i. Results in C), D) and E) are shown as mean ± SEM. P-values are indicated.*

5.4.3 *Worm burden, sex-ratio and co-infections*

The worm burden was assessed at slaughter at week 12 p.i. for the four experimental groups (n = 189 animals) (Figure 14). The majority of the worms were collected from the jejunum, but a small amount was found in the ileum.

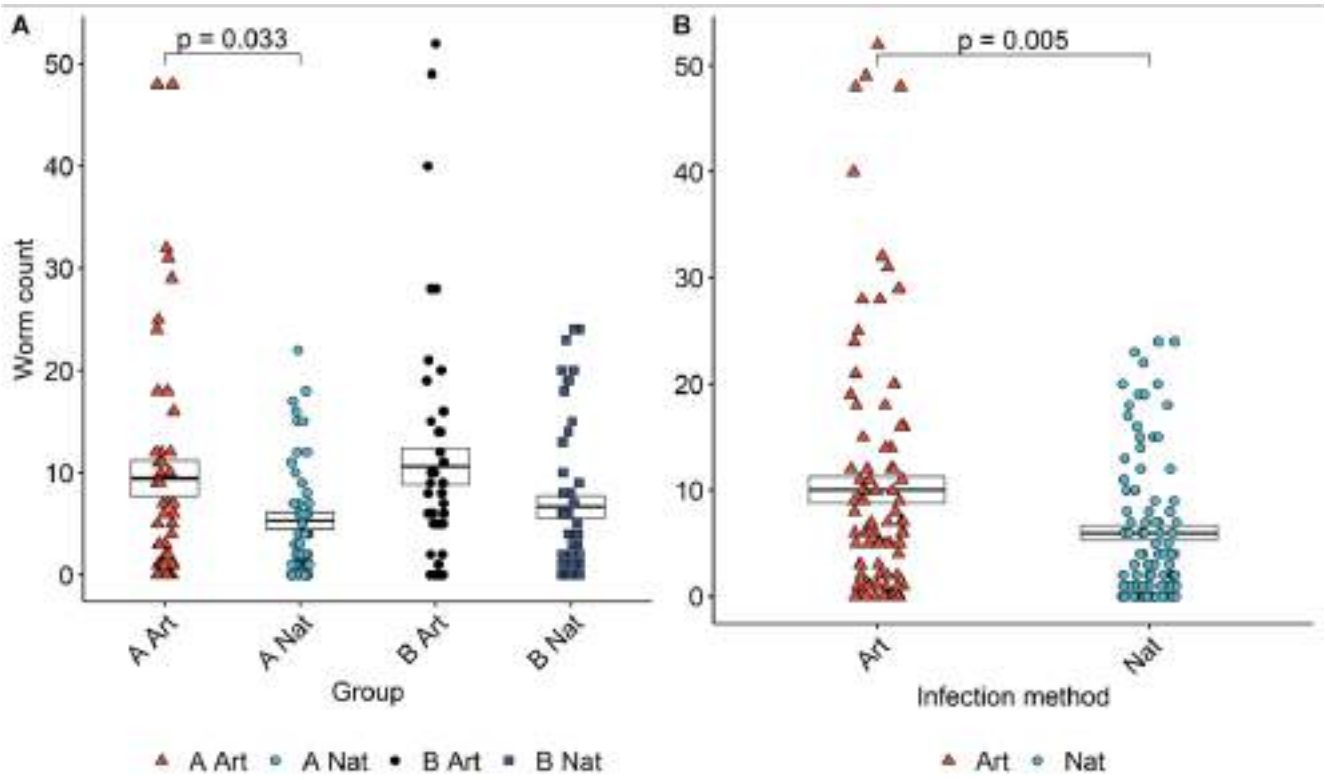

Figure 14: A) A. galli worm count at week 12 p.i. Results are shown as individual worm burdens and as mean ± SEM. P-values are indicated. B) A. galli worm count at week 12 p.i. in relation to infection methods. Results presented as mean ± SEM. P-values are indicated.

The mean of the worm burden in the treatment group, B Art, was the highest followed by A Art, B Nat and at last A Nat, which had the lowest worm burden mean (Figure 14A). Considerable variation within each group and even within each pen was observed. However, there were significant differences between artificially and naturally infected hens (Figure 14B).

Furthermore, the sex ratio of the worms was determined in the worm burden from each of the 64 birds that were also used for blood sampling (Table 7). The percentage of female worms had a large

variation between chickens, ranging from 0-100%. Artificially infected hens had slightly lower mean female worm burden than the naturally infected hens, but no differences were significant (Table 7).

Table 7: Female worm burden presented as mean ± SEM with p-values. [ab] Different letters within a row indicates statistically significant difference.

Group	**A - Art**		**A - Nat**		**B - Art**		**B - Nat**		**p-values**		
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Infection**	**Feed**	**Infection*feed**
Female worm burden (%)	43.8	9.49	52.6	8.34	44.8	5.53	53.7	7.57	0.257	0.894	0.733

Co-infection with other parasites was observed at slaughter. When EPG was counted under the microscope, the pens where *Capillaria spp.* eggs were present in faecal samples were marked. Over the entire experimental period, *Capillaria ssp.* were observed at some point in 62.5% of the pens belonging to group B Art, 100% of the A Art pens, 87.5% of the A Nat pens and 100% of the B Nat pens.

At slaughter, *Heterarkis spp*. were observed in caeca from several chickens, but no counting attempted, however the number of tapeworms in the intestine was counted. Tapeworms were found in 12.5% of the A Nat hens, in 17% of the A Art hens, in 16.7% of the B Nat hens and in 28.3% of the B Art hens. Results were not treated statistically. See appendix E for table with overview of observed co-infections.

5.5 Immunocompetence

5.5.1 Absolute counts of blood cell subsets in peripheral blood

An absolute count flow cytometric protocol was used for quantifying blood cell subsets in peripheral blood during the experimental period (Figure 15).

