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IMMUNOMODULATORY EFFECTS OF GARLIC AND
ARONIA BERRY AND THEIR ANTIPARASITIC
POTENTIAL AGAINST ASCARIDIA GALLI IN CHICKENS

Master Thesis (60 ECTS) by Geng Pan

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Preface

This master thesis was written from September 2021 to June 2022, when I participated in the EU-funded project focusing on disease control in e.g. organic chicken production. The master project consists of three months of *in vivo* and three months of *in vitro* study to investigate the immunomodulatory effect and antiparasitic effect of garlic and aronia berry. This experience gave me a perfect introduction to my “potential” academic career, and I would like to extend my sincere thanks to everyone on this journey.

I deeply appreciate the enlightenment from Tina Sørensen Dalgaard, who is always enthusiastic about sharing knowledge and discussing ideas with me, inspiring my passion for research. I want to express my gratitude to Rikke Brødsgaard Kjærup, who always answered my question patiently and taught me data analytics and writing skills, providing me with a solid background for future study. I would also like to thank Lene Rosborg Dal, who taught me lots of Danish, and your humor is beneficial for my mental health.

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List of abbreviations

A. galli	Ascaridia galli
ConA	Concanavalin A
DP	Double-positive (CD4+CD8+)
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immune sorbent assay
EPG	Eggs per gram faeces
FACS	Fluorescence-activated cell sorting
GALT	Gastrointestinal-associated lymphoid tissue
H/L-ratio	Heterophils/lymphocytes ratio
IEL	Intraepithelial lymphocytes
LPL	Lamina propria leukocytes
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgY	Immunoglobulin Y
IL	Interleukin
MD	Meckel's diverticulum
MHC	Major histocompatibility complex
OD	Optical density
p.i.	Post infection
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
RT	Room temperature
Tc	Cytotoxic T cell
Th	T helper cell

Summary

Aronia berry and garlic are known for their immunomodulatory effects in mammals. The immunomodulatory effect of these plants in chickens has only been sporadically studied. However, we hypothesize that the plants may have both immunomodulatory and antiparasitic effects on chickens infected with the roundworm *Ascaridia galli* (*A.galli*). We aimed to investigate both these effects *in vitro* with a follow-up study of the antiparasitic effect *in vivo*. When plant extracts were incubated with ConA stimulated peripheral blood mononuclear cells (PBMC), both water and ethanol extract of garlic tended to increase the proliferation of CD4⁺T cells (Th) and CD8⁺ T cells (Tc). The garlic ethanol extract specifically was shown to significantly promote the activation of CD4⁺ T cells. When a chicken macrophage cell line was incubated with plant extracts, garlic and aronia berry generally showed anti-inflammatory effects by significantly reducing macrophage production of nitric oxide(NO). In a ten-week animal trial, hens were fed with garlic or aronia berry powder and experimentally infected with *A. galli*. The tested feed inclusion levels of the plants showed no or limited effect on the burden of adult worms in infected hens. However, there was a numeric garlic-induced reduction of worm burden in one experimental room, and also a potential impact of garlic was shown on the larvae stage of *A. galli* as IgY titers remained very low in the histotropic phase of the worm development where a peak in antibody titer was observed in control animals not fed any plant powder. Furthermore, the garlic and aronia berry may potentially facilitate the development of adaptive immunity against *A. galli*, as increased levels of parasite-specific T cells were observed in these groups four weeks after infection. The obtained result also implied that garlic and aronia berry have potential impacts on phenotype and activation state of intestinal immune cells, which are essential for helminth control and gut health in general.

Sammendrag(Danish summary)

Det er velkendt fra studier i pattedyr at planter som hvidløg og Aroniabær kan have immunmodulatoriske egenskaber. Om det samme gør sig gældende i høns er endnu ikke undersøgt til bunds. Vores hypotese var at hvidløg og Aroniabær muligvis kan besidde både immunmodulatoriske og anti-parasitære egenskaber over for infektion med rundormen *Ascaridia galli* (*A. galli*) i æglæggende høner. Projektets formål var således at undersøge begge disse planters effekter *in vitro* og at lave et opfølgende dyreforsøg med speciel fokus på den anti-parasitære del.

In vitro blev det vist at både vandig og alkohol ekstrakt af hvidløg havde en positiv effekt på T celle proliferation, når oprensede celler fra perifert blod blev stimuleret med mitogenet ConA, der fremmer celledeling. Effekterne sås både på T hjælper celler (CD4+) og på cytotoksiske T celler (CD8+) men var dog ikke statistisk signifikante. Mht. til T hjælper cellerne var der dog en signifikant øgning af aktiveringsmarkøren CD25 på cellernes overflade når alkohol ekstrakt af hvidløg var tilstede i cellekulturen. Plante ekstrakterne blev også undersøgt i en hønse makrofag cellelinje og her viste begge planter et anti-inflammatoriske potentiale idet produktionen af nitrogenoxid fra aktiverede makrofager var stærkt reduceret når plante ekstrakterne var tilstede i cellekulturen.

Planterne blev desuden undersøgt i et 10 uger langt dyreforsøg hvor æglæggende høner blev eksperimentelt inficerede med *A. galli* æg. Dyrene var opdelt i fire grupper, en kontrolgruppe (uden planter tilsat foderet), en gruppe med hvidløgpulver som fodertilsætning, en gruppe med Aroniabær pulver som fodertilsætning samt en gruppe der fik foder tilsat pulver fra begge planter. I de testede koncentrationer viste planterne kun en begrænset indflydelse på antallet af voksne orm/ormeburden da hønernes tarm blev undersøgt ved forsøgets afslutning. Der var dog en numerisk reduktion i antallet af orm i gruppen fodret med hvidløg i det ene forsøgsrum og der blev også vist en potential indflydelse af hvidløg på ormenes larvestadie idet der ikke kunne påvises et peak i *A. galli* specifikke IgY antistoffer i hønernes blod to uger efter infektion. Et peak blev observeret i de tre andre grupper inklusiv kontrol gruppen uden plante tildeling.

Endelig blev det vist at begge planter havde en potentiel indflydelse på antallet, fænotypen og aktiveringsstatus af immuncellerne som var til stede i hønernes tarm. Netop tarmens immunceller er vigtige for kontrol af ormeinfektioner men også for generel tarmsundhed.

1. Introduction

1.1 Challenge of *A. galli* infection in organic chicken production

Organic egg production, which gives layers the freedom to move around and forage outdoors, has steadily increased its market share in Denmark during the last ten years (Statistics Denmark, 2021). Outdoor access is often considered to provide animals with an environment that accommodates their health and naturalness. However, the hens are also in close contact with their feces and soil, increasing the risk of being exposed to and spreading pathogens (Jansson et al., 2010). Outdoor access reduces the protection against the intestinal roundworm *Ascaridia galli* (*A. galli*), a parasite which causes little or no problems in caged animals. Hence, the prevalence of *A. galli* infection in Danish organic layers is more than 75%, making *A. galli* the most prevalent helminth infection in Denmark (Thapa et al., 2015). For Danish organic layers, deworming occurs in two circumstances: if the test result exceeds the farmers' threshold for acceptable parasite egg numbers per gram feces or if the veterinarian directly recommends treatment. However, not all farmers will test for the helminth infection regularly, and the treatment thresholds of farmers may vary, which gives problems associated with higher mortality of the chickens (Hinrichsen et al., 2016). Avoidance of tests and treatment may be due to the severe consequence of using anthelmintics in organic production. Using synthetic chemical anthelmintics will result in a 14 days egg withdrawal period (Commission, 2001). Besides, the eggs cannot be labelled as organic for six weeks (Commission, 2008). Thus, *A. galli* infections have become a challenge in organic egg production, especially because it is more difficult to prevent and control them under outdoor conditions.

1.2 The opportunity of using medicinal plants as anthelmintics

According to the EU organic regulation, using internal natural resources is one of the principles of organic agriculture, and the external inorganic input is strictly limited (Council, 2007). Various medicinal plants have been identified to have potential efficacy against *A. galli* infection (Zaman et al., 2020). Using carefully selected plants

in *A. galli* control as a natural input and as an alternative to synthetic drugs would support the prevention of animals losing their organic identity and thereby support farmer revenue and animal welfare.

Anthelmintic residues are indeed a challenging problem in egg production. A study by Moreno et al., showed that residues of albendazole in the egg is higher than in plasma, and higher than the maximum residue level (MRLs) within seven days of treatment (Moreno et al., 2018). The withdrawal period can reduce the residue concentration in eggs, but during the withdrawal, the anthelmintic residues still have a potential impact on the aquatic and terrestrial ecosystems (Lee and Choi, 2019), which indirectly may influence human health. Using medicinal plants as a substitute for synthetic anthelmintic products may reduce the drug residue issues within the whole system (Abo-EL-Sooud, 2018).

Medicinal plants may also be a solution to reduce drug resistance. Since 2000, only three synthetic anthelmintics have been developed for helminth control (Nixon et al., 2020). Lack of rotational use of anthelmintic type may lead to drug resistance. However, there are plenty of plant-based anthelmintic resources, causing no resistance (Zaman et al., 2020). Furthermore, various plants have been found to boost the function of different immune cells, protecting the animal in a long-term perspective also against other pathogens in other species like lamb (Tzamaloukas et al., 2006, Niezen et al., 2002)

The EU regulation emphasizes that animal-health management should be based on prevention (Council, 2007). Highly in accord with the regulation, medicinal plants may enhance the health of layers by controlling parasite infection, support general immune competence effects (Tzamaloukas et al., 2006, Niezen et al., 2002) and provide beneficial interactions with host-microbiota (Kalaiselvi et al., 2017).

Despite various advantages of applying medicinal plants to prevent and treat *A. galli* infection, application method, appropriate dosage, efficacy, and toxicity are still not characterized in dept. Thus, further investigation is needed to characterize/optimize the efficacy and distinguish from potential side effects of medicinal plants. Furthermore,

choice of plants that are cost-efficient and can be locally grown is preferable.