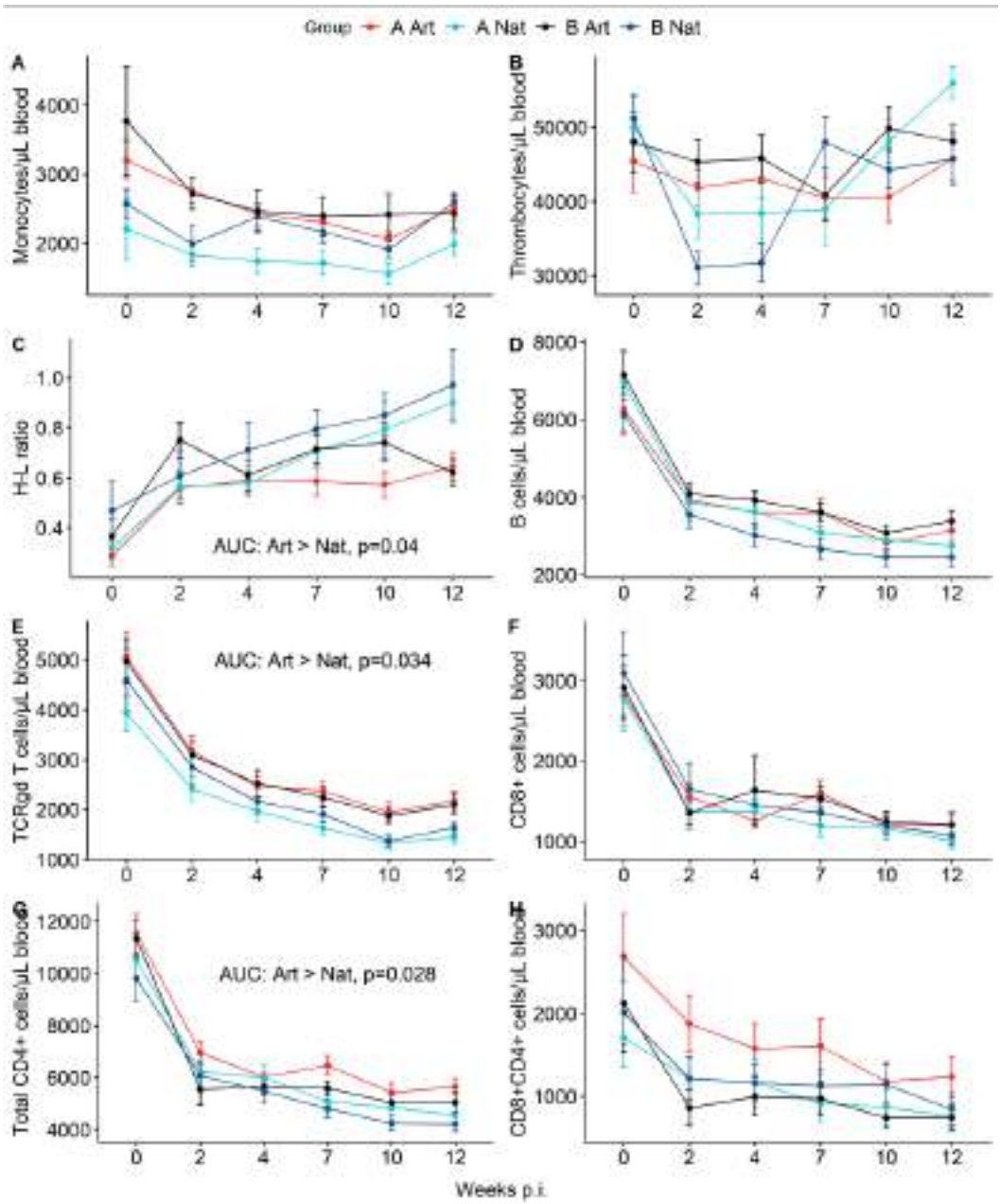

Figure 15: Blood cell subsets in peripheral blood during the experimental period. Gating of blood cell subsets were performed as shown in Appendix A. Results are shown as mean values ± SEM, n= 3-4 chickens for all groups. Statistical analyses were performed on area under curve (AUC). A) Absolute counts of monocytes. B) Absolute counts of thrombocytes. C) Heterophil-lymphocyte (H/L) ratio. D) Absolute counts of B cells. E) Absolute counts of $\gamma\delta$ T cells. F) Absolute counts of CD8+ T cells. G) Absolute counts of Total CD4+ T cells. H) Absolute counts of CD8+CD4+ (DP) T cells.

The area under curve (AUC) is the definite integral of a curve, which can help describe the variation in different concentrations of blood cell subsets over time. The limitation of AUC is that significant differences between groups is in relation to the concentration over the entire defined time period, hence significant differences between groups in a specific week is not necessarily discovered. A large AUC corresponds to a high concentration of the specific blood cell subset during the experiment, and a small AUC corresponds to a low concentration of the specific blood cell subset. No significant differences between the AUC for the four groups ($p>0.05$) was observed for monocytes thrombocytes, B cells, CD8+ T cells or CD8+CD4+ T cells (DP). For the heterophile/lymphocyte ratio (H/L-ratio), $\gamma\delta$ T cells and CD4+ T cells, a significant difference was observed between the AUC for the two different infection methods. The AUC was significantly higher in the artificially infected birds compared to the naturally infected birds ($p=0.04$, $p=0.034$ and $p=0.028$ for H/L-ratio, $\gamma\delta$ T cells and CD4+, respectively). The number of monocytes seemed to decrease from week 0 to week 2 p.i. for all four groups and appeared relatively stable during the rest of the experiment (Figure 15A). The number of thrombocytes in the naturally infected hens seemed to decrease during weeks 2 and 4 p.i. (Figure 15B). The heterophil/lymphocyte ratio seemed to increase during the experimental period (Figure 15C). The number of B cells dropped from week 0 to week 2 p.i. and then decreased continuously during the rest of the experiment (Figure 15D). The same pattern is observed for the numbers of $\gamma\delta$ T cells, CD8+ T cells, CD4+ T cells and DP T cells (Figure 15E, Figure 15F, Figure 15G and Figure 15H, respectively).

5.5.2 In vitro antibody production of intestinal lymphocytes

At slaughter, intestinal tissue was removed from the chickens and *in vitro* antibody production from the intestinal B cells was measured after 0 and 16 hours of incubation (Figure 16).

For *in vitro* produced IgY, significant opposite effects of the infection methods between the two different diets were observed. For diet A, the naturally infected group had the highest change in IgY concentration, whereas for diet B, the artificially infected group had the highest change in IgY concentration (Figure 16A). The results indicate an interaction between diet and infection method.

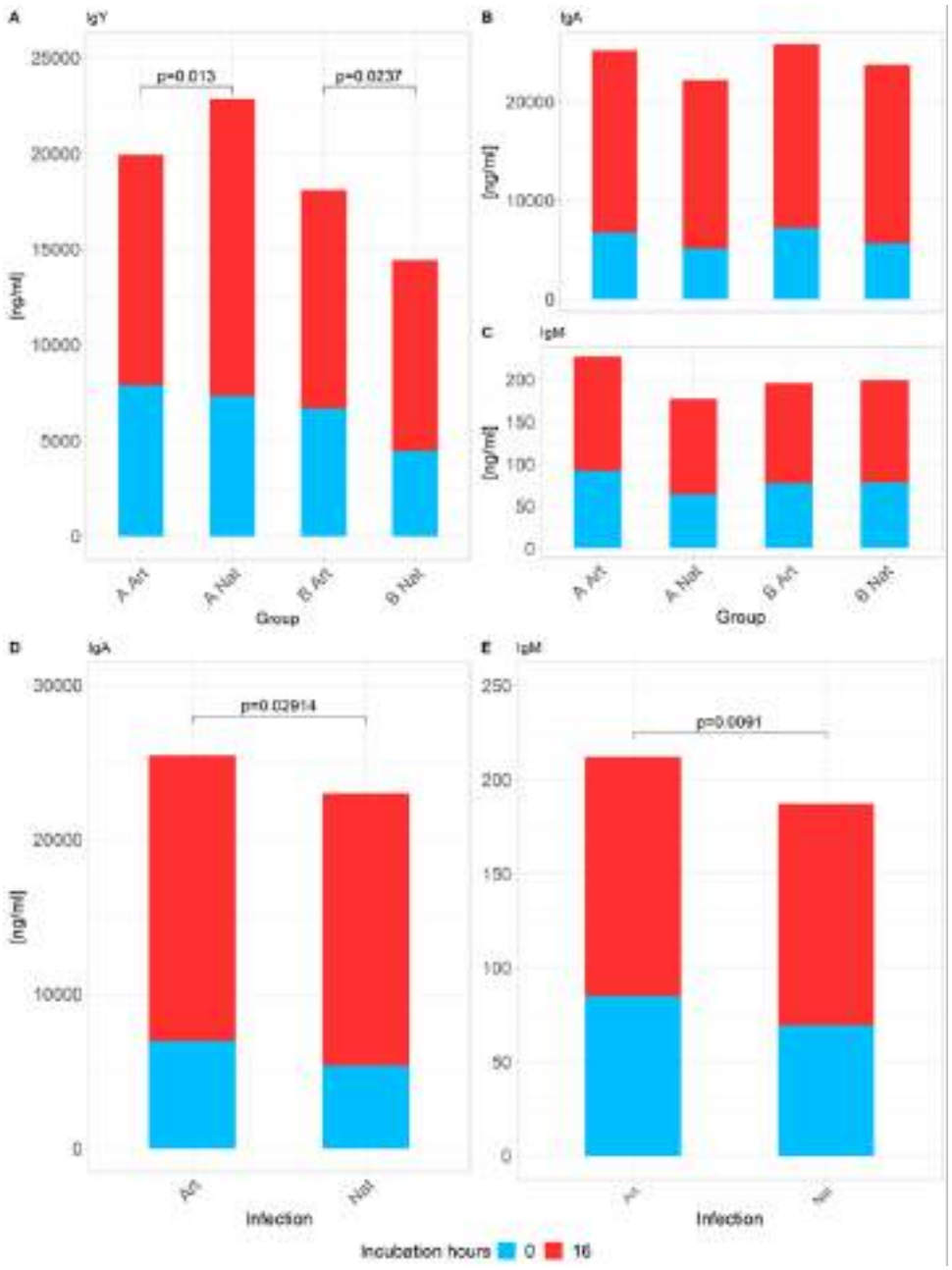

Figure 16: In vitro antibody production from intestinal lymphocytes. Results are shown as means and statistical analyses performed on fold change from 0 (blue bars) to 16 hours (red bars) of incubation. P-values are presented when relevant. A) IgY for the four groups. B) IgA for the four groups. C) IgM for the four groups. D) IgA production potential in relation to the two infection methods. E) IgM production potential in relation to the two infection methods.

No significant differences were observed between the groups for *in vitro* produced IgA or IgM (Figure 16B and 16C). However, for both IgA and IgM, an effect of infection method was observed (Figure 16D and 16E). The change in Ig concentration was highest for the hens, who were artificially infected ($p=0.029$ and $p=0.009$ for IgA and IgM, respectively).