1.3 *A. galli*

1.3.1 Morphology

A. galli is a nonmigrating parasite species in the avian small intestine (Urquhart et al., 1999). Adult worms are white and stout, with 42 ~76 mm in length for males and 72~108 mm for females (Ramadan and ABOUZNADA, 1992). Male worms have an ellipsoidal preanal sucker and a tail with narrow caudal alae. Female worms have straight, conical tails. Their eggs are covered by smooth and thick shells and are not embryonated when first laid but needs to mature in the environment outside the host (Levine, 1968) (Figure 1).

1.3.2 Life cycle

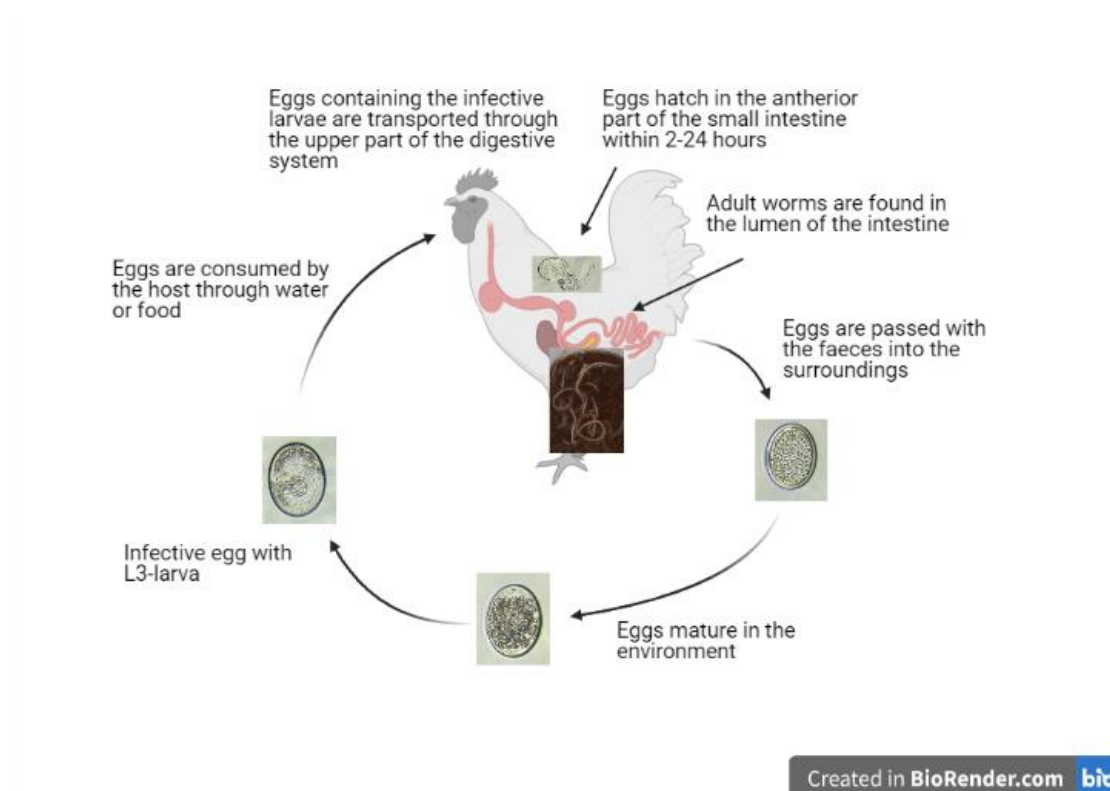


Figure 1 Life cycle of *A. galli*. Made in BioRender with own pictures.

Exposed to outdoor conditions, the chickens have a risk of taking in the infective L3 stage larva in egg from contaminated food or soil. After ingestion, the eggs will hatch

within 24 hours, and larvae will reside the proventriculus or duodenum. Within 1-9 days, the larvae will penetrate the mucosa, which may cause slight bleeding, and grow and mature in the mucosa – the so-called histotropic phase of the *A. galli* life cycle (Luna-Olivares et al., 2012, Tugwell and Ackert, 1952). After 2-3 weeks, the larvae will usually return to the lumen, where mature larvae are typically located in the jejunum and will become adult worms within 10-13 days (Luna-Olivares et al., 2012). The adult female worms are capable of producing eggs, and most of the eggs are excreted into the environment (Levine, 1968) Totally, it takes 5-8 weeks from the chickens are infected by worm eggs until they first start to shed *A. galli* eggs in feaces – this period is the so-called pre-patent period (Herd and McNaught, 1975). The thick, albuminous shells keep the eggs hydrated and they may persist long in different environments (Ackert, 1931). In 8~21 days, the excreted *A. galli* eggs will develop into the L3 stage and become infective (Urquhart et al., 1999).

1.3.3 The impact of *A. galli* infection on production animals

A. galli infection has negative impacts on animal health, productivity and welfare.

From an intestinal health perspective, *A. galli* will cause damage to intestinal mucosa, causing the hens to be more susceptible to bacterial infections from e.g. *E. coli*, *Salmonella* and *Pasteurella* (Hinrichsen et al., 2016). Besides the mucosal damage, the increased risk of bacterial and virus secondary infections may also be due to the cytokine environment changes induced by *A. galli* infection (Pleidrup et al., 2014). The parasite induced dominant Th2 response (Schwarz et al., 2011) may suppress the ability to mount a Th1 response, which is usually required for effective immunity against viruses and some bacteria (Guenova et al., 2013).

A. galli infection may also hinder the absorption of nutrients, suppressing the growth of the animals (Daş et al., 2010). The body weight gain was shown to be repressed 0-10 weeks after infection in an experimental trial (Malviya et al., 1988). In addition, higher infectious severity and higher worm burden have been found in the chickens with lower body weight (Daş and Gauly, 2014), revealing that the use of energy by an

immune response against *A. galli* may also affect animal growth. Compared to the healthy chicken, the mortality rate of *A. galli* infected chickens was significantly increased at the peak of lay (Hinrichsen et al., 2016)

A. galli infection can have additional negative consequences for animal welfare. The infected chickens may display more agonistic behavior, such as pecking directly toward each other's heads, or leading to chickens avoiding other animals (Gauly et al., 2007). Furthermore, locomotion activity and the ground pecking of hens were significantly decreased after *A. galli* infection, revealing that *A. galli* infection may suppress the natural behavior of the animals (Gauly et al., 2007).

1.4 The avian intestinal immune system

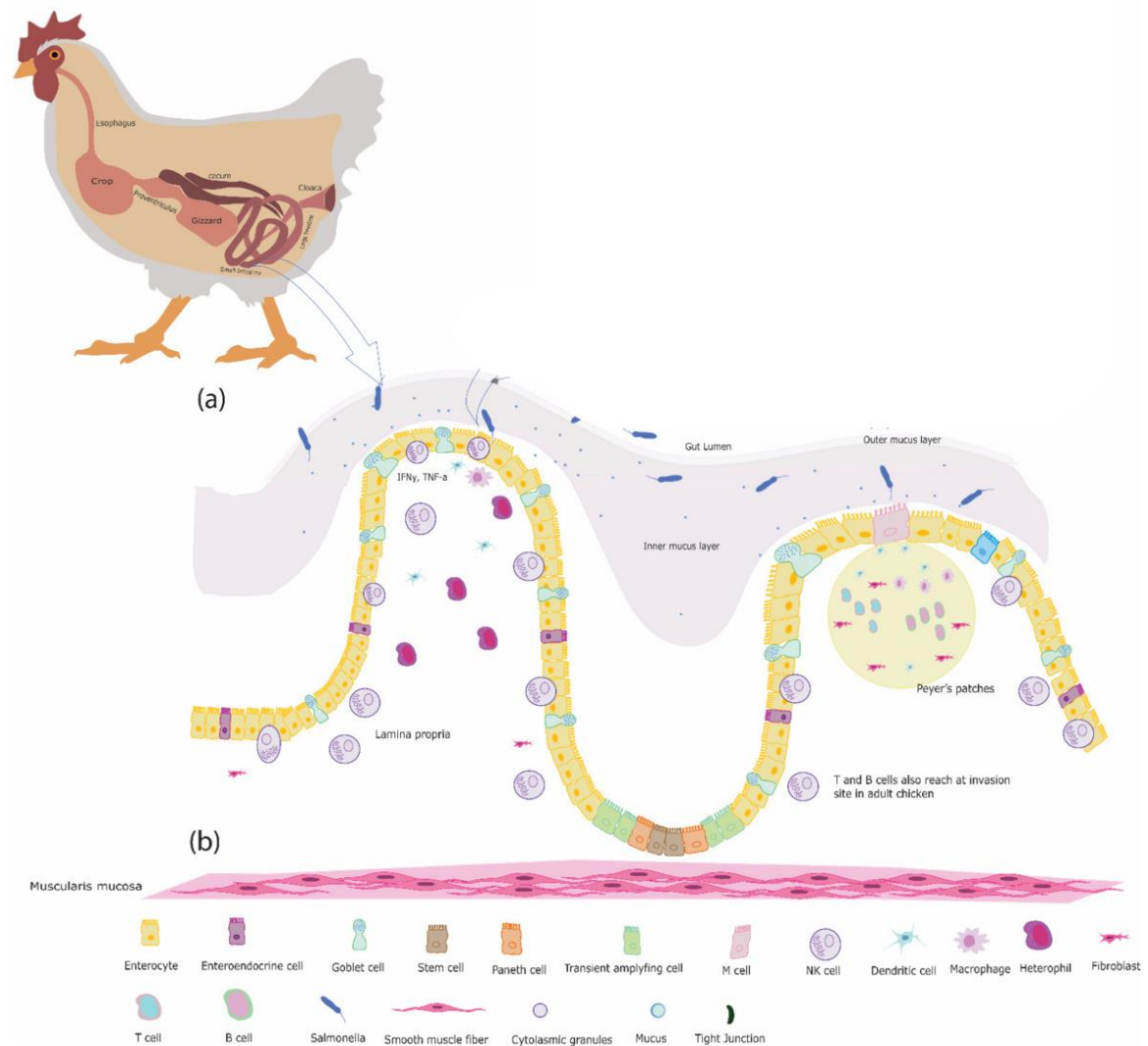


Figure 2: (a) Schematic representation of the chicken gastrointestinal tract, including the avian specific crop, gizzard, ceca, and cloaca b) Anatomical features of chicken intestinal epithelium and innate immune cells embedded in intestinal epithelium, lamina propria, and Peyer's patches (adapted from Ijaz et al., 2021)