5.5.3 Mitogen stimulation of peripheral blood mononuclear cells

To assess if the different diets and infection methods affected the immunocompetence of the chickens, peripheral blood mononuclear cells (PBMCs) were isolated from blood at week 11 p.i. and stimulated with ConA or PMA. The cells were analysed by flow cytometry 48 (PMA) or 72 (ConA) hours after incubation with the mitogen. A heatmap was made to visualize the differences between stimulated and non-stimulated cells for selected markers and proliferating cells (blasts) (Figure 17). Results were analysed by principal component analysis (PCA) (Figure 18) and no significant differences between groups were observed.

For the PBMCs stimulated with ConA, it was observed that there was a larger variation in the data after stimulation compared to the PBMCs receiving medium only. This was not observed to the same extent for the PBMCs stimulated with PMA (Figure 18). There were no significant differences in relation to feed or infection method.

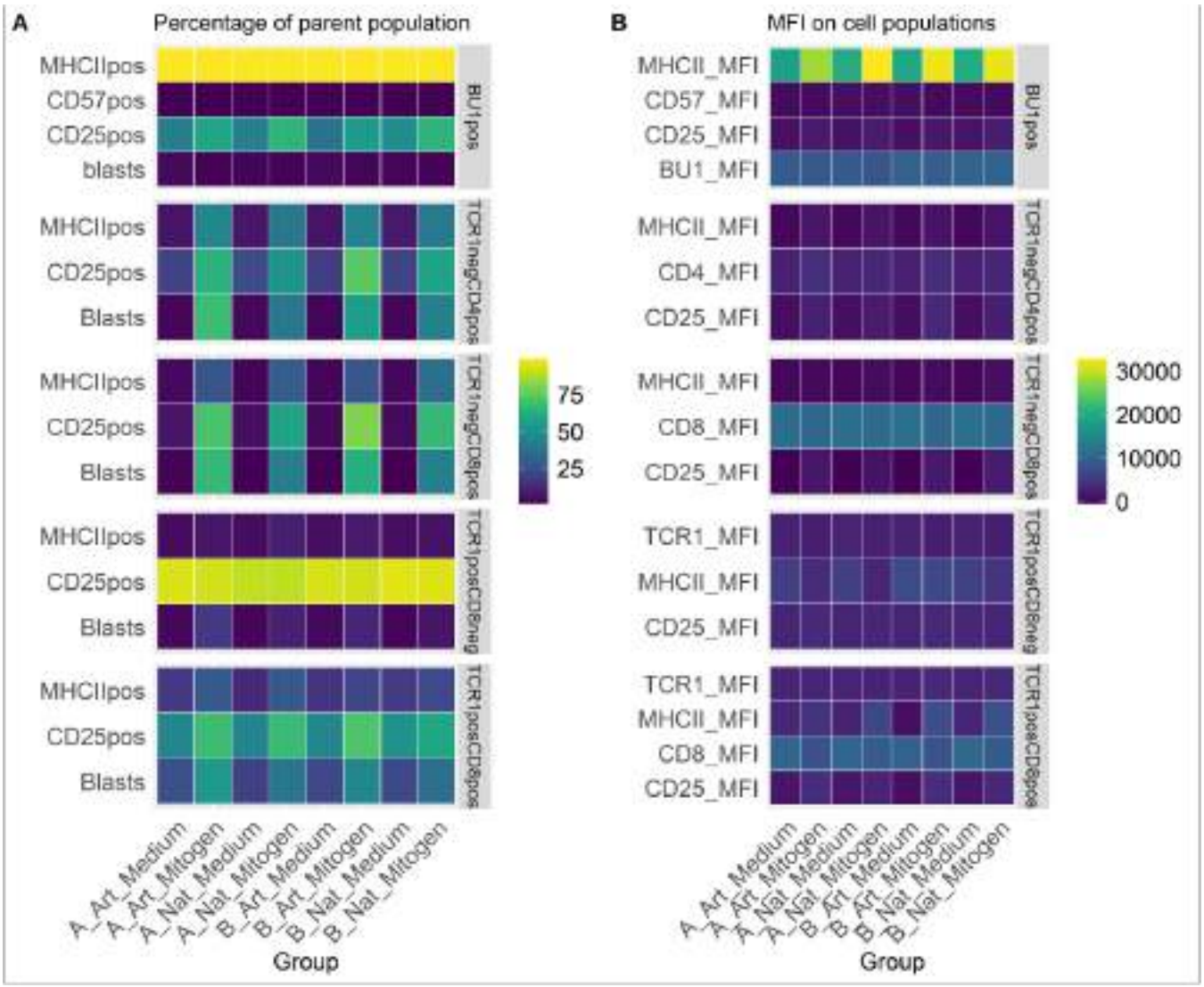

Figure 17: Heat map of blasts and selected markers obtained from flow cytometric analysis of mitogen stimulated PBMCs. A) Frequency of subpopulations for the four groups receiving medium or mitogen blasting or positive for activation markers. Colours vary from dark blue (equivalent to 0% of the parent population being blasts or expressing the marker) to yellow (equivalent to 100% of the parent population being blasts or expressing the marker). B) Mean Fluorescent Intensity (MFI) of selected markers on indicated cell populations. Colours vary from dark blue (equivalent to an MFI of 0) to yellow (equivalent to an MFI of 30,000).

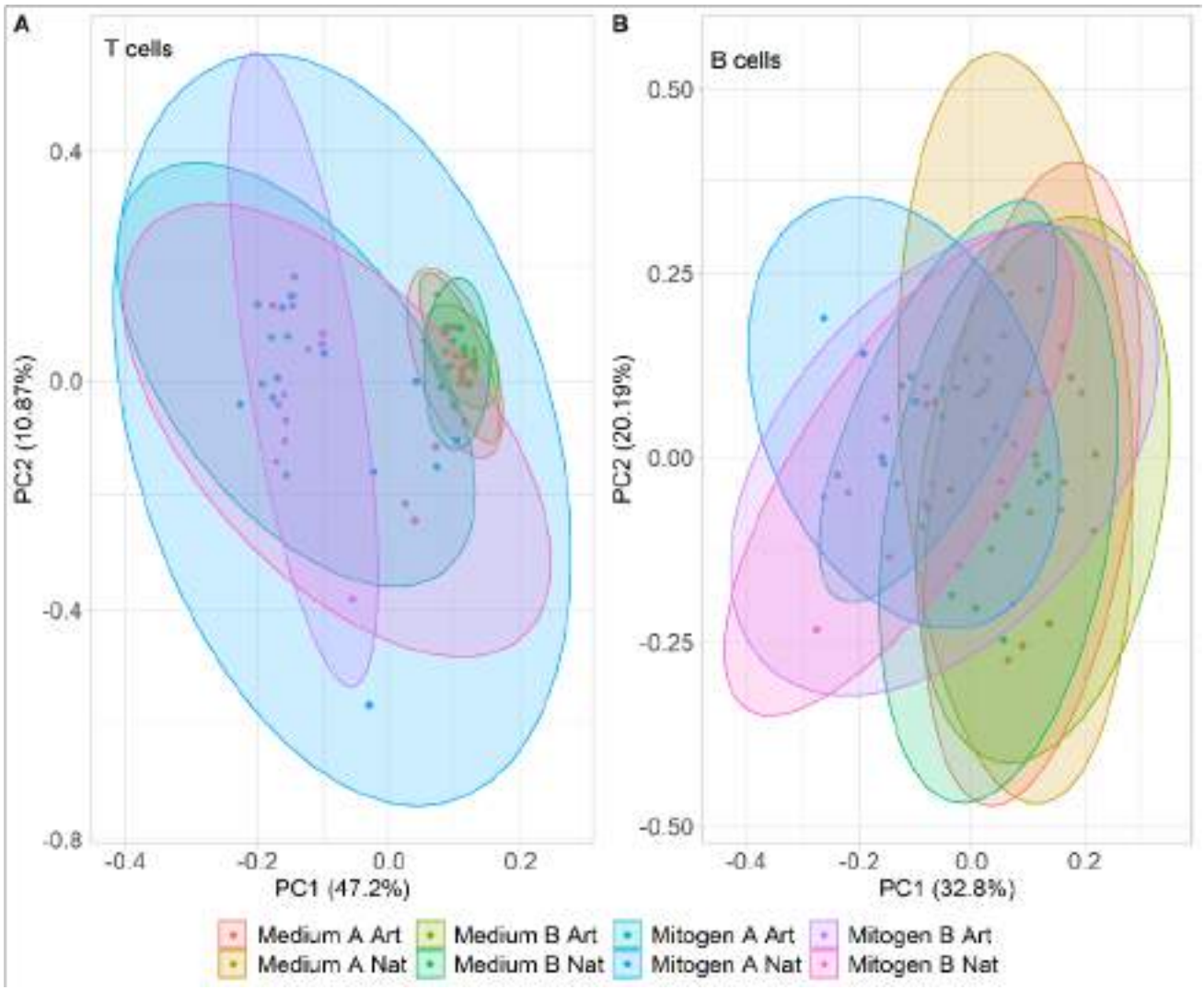

Figure 18: Principal component analysis (PCA) plots illustrating the multivariate variation among data obtained by flowcytometric analysis of mitogen stimulated PBMCs at week 11 p.i. PC1, principal 1, PC2, principal 2. A) PCA plot of multivariate variation for T cell markers (panel 3) 72 hours after ConA stimulation. Staining and gating of flow cytometric analysis of ConA stimulated PBMCs was performed (Appendix B). B) PCA plot of multivariate variation for B cell markers (panel 4) 48 hours after PMA stimulation. Staining and gating flow cytometric analysis of PMA stimulated PBMCs was performed (Appendix C).