Mammalian intestinal immunity has been extensively studied. Though the avian intestinal immune system shares similarities with that of mammals, the interactions and mechanisms of immune cells with intestinal pathogens still need further investigation. The gut-associated lymphoid tissues (GALT) in the chicken are mucosal-associated and diffused (Ijaz et al., 2021), consisting of Peyer's patches (PP), cecal tonsils (CT), Meckel's diverticulum (MD), intraepithelial lymphocytes (IELs), and lamina propria lymphocytes (LPL) (Befus et al., 1980). Located at the surface of the intestine, the

intestinal epithelial cells (IE) secrete mucus to shape a diffusive barrier to the pathogen, producing cytokines, which can initiate recruitment and shape the activity of immune cells (Li et al., 2009, Withanage et al., 2005). IELs, interspersed between the IE, comprise NK cells, $\alpha\beta$ T cells, and $\gamma\delta$ T cells, which are highly associated with the cytotoxicity of infected host cells. Underneath the intraepithelial tissue, there is the lamina propria tissue. In mammals, this layer contains various immune system components, with a massive amount of B cells, plasma cells, dendritic cells, macrophages, and T cells (Mowat, 2003). The avian lamina propria has a similar cellular pattern. When intestinal infection occurs, macrophages, and T-cells invade the lamina propria tissue, followed by the B-cells recruitment at the infectious sites (Van Immerseel et al., 2002). In the small intestine submucosa, PP plays a crucial role in antigen sampling and activation of T cells and B cells. The antigen presentation is highly associated with the major histocompatibility complex class I and II (MHC-I, MHC-II) presented by antigen-presenting cells (APCs) such as dendritic cells and macrophages. Furthermore, naïve B cells are primarily located in the follicles of PP, and the T cells are located in the intrafollicular region (Qu, 2018).

1.5 Mechanisms of helminth expulsion

Though there is increasing research output on the pathways responsible for helminth expulsion, a comprehensive understanding of the involvement of the avian immune response against *A. galli* is still lacking.

In mammals, Th2 (T helper 2) is considered highly responsible for the protective immunity to helminths (Perrigoue et al., 2008). During helminth infection, Interleukin-25 (IL-25) is consecutively produced by CD4⁺ and CD8⁺ T cells in the gut, which play an essential role at the onset of worm expulsion (Owyang et al., 2006). Induced by IL-25, naïve CD4⁺ T cells will differentiate into CD4⁺ Th2 cells and produce type 2 cytokines such as Interleukin-4 (IL-4), Interleukin-13 (IL-13), Interleukin-5 (IL-5) and Interleukin-9 (IL-9) (Anthony et al., 2007).

Regarding humoral immunity in mammal, IL-4 and IL-13 can activate B cells and

promote IgE secretion to eliminate the helminths (Fallon et al., 2006). In chickens, *A. galli* was also shown to induce a Th2 response 14 days after infection, producing IL-4 and IL-13 (Degen et al., 2005). In chickens, even though the Th2 response may promote *A. galli* specific IgY (IgG) production, no evidence shows that the IgY can significantly reduce the worm burden (Andersen et al., 2013). Additionally, IL-4 and IL-13 can increase the contractility of intestinal smooth muscle cells in mice (Zhao et al., 2003), reducing intestinal transit time (Masure et al., 2013). Increased intestinal motility combined with mucin secretion may hinder the parasitization in the early stage, promoting worm expulsion (Stehr et al., 2018). In terms of IL-5 and IL-9, these interleukins may respectively accelerate the recruitment of eosinophils and mast cells. Meanwhile, these recruitments help to promote the polarization to a Th2 response (Cortés et al., 2017).

In mammals, cytotoxic T cells may also participate in eliminating helminths. The worm expulsion peak coincided with the peak of cytotoxicity related mRNA transcription in IELs in pigs (Masure et al., 2013). Molecules like granzymes, perforin and NKG2D expressed by IELs points to a role of cytotoxic T cells (van Wijk and Cheroutre, 2009). Innate molecules like nitric oxide (NO) may also be beneficial to worm expulsion. Nitrite is one of the stable end products of NO catabolism. In culture of murine peritoneal lavage cells with LPS and naïve *Fasciola hepatica*, the nitrite level was negatively correlated with the survival rate of the parasites (Piedrafita et al., 2001). There is a similar phenomenon described in avian species as chickens infected with *Escherichia coli* (*E. coli*) before *A. galli* infection had a significantly lower worm burden than those solely infected with *A. galli* (Permin et al., 2006). LPS on the bacterial membrane of *E. coli* is also a potent inducer of NO production from chicken macrophages (Crippen et al., 2003). Thus, there is a possibility that NO is correlated to *A. galli* expulsion in the chicken.

1.6 Anthelmintic effect of plant compounds

A number of *in vitro* and *in vivo* studies of various medicinal plant have been carried

out to study the anthelmintic effect against *A. galli* (Table 1) However, some studies have been contradictory and others are not repeatable.