5.5.4 *Immunoprofiling of intestinal tissue*

Lymphocytes were isolated from intestinal tissues sampled from hens slaughtered at week 12 p.i. and analysed by flow cytometry (Figure 19).

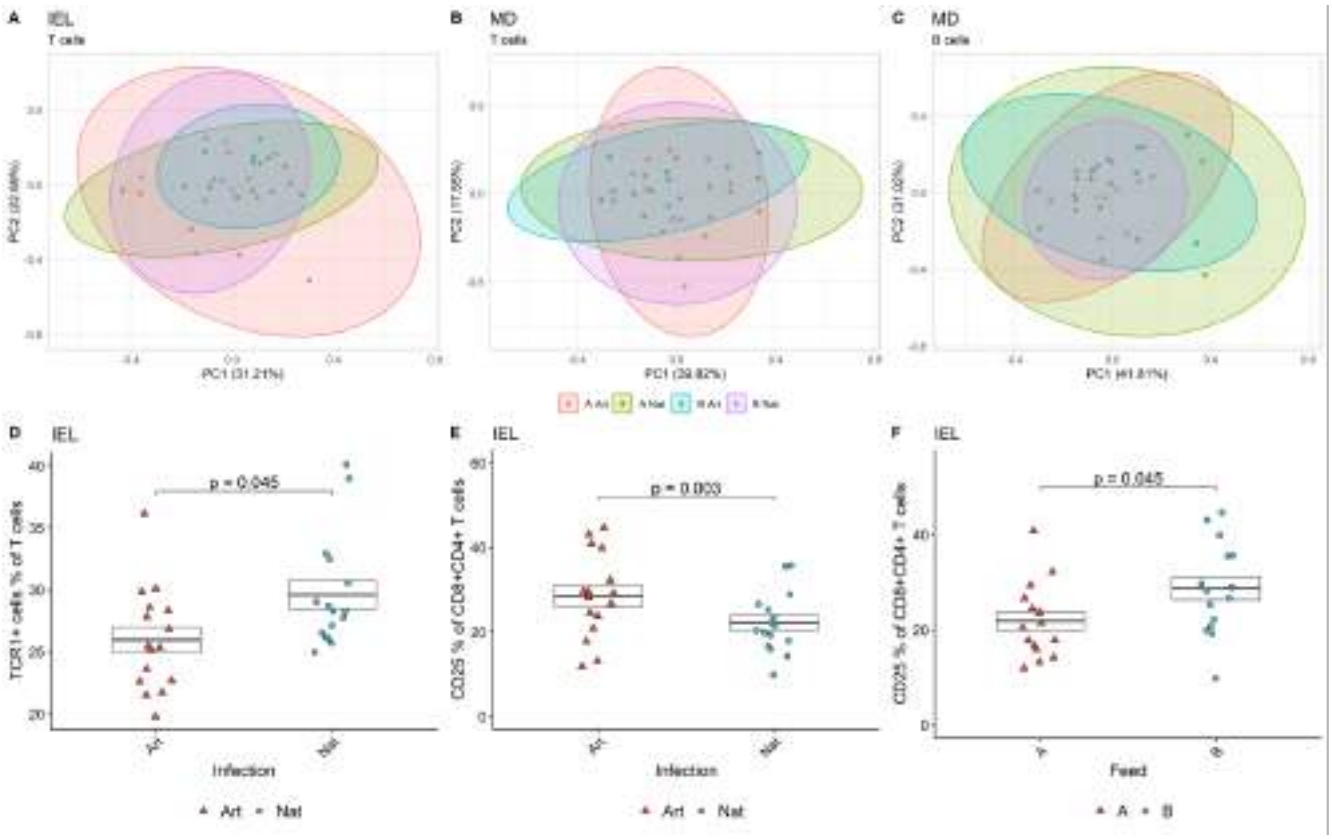

Figure 19 : Immunoprofiling of lymphocytes isolated from intestinal tissue at week 12 p.i. Principal component analysis (PCA) plots illustrating the multivariate variation among data obtained by flowcytometric analysis of intraepithelial lymphocytes (IEL) and Meckel's Diverticulum (MD). PC1, principal 1, PC2, principal 2. Staining and gating of lymphocytes were performed (Appendix D, panel 5). A) PCA plot of multivariate variation for T cell markers in IEL. B) PCA plot of multivariate variation for T cell markers in MD. C) PCA plot of multivariate variation for B cell markers in MD. D) The percentage of T cells expressing TCR $1\gamma\delta$ (TCR1+) in IEL in relation to infection method. E) Percentage of the double-positive CD8+CD4+ T cells expressing CD25 in IEL in relation to infection method and F) Percentage of the double-positive (CD8+CD4+) T cells expressing CD25 in IEL in relation to diet. Results in D), E) and F) are shown as mean ± SEM. P-values are indicated.

Results from the flow cytometric analysis of cells from IEL and MD were analysed by principal component analysis (Figure 19). No differences were observed between the four groups for data obtained for cells isolated from MD (Figure 19B and 19C). Regarding T cells isolated from IEL, naturally infected hens had a significant higher percentage of T cells expressing TCR1$\gamma\delta$ than artificially infected hens (p=0.045) (Figure 19D). For DP T cells (CD8+CD4+) in IEL expressing CD25, the infection method showed the opposite effect, where the artificially infected hens had a higher percentage of DP T cells expressing CD25 compared to artificially infected hens (p=0.003) (Figure 19E). Also, the diet affected the percentage of T cells expressing CD25 (Figure 19F). Diet B had a larger percentage of the DP T cells expressing CD25 (p=0.045).

6. Discussion

Organic and free-range egg production systems promote the opportunities of performing natural physiological behaviour and provide the hens with more space compared to other egg production systems. This may increase the animal welfare in relation to the expression of natural behaviour, but it also comes with an increased risk of infections with endoparasites, like infection with *A. galli* (Hinrichsen et al., 2016).

6.1 Performance

Egg production rate (Figure 9) was not affected by the dietary feed supplement, but it appears that the infection method affected the onset of lay at week 1 and 2 p.i., which corresponds to a hen age of 21 and 22 weeks (Figure 9B and 9C). Artificially infected hens had a significantly faster onset of egg production than the naturally infected hens (p=0.041 and 0.042 for week 1 and 2 p.i., respectively). The same observation was made for hens (20 weeks of age), where egg production was lower for the low infection group (inoculated with 250 embryonated *A. galli eggs*) compared to high infection groups (inoculated with 1,000 and 2,500 embryonated *A. galli* eggs) (Sharma et al., 2018a). Several earlier studies showed no significant differences in egg production between *A. galli* infected hens and uninfected control groups (Gauly et al., 2007, Sherwin et al., 2013), and neither in egg mass, egg quality, BWG, FI or feed conversion ratio (Sharma et al., 2018b). Another study reported a decrease

in egg production from 84% to 60% after 6 weeks of infection with *A. galli*, but this could be due to the fact that the hens were co-infected with *P. multocida* (Dahl et al., 2002). There were no significant differences between the other performance data (Table 4). It should be noted that the performance results were subject to uncertainty. Some eggs were laid on the floor instead of in the nest box, which resulted in some eggs being crushed, and in some cases, even eaten by the hens, which made the exact egg enumeration difficult and potentially give an underestimated egg production rate. A considerable feed spillage was observed in some pens, which potentially resulted in an overestimation of the feed intake. However, the primary purpose of this experiment was not to investigate the effect on the performance, and the study was not designed to measure robust production data due to the sample size.