Table 1. Examples of plants with effects on A. galli used for in vitro or in vivo studies (adapted from Zaman et al., 2021)

<i>In vitro</i> studies	<i>In vivo</i> studies
<i>Citrus limon</i>	<i>Citrus limon</i>
<i>Zingiber officinale</i>	<i>Zingiber officinale</i>
<i>Ocimum sanctum</i>	<i>Azdirachta indica</i>
<i>Momordica charantia</i>	<i>Carica papaya</i>
<i>PolygonumHydropiper</i>	<i>Momordica charantia</i>
<i>Swietenia macrophylla</i>	<i>Morinda citrifolia</i>
<i>Morinda Citrifolia</i>	<i>Tephrosia vogelli</i>
<i>Thespesia Lampas</i>	<i>Vernonia Amygdalina</i>
<i>Sesbenia grandiflora</i>	<i>Anasus comosus</i>
<i>Murraya koenigii</i>	<i>Datura metel</i>
<i>Cassia occidentalis</i>	
<i>Acanthus ilicifolius</i>	
<i>Acacia oxyphylla</i>	
<i>Securidacalolongepedunculata</i>	
<i>Achillea wilhelmsii</i>	
<i>Teucriumstocksianum</i>	
<i>Alium sativum</i>	
<i>Cleome viscosa</i>	
<i>Carica papaya</i>	
<i>Mentha longifolia</i>	
<i>Cassia angustifolia</i>	
<i>Curanga fel-terrae</i>	
<i>Aloe secundiflora</i>	

The anthelmintic effect of medicinal plants may be associated with secondary metabolites of plants (Zaman et al., 2020) (Figure 3). The secondary metabolites of plants can usually be divided into four groups regarding their biosynthetic origin and chemical structure, which are terpenes, phenolics, sulphur and nitrogenous compounds (Hrckova and Velebny, 2012)

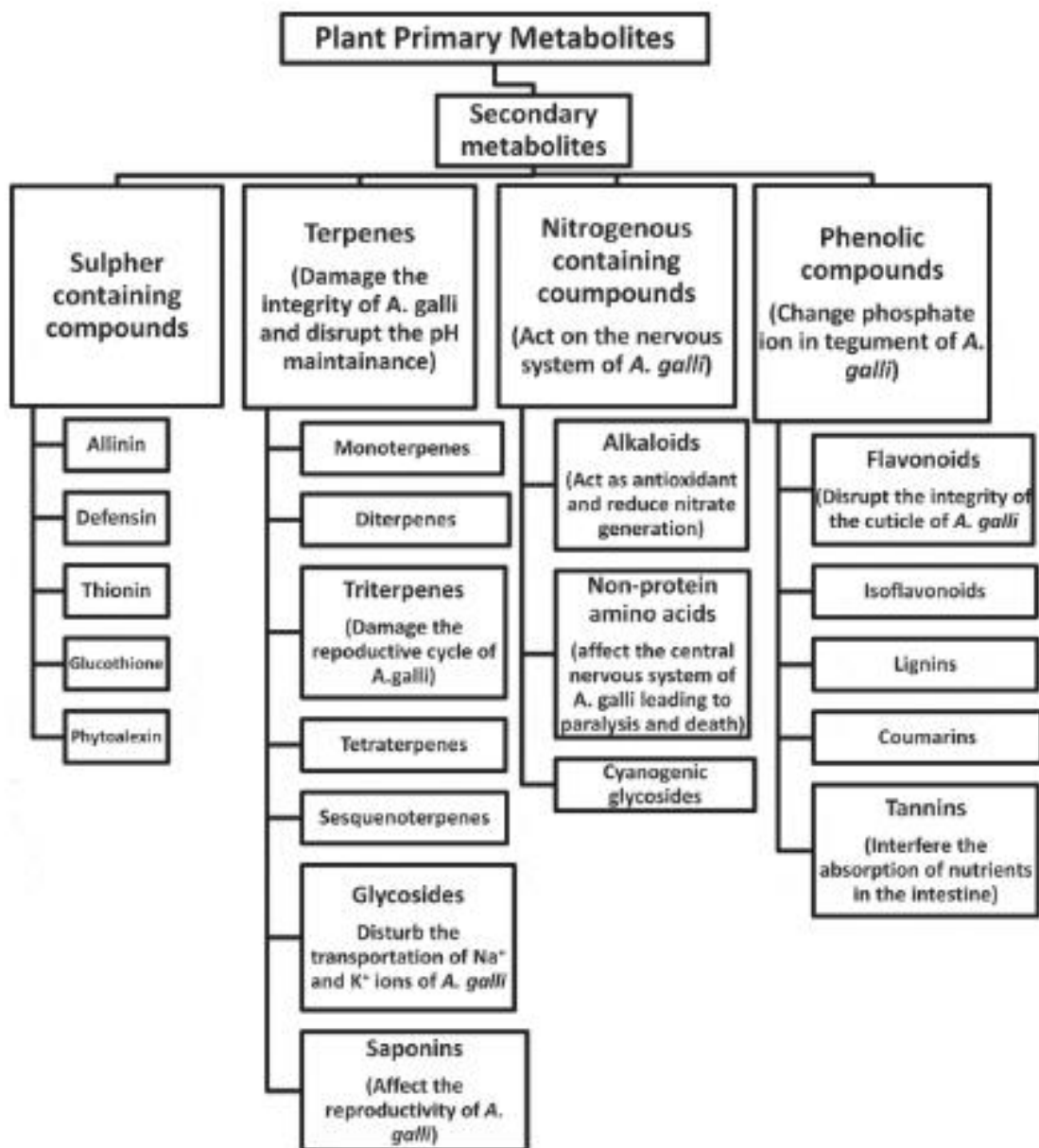


Figure 3. Examples of anti-parasitic secondary metabolites from medicinal plants (Zaman et al., 2020)