6.2 Gut health and intestinal integrity

Several aspects are important for a healthy and well-functioning gut. These are a well-balanced diet, well-functioning digestion and absorption, a normal and stable microbiota, an effective immune status, healthy gut mucosa, and well-working neuroendocrine and motor function (Celi et al., 2017). In this experiment several parameters were investigated to characterize gut health of the hens. These include measurements of faecal pH and of ileal and caecal contents, the barrier status of the gut epithelium using serum coloration as indicator and the concentrations of SCFA and lactic acid as indicators of microbial composition and activity.

SCFA are the major bacterial fermentation product in the intestine and the concentration has subsequently influence on the pH of intestinal and faecal contents.

A large difference between ileum and caeca was observed with respect to the SCFA profile, which corresponds to differences in microbial composition as reviewed by (Khan et al., 2020). In ileal content, lactic acid bacteria dominate, which is reflected by the presence of lactic acid and acetic acid. In caeca, a high diversity of bacteria is present as reflected by a high diversity of SCFA (Table 5). Lactic acid is not measured at this location, as lactic acid is a substrate for caecal bacteria, i.e. butyrate producers (Engberg et al., 2009).

The concentrations of the most dominant SCFAs, acetic acid, propionic acid and butyric acid, were higher in caecal content of hens receiving the fermented feed supplement (Table 5). Caecal fermentation is a reflection of nutrient digestion in the small intestine, which means that the nutrients, which having escaped digestion and absorption in the small intestine, serve as substrate for bacteria in the caeca. These results could indicate that nutrient digestibility was lower in hens fed the diet with the feed supplement. However, nutrient digestibility was not measured in the present experiment.

An effect of infection method was observed with respect to the concentration of isobutyric and isovaleric acid, where artificially infected hens had higher concentrations of these acids in caecal content. Isobutyric and isovaleric acid are branched chain SCFAs deriving from the fermentation of protein. The reason for this is not clear.

Artificially infected hens had a significantly higher faecal pH than naturally infected hens 7 and 12 weeks p.i. (Figure 10A). However, no effect of the infection method on ileal pH and caecal pH was observed (Table 5). Therefore, these results are difficult to explain.

Serum coloration was measured as an indicator of intestinal integrity and the absorption capacity of nutrients (Figure 11, A-F). Carotenoids, or vitamin A, are fat-soluble vitamins, which are absorbed in the small intestine to the peripheral blood. Carotenoids affects the serum coloration, which is reflected in OD. Chickens with damaged intestinal epithelium caused by coccidiosis had lower OD of serum compared to uninfected chickens (Hamzic et al., 2015). In addition, the concentration of carotenoids in serum was negatively correlated with coccidian infestation in house sparrows (Pap et al., 2009). This might be related to a lower absorption of carotenoids, because intestinal damage of the epithelium leads to a reduced absorption of fat-soluble vitamins in the intestine (Breithaupt et al., 2003). In the current experiment, significant differences at week 2 p.i. could be related to the histotrophic phase of the *A. galli*, where the larvae penetrate and migrate the intestinal mucosa, which may lead to intestinal epithelial damage and give impaired absorption of fat-soluble vitamins. Naturally infected hens had lower OD of serum compared to artificially infected hens at week 2 p.i. (only seen for diet A), which was unexpected, since naturally infected hens had lower worm burden

and lower A. galli specific titre at week 12 p.i., which may indicate that they would have had lower larval burden at week 2 p.i.

6.3 *Ascaridia galli* burden

The titres of *A. galli* specific IgY in serum, EPG and worm burden was measured to determine the impact of *A. galli* infection (Figure 12, Figure 13, Figure 14).

There were no significant differences between the worm burdens at week 12 p.i. in relation to the feed, but there was a significant difference in relation to infection method (p=0.005) (Figure 14A and B). Artificially infected hens had more worms than naturally infected hens (Figure 14B). However, this is the opposite of the results found by Sharma et al., (2018), where naturally infected hens had a larger worm burden at week 14 p.i. compared to artificially infected hens inoculated with 2,500 embryonated *A. galli* eggs.

EPG is an indication of the severity of the *A. galli* infection, but because it only refers to the female worm burden, it comes with some uncertainty, which is also indicated in the correlation plot (Figure 12B). The female percentage for the artificially inoculated hens tended to be lower compared to naturally infected hens, although results were not significant (Table 7), which is accordance to Permin et al. (1997), who found that the infection dose correlates with the sex ratio in the worm burden, which means a high infection dose favours the male worms.

Further, there were no significant differences in EPG in relation to feed, but it seems that the EPG development during the experiment might have been affected by the feed. The EPG of hens fed the control diet (feed A) tended to have the highest EPG at week 7 p.i., where the EPG for hens fed the diet supplemented with fermented rapeseed meal (feed B) tended to have the largest EPG at week 10 p.i. (Figure 12A). This might indicate that the duration of the prepatent period can be affected by the diet.

The *A. galli* specific IgY titre was significantly higher in artificially infected hens compared to the naturally infected hens at week 2 p.i. (Figure 13C) and hens receiving the supplemented diet had lower *A. galli* titres compared to hens given the control diet at week 10 p.i. and 12 p.i. (Figure 13D and 13E). Elevated titre from ELISA analysis detects the magnitude of the antibody response from the

host, but may not correlate to infection intensity (Ruhnke et al., 2017). Results showed that the *A. galli* specific IgY titres at week 12 p.i. are not correlated with the worm burden at week 12 p.i. (Figure 13B), and this poor correlation was also confirmed in another study at 6 weeks p.i. (Schwarz et al., 2011). However, the number of larvae has been proven to affect the *A. galli* specific IgY in serum more than the count of adult worms (Das et al., 2018). This might indicate that the artificially infected hens may have had more larvae embedding in the intestinal mucosa in the histotrophic phase 2 weeks p.i. compared to naturally infected hens, which may explain the higher titre in week 2 p.i. (Figure 13C). In the histotrophic phase the parasite is more exposed to the host immune cells compared to when the adult larva is present in the intestinal lumen. If the elevated titre in this case is related to the infection intensity of embedded larvae, it corresponds well with the larger final worm burden observed in artificially infected hens compared to naturally infected hens.

At week 10 and 12 p.i., hens receiving the supplemented diet (feed B) had lower *A. galli* titres compared to hens given the control diet (Figure 13D and 13E). There were no significant differences in worm burden in relation to feed, which might indicate that diet B may reduce the immune response towards *A. galli* in terms of *A. galli* specific antibodies production.

Antibodies produced by B memory cells can stay elevated for months p.i. The hens were naturally infected with *A. galli* before the beginning of the experiment and deworming, which explains why the titres at week 0 p.i. were larger than zero.

Co-infection with other nematodes is common in *A. galli* infected hens. This involves *Heterakis spp*., *Raillietina spp.* and *Capillaria spp.* (Thapa et al., 2015, Sharma et al., 2019), which corresponds with the results in our experiment, where *Capillaria spp.* eggs were found in 28 pens out of 32. Tapeworms were observed in 22 pens out of 32 pens, but only in 1-3 animals within a pen consisting of 6 animals. Tapeworms requires an intermediate host, like for instance a snail, beetle or fly, which makes the life cycle more complicated in an indoor system, and that might explain why some hens had not been infected with tapeworms. The incidences of *Heterarkis spp.* were not counted, but observed in the caeca of several animals.

6.4 Immunocompetence

To investigate the effect of the feed and the *A. galli* infection on the immune system as well as the general immunocompetence of the hens several parameters were measured. The absolute counts of blood cell subsets were determined, general antibody production potential from intestinal B cells were quantified, the PBMC activation potential was assessed and lymphocytes from intestinal tissue were phenotyped.

Heterophils are the first cells involved in an inflammatory response in response to detection of pathogens and show different killing mechanism by phagocytosis, cellular degranulation, production of an oxidative burst and use of heterophil extracellular traps (HETs), which contains DNA and histones (Genovese et al., 2013, Chuammitri et al., 2009). The H/L-ratio in the peripheral blood is a reliable parameter to measure long-term stress in domestic fowls (Gross and Siegel, 1983, Hofmann et al., 2020). Corticosterone, which is the major adrenal glucocorticoid in avian, is released by stress. Corticosterone binds to specific adrenergic receptors that are expressed in almost any tissue of the body. In mammalian species, these specific receptors are highly expressed on lymphocytes, but in avian species, they are more often expressed on cells of the innate immune system, especially heterophils (Hofmann et al., 2020). Glucocorticoids like corticosterone have immunomodulatory properties in terms of up-regulating expression of pro-inflammatory cytokines and chemokines as well as supress proliferation of lymphocytes. Corticosterone is released into the peripheral blood in chickens, which increases the heterophil concentration and decreases the lymphocyte concentration, which ultimately increases the H/L-ratio. Corticosterone levels was not measured in this experiment, but all the groups had elevating H/L-ratio and decreasing lymphocyte concentrations after inoculation or exposure to contaminated litter at week 0 p.i. The H/L ratio for the artificially infected hens kept increasing over the 12 weeks, whereas the H/L ratio for the naturally infected hens increased at week 2 p.i. and then stabilized (Figure 15C). The overall response (AUC) for artificially infected hens was significantly higher than for the naturally infected hens, which may indicate that the artificially infected hens had experienced more stress than the naturally infected group. Stressed or fearful chickens have larger EPG compared to unstressed chickens (Sherwin et al., 2013). In this experiment, there was no connection between the results from EPG and the H/L-ratio. However, the higher H/L-

ratio in artificially infected hens may be explained by the larger worm burden, because parasite infection induce stress in the host (O'Dwyer et al., 2020). Further, the artificially infected hens also had higher concentration of γδ T cells and more CD4+ T cells than the naturally infected hens (Figure 15E and 15G), which may also indicate a more activated immune system.

Earlier studies showed that there is a time-dependant increase in intraepithelial CD4+ T helper cells, which peaks at day 20 p.i. in *A. galli* infected hens compared to uninfected control birds (Ruhnke et al., 2017). This trend was confirmed by another study (Schwarz et al., 2011), who also found increased amount of T helper cells in *A. galli* infected hens. In our experiment, the artificially infected hens, who had the largest worm burden, also had the highest concentration of CD4+ T helper cells in the peripheral blood during the experiment compared to the naturally infected hens with lower worm burdens (Figure 15G).

Intraepithelial lymphocytes represent a population of different lymphocytes, including T cells and lymphocytes similar to natural killer cells, which are located above the intestinal membrane between epithelial cells. In this experiment, naturally infected hens, who had the lowest worm burden, had significantly more γδ T cells (TCR1+, Figure 19D). Chickens have high frequencies of γδ T cells compared to mice and human but their exact roles in various infection models are still unknown. There is a broad tissue distribution of chicken γδ T cells emphasizing their important innate immune surveillance functions and recent data suggest that especially IEL γδ T cells have high cytotoxic potential (Fenzl et al., 2017). Others have reported influx of γδ T cells in the jejunal mucosa during the mucosal phase on *A. galli* infection indicating a potential role in the parasite induced immune response. In mouse, it was earlier reported that γδ T cells play a protective role during nematode infection by promoting intestinal goblet cell function (Inagaki-Ohara et al., 2011). To understand the meaning of this observation, the function of γδ T cells under infection must be investigated in future studies in relation to cytokine production and cytotoxic immune response in the *A. galli* infection model.

Interestingly, the increased frequency of IEL γδ T cells in naturally infected hens co-incited with a lower percentage of DP T cells expressing CD25 (activated DP cells) than in artificially infected hens (p=0.003) (Figure 19E). In chickens, DP T cells are observed in the peripheral blood (20–40%), spleen

(10–20%), and intestinal epithelium (5–10%), but their functions have not yet been studied in detail. Hence, little is known of *A. galli* influence on activation of this cell population but interestingly microbiota was shown to affect IEL DP activation status in chickens e.g. via SCFA levels (Lee et al., 2018).

Immunoglobulins are produced by B cells, and intestinal tissue was sampled to investigate the antibody production potential of B cells located in lamina propria. The assay investigated the total immunoglobulin production potential at week 12 p.i. The potential of IgA and IgM production from intestinal B cells was larger in artificially infected chickens than in naturally infected chickens (Figure 16D and 16E), which may be related to the larger worm burden, but follow-up studies of the *A galli* specific production potential is needed in order to clarify this point.

ConA is an antigen-independent mitogen and leads T cells to polyclonal proliferation. PMA is a B cell specific mitogen and stimulates division of B cell and induces activation of different cell types to produce cytokines. The mitogen stimulation of the PBMCs did not show any significant differences between the four groups (Figure 18). However, the variation in the results between the ConA stimulated cells and the non-stimulated control was larger than the variation between the PMA stimulated cells and their non-stimulated control (Figure 18A and 18B), which indicated that ConA gave a stronger response from the T cells compared to PMA's ability to stimulate the B cells. However, the study provided a comprehensive overview of the relative value of different lymphocyte activation markers (Table 17). PMA had limited impact on activation markers on the B cells, except for a minor increase in B cells expressing the CD25 marker and MHC-II (Figure 17A). For the different T cell populations, the frequency of cells with activation markers increases after stimulation with the mitogen, ConA, especially in the CD4+, CD8+ and TCR1$\gamma\delta$+CD8+ T cell populations (Figure 17A). The TCR1$\gamma\delta$+CD8- T cell population seemed less affected by the ConA stimulation (Figure 17A). Only small changes in MFI for the different markers on the T cell populations were observed after stimulation with ConA (Figure 17B). This is in accordance with a recent study of several chicken lymphocyte activation markers and their expression kinetics upon mitogen induced in vitro activation (Naghizadeh et al., 2021).

7. Conclusion and future perspectives

The aim of this study was to investigate the effect of supplementing the diet with pre-fermented rapeseed meal and seaweed (6%) on *A. galli* infection compared to a non-supplemented control diet. Based on the research performed during this master thesis project, the following conclusions can be drawn.

Effects observed in relation to feed

- Feed B had no significant effect on performance, worm burden or immune competence compared to the control feed.
- Chickens receiving the diet with the fermented feed supplement had higher concentrations of acetic acid, propionic acid and butyric acid in caecum at week 12 p.i.
- Chickens receiving the diet with the fermented feed supplement had lower *A. galli* specific IgY titre at week 10 p.i. and at week 12 p.i.
- Chickens receiving the diet with the fermented feed supplement had a larger percentage of DP T cells expressing the CD25 surface marker at week 12 p.i.

Effects observed in relation to infection method

- At 12 weeks p.i., artificially infected chickens had a larger worm burden than naturally infected chickens.
- Naturally infected chickens had lower OD of serum coloration than artificially infected chickens at week 2 p.i.
- Artificially infected chickens had higher *A. galli* specific titres at week 2 p.i. than naturally infected chickens.
- Artificially infected chickens had a higher H/L-ratio, higher concentration of $\gamma\delta$ T cells and CD4+ T cells circulating in the peripheral blood, which related to the larger worm burden naturally infected chickens.
- In the intraepithelial lymphocytes, artificially infected chickens had more CD25+ activated DP T cells, but a lower amount of TCR1+ T cells at week 12 p.i. compared to naturally infected chickens.

- The potential of total IgA and IgM production from intestinal B cells was larger in artificially infected chickens than in naturally infected chickens.

Further investigations must be conducted to understand the functional mechanisms in the immune response towards *A. galli* infection. It can be concluded that supplementing the layer diet with 6% fermented rapeseed meal with seaweed (EP 199, Fermentation Experts) did not have potential to control *A. galli* infection in chickens. Future research must be conducted on other feeding strategies to control *A. galli* infection, e.g. plant additives.