Terpenes are primary components of the essential oil from various flowers and plants, such as sage, lavender and rosemary (Omar et al., 2016). Their efficacy against *A. galli* mainly depends on their effect on damaging the integrity of *A. galli* and disrupting the pH and maintenance of organic ions (Zaman et al., 2020). Citron peels extract, which contains a large amount of terpenes, was in a single study shown to reduce *A. galli* worm burden with up to 87.5% (Abdelqader et al., 2012).

Nitrogenous compounds are present in plants in the forms of alkaloids (AK) and non-

protein amino acids (NPAA). The plants rich in AK impact the *A. galli* nervous system, and suppress sugar uptake (Roy et al., 2010, Badarina et al., 2017). NPAA works on the central nervous system of *A. galli* as well, leading to paralysis and death of the worms (Symeonidou et al., 2018).

Phenolic compounds (PC) produced by plants are also regarded as anti-herbivore compounds (Pratyusha, 2022). Plants containing PC show an antiparasitic effect against *A. galli* (Ohri and Pannu, 2010), with the possible mechanism relying on disrupting the phosphatase enzyme in the tegument of the parasites (Swargiary and Roy, 2015). Flavonoids, one of the abundant PC, commonly presented in various plants like onion, aronia berry garlic and broccoli (Dai and Mumper, 2010), can block the phosphorylation reaction in parasites, inhibiting the energy supply (Sharma and Prasad, 2014).

The toxicity and odor of sulfurs-containing compounds (SCC) are essential for plants to defend themselves against pathogens and pests (Nwachukwu et al., 2012). Garlic contains about 33 different kinds of organosulfur compounds (Omar and Al-Wabel, 2010), and some studies suggest that garlic has anthelmintic effects (Gharavi et al., 2011, Kavindra and Shalini, 2000). However, the specific knowledge on the mechanism of SCC in garlic against *A. galli* is still lacking. In the current study the effect of garlic and aronia berry extracts on *A. galli* were studied.

1.7 Antiparasitic and immunomodulatory effects of garlic

Even though garlic contains various kinds of organic sulfides, saponins, phenolic compounds and polysaccharides (Newall, 1996) that provides garlic with massive potential for anthelmintic and immunomodulatory effects, preparation of extracts is a key point. Prepared in different solutions, the relative amounts of the bioactive garlic compounds will vary and hence modify the immune response in versatile ways (Arreola et al., 2015)

Allixin is the main compound of garlic in ethanol and water extracts (Jiang et al., 2020). Studies of murine peritoneal macrophages showed that allixin can increase the pro-

inflammatory cytokine and NO production (Kang et al., 2001), induce a Th1 response in low doses and induce a Th2 response in high doses (Liu et al., 2009). Regarding parasite infection, allicin showed a promising antiparasitic effect, but the stimulated immune responses are diverse. In mice, administration of allicin can significantly reduce the worm burden of *schistosomes* but it reduces pro-inflammatory cytokines such as Tumor necrosis factor- α (TNF- α) and Interleukin 6 (IL-6) (Metwally et al., 2018). However, in another study allicin increased the pro-inflammatory cytokines and NO production, promoting the recruitment of macrophages and dendritic cells in mice (Feng et al., 2012). Thus, garlic has different immunomodulatory and antiparasitic potential in the presence of different parasitic infections, and the effect is highly dependent of dose and extraction method.

Even though convincing *in vivo* studies of using garlic against *A. galli* infection are still lacking, several *in vitro* studies reveal that garlic is a potential candidate for *A. galli* control *in vivo*. Prepared by water extract, garlic showed an antiparasitic effect of *Leishmania major* promastigotes and increased IL-12 and IL-10 levels in an infected J774 macrophages cell line (Gharavi et al., 2011). By investigating adult worms *in vitro* it was shown that garlic oil can significantly increase the mortality of *A.galli*, along with the reduction of glucose uptake, glycogen content, and oxygen consumption (Kavindra and Shalini, 2000). Dichloromethane extract of garlic bulbs, which mainly contain organic sulfides, also killed *A. galli* efficiently in an *in vitro* parasitology study (Krstin et al., 2018). Despite promising evidence, the interaction between garlic and the intestinal immune system as well as dosage/preparation for optimal antiparasitic effect during *A. galli* infection still needs further investigation.