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

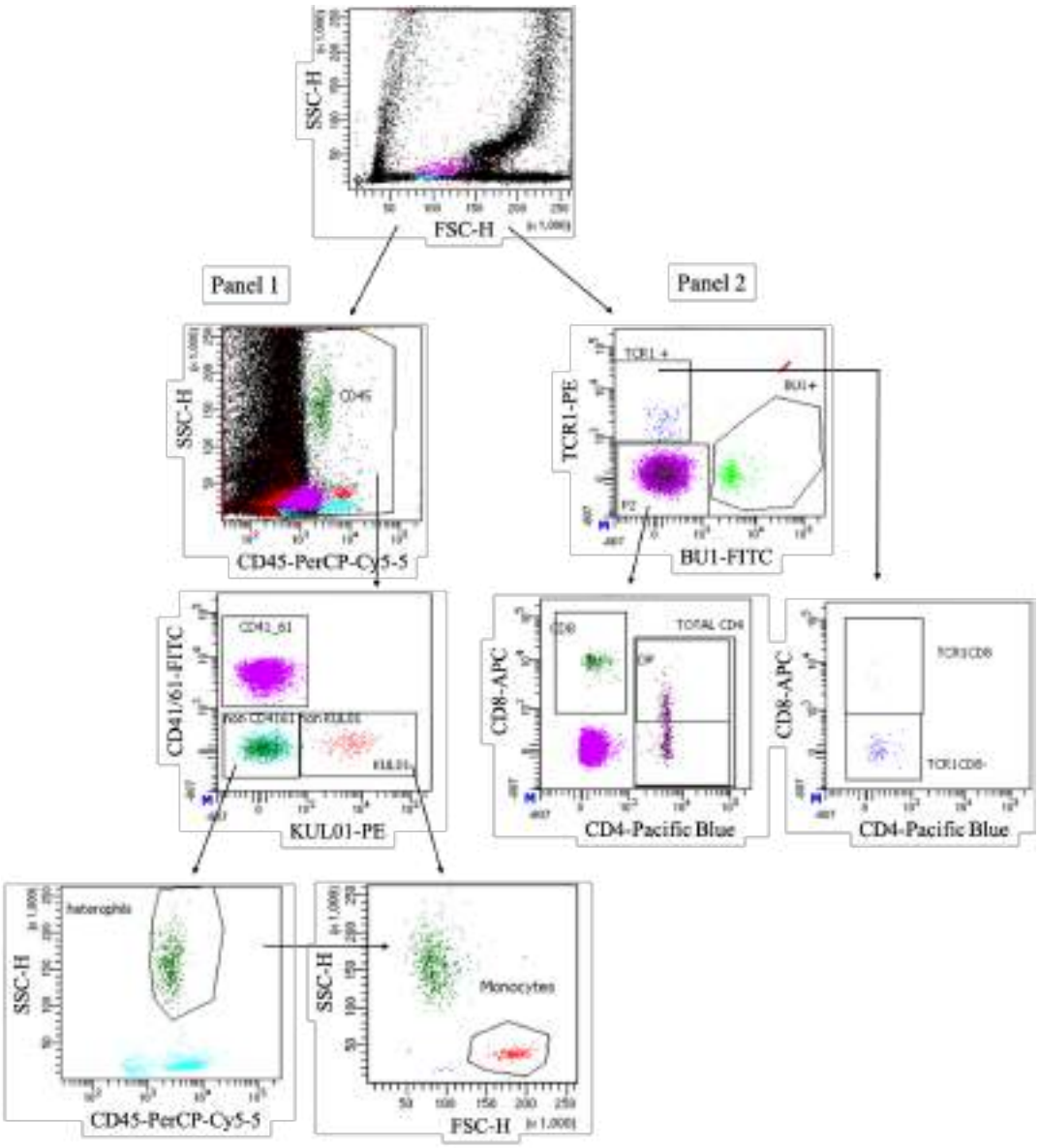

Figure 20: Gating strategies for flow cytometric analysis of absolute counts of blood cell subsets. The gating strategy used for Figure 15 for Panel 1 was: CD45^{+} cells, CD45+CD41/61+ cells / CD45^{+}KUL01^{+} cells / CD45+CD41/61-KUL01- heterphils and CD45+KUL01+ FSC-H monocytes. Panel 2 was: BU1+ 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^{TM} counting beads. Staining from 10 weeks p.i. is shown as an example, but the gating strategy was identical for all weeks.

Appendix B – Gating strategy for flow cytometric analysis (ConA)

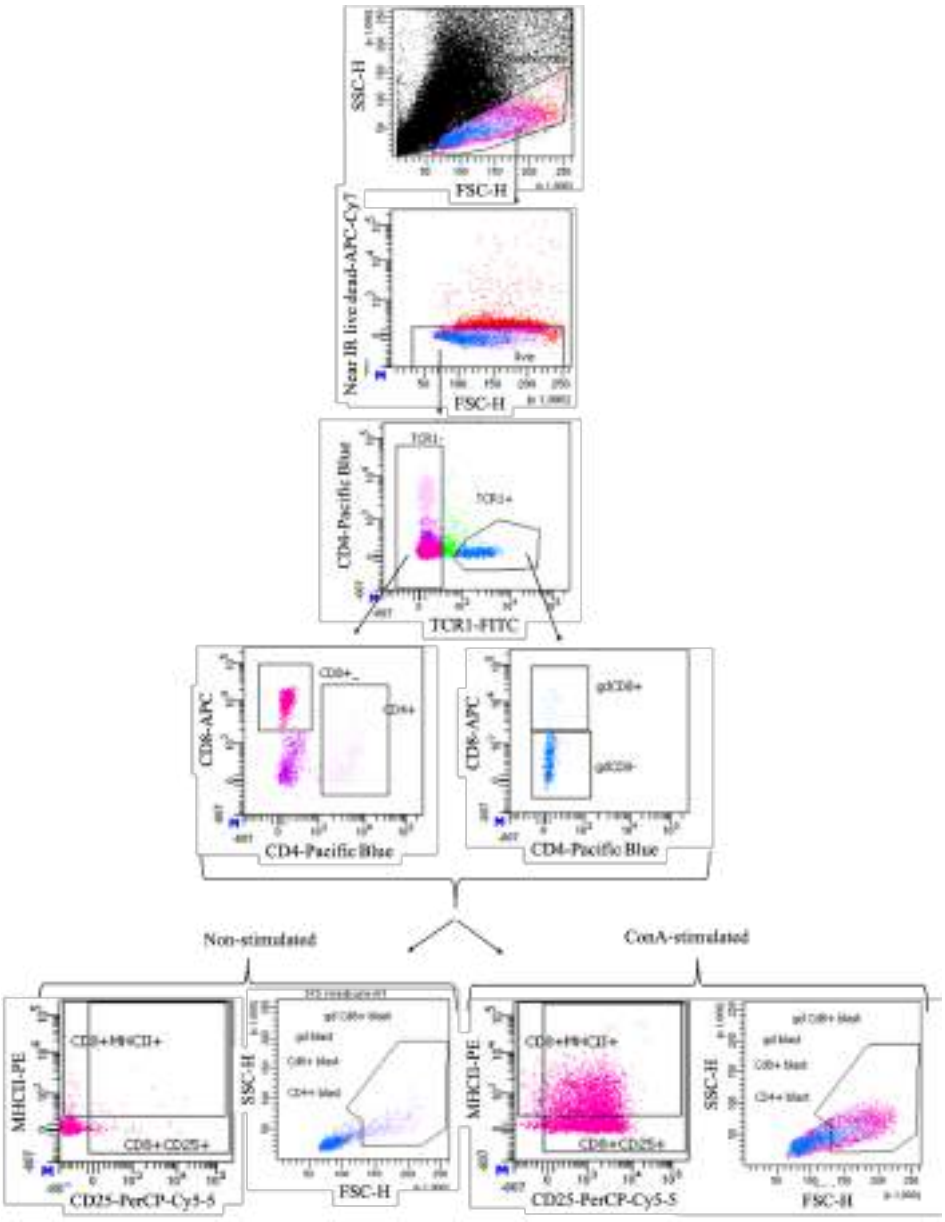

Figure 21: Gating strategies for flow cytometric analysis of ConA stimulated PBMCs. The gating strategy used for Figure 18 A was: SSC-H/FSC-H defined lymphocytes, live cells, TCR+ or TCR- cells, CD8+ cells, CD4+ cells CD45+CD41/61-KUL01- heterophils and CD45+KUL01+ FSC-H monocytes. Panel 2 was: BU1+ B cells, TCR1+CD8+ T cells ($\gamma\delta$ CD8+), TCR1+CD8- T cells ($\gamma\delta$ CD8-), CD8+MHCII+ T cells, CD8+CD25+ T cells and blasting CD8+ T cells. Gating of CD8+MHCII+ T cells, CD8+CD25+ T cells and blasting CD8+ T cells are shown for both non- and ConA stimulated cells. Gating for all subsets with the MHCII or CD25 markers as well as the blasting cells was performed as shown for CD8+MHCII+ T cells and CD8+CD25+ T cells. Staining of PBMCs from a representative animal is shown as an example.

Appendix C – Gating strategy for flow cytometric analysis (PMA)

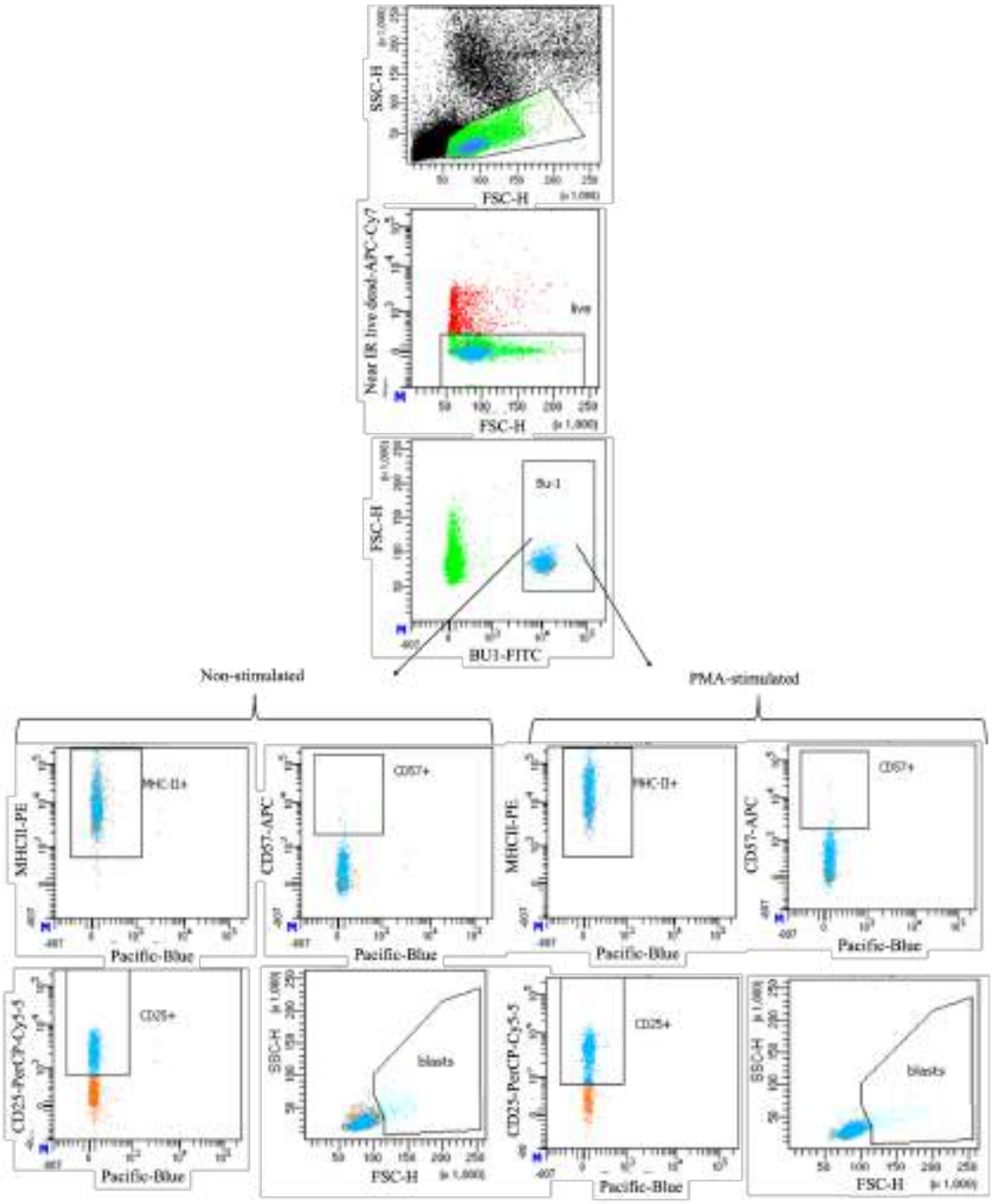

Figure 22: Gating strategies for flow cytometric analysis of PMA stimulated PBMCs. The gating strategy used for Figure 18 B was: SSC-H/FSC-H defined lymphocytes, live cells, BU1+ B cells, BU1+MHCII+ B cells, BU1+CD57+ B cells, BU1+ CD25+ B cells and blasting BU1+ B cells. Gating of BU1+MHCII+ B cells, BU1+CD57+ B cells, BU1+ CD25+ B cells and blasting BU1+ B cells are shown for both non- and PMA stimulated cells. Staining of PBMCs from a representative animal is shown as an example.

Appendix D – Gating strategy for flow cytometric analysis (IEL and MD)

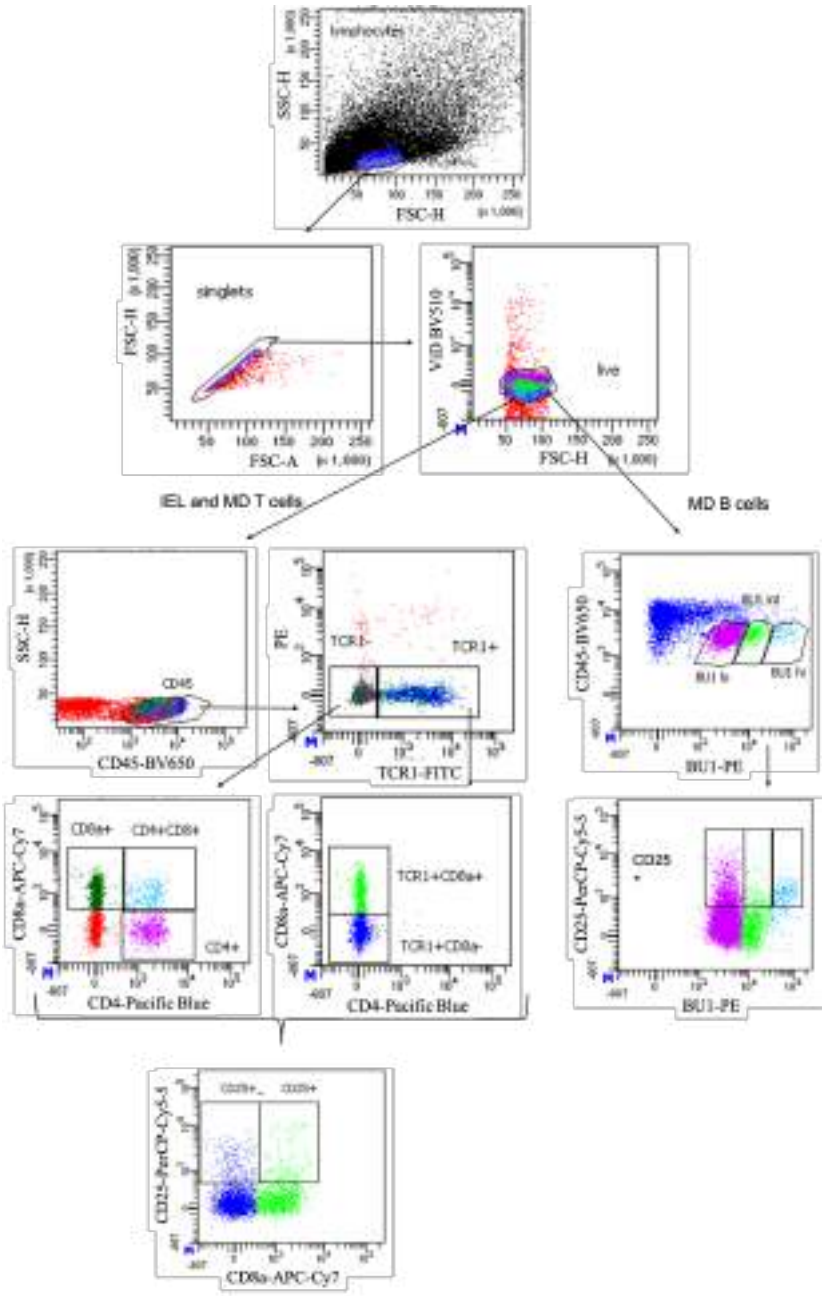

Figure 23: Gating strategies for flow cytometric analysis of IEL and MD lymphocytes. The gating strategy used for Figure 19 was for T cells: SSC-H/FSC-H defined lymphocytes, FSC-H/FSC-A defined singlets, live cells, CD45+ cells, CD45+TCR1- cells / CD45+TCR1+ T cells, CD45+TCR1-CD8a+ T cells / CD45+TCR1-CD4+ T cells / CD45+TCR1-CD4+CD8a+ T cells, CD45+TCR1+CD8a+ T cells / CD45+TCR1+CD8a- T cells. Gating for B cells was: SSC-H/FSC-H defined lymphocytes, FSC-H/FSC-A defined singlets, live cells, BU1+lo B cells / BU1+int B cells / BU1+hi B cells, BU1+lo CD25+ B cells / BU1+int CD25+ B cells / BU1+hi CD25+ B cells. Staining of PBMCs from an representative animal is shown as an example.

Appendix E – Co-infections with other parasites (overview)

Table 8: Overview of co-infections with other parasites observed within the four groups in % of pen or % of animals.

	A Nat	**A Art**	**B Nat**	**B Art**
Presence of *Capillaria spp.* eggs (% of pens)	87.5%	100%	100%	62.5%
Presence of tape worms (% of pens)	62.5%	62.5%	75%	75%
Presence of tape worms (% of animals)	12.5%	17.0%	16.7%	28.3%