1.8 Antiparasitic and immunomodulatory effects of aronia berry

Aronia berry (AB) have shown beneficial effects as immunomodulatory agent due to its polyphenolic compounds (Ho et al., 2014). In a recent study, the best source of phenolic acids in berries other than rowanberry was identified as aronia berry(96 mg/100 g FW) (Mattila et al., 2006). The polyphenols in aronia berry includes proanthocyanidins,

anthocyanins, phenolic acids, flavonols, and flavanols (Oszmiański and Lachowicz, 2016, Denev et al., 2013). As mentioned previously, flavonoids are known to specifically block the phosphorylation reaction of parasites, inhibiting the energy supply. Furthermore, plants containing phenol compounds was shown to have inhibitory effect on the development of *A. galli*. For instance, Aloe Secundiflora Crude Extracts, which mainly consist of mixture of phenolic compound such as athrones, chromones and phenyl pyronesm can hinder the the development of *A. galli* eggs to L3 stage (Kaingu et al., 2013).

Generally, AB show anti-inflammatory effects, which are associated with polyphenol compounds like anthocyanins (Joseph et al., 2014). AB was reported to alleviate Colitis in a murine model, specifically reducing oxidative stress (Pei et al., 2019). Furthermore, feeding the mice with 2g/kg AB extract show a 50% reduction of ethanol-induced gastric mucosal damage (Matsumoto et al., 2004). When RAW 264.7 mouse macrophages incubated with AB, the NO production was inhibited (Ho et al., 2014). However, another study reported that AB increased NO production of macrophages isolated from C57BL/6 mice and promoted phagocytosis (Gajic et al., 2020). Especially, the abundance of phenolic compounds makes AB an interesting plant candidate with both immunomodulatory and anthelmintic potential for use in chickens.

2. Materials and Methods

2.1 Preparation of plant extracts or feed additives

Powder from two plant types were used in the study: garlic (*Allium sativum*) and aronia berry (*Aronia melanocarpa*). Aronia berry powder, consisting of dehydrated pulp, peel and pips from the berry, was purchased from <https://www.elkaerholm.dk/>. Garlic, a kind gift from Martin Jensen, FOOD, Aarhus University, was sliced and freeze-dried before pulverization. For *in vitro* studies, the plant extracts were either prepared with autoclaved MilliQ water (w) (0.05 g/ml) and filtered through Whatman paper with the pore size of first 11 µm and second with 5 µm or being a pre-made 70% ethanol extract (e) (garlic 70% ethanol extract was a Gift from Dr Marina Spinu, USAMV Faculty of Veterinary Medicine, Romania). For the *in vivo* study, plant material was formulated into the chicken feed.

2.2 Isolation of peripheral blood mononuclear cells (PBMC)

Blood samples were obtained from the jugular vein and collected into Vacuette® tubes coated with 18 IU/ml Sodium Heparin (Greiner Bio-One, cat. no. 454030). 4ml of peripheral blood from each animal were diluted with an equal amount of phosphate-buffered saline (PBS, pH 7.4). Diluted blood was added into two tubes each with 3ml of Ficoll-Paque® reagents and centrifuged 400×g for 25 minutes. Then, the interphase was transferred to two tubes each with 10ml of PBS and centrifuged 300×g for 10 minutes. After discarding the supernatant, the cells were washed with 5ml of PBS and centrifuged 300×g for 10 minutes. Afterwards, the supernatant was discarded, and cells were resuspended in RPMI 1640 culture medium (cat. no. BE12-115F/U1, Lonza) at a concentration of 1×10^7 cells/ml.

2.3 HD11 cell line culture

The avian MC29 virus-transformed macrophage cell line, HD11 was a gift from Dr. Bernd Kaspers, Ludwig Maximilians Universität, München, Germany). Initially, 1×10^6 HD11 cells were placed in a T75 culture flask with 15ml of Culture Media (CM):

RPMI 1640 medium with glutamine and 10% Fetal bovine serum (FBS, Gibco, cat. no. 10082147), 100U/ml penicillin and 100µg/ml streptomycin (PEST, Gibco, cat. no. 15140-122). The cells were maintained at 41°C, 5% CO₂ and regularly passaged when reaching 80 - 90% confluence.

2.4 Plant effect on PBMC mitogen stimulation

The experiment was based on blood samples from the inbred white leghorn chicken line L133 kept at Aarhus University homozygous for the Major histocompatibility complex (MHC) haplotype B13. Breeding and MHC haplotyping of the chickens were as earlier described (Larsen et al, 2019). PBMC were isolated from three adult animals with the Ficoll method described above. To decrease assay background viability of the PBMC was tested using decreasing amounts of added FBS or chicken serum (CS). It was impossible to reduce serum content to below 2.5% and CS provided the best viability for the cells. Hence, the PBMC (1×10^6 cells/well) was incubated in RPMI with 2.5% CS with or without 5 µg/ml Concanavalin A (ConA) (Sigma-Aldrich Merck, cat. no. C5275) and different plant extracts in two identical 96-wells plates (Nunclon Delta Surface plates, ThermoScientific, cat. no. 163320). In advance, cell viability was also determined with varying plant concentrations and the highest volume/volume %(v/v%) that did not jeopardize cell viability was chosen for the experiment. The experimental setup is shown in Table 3 below, and each treatment was set up with three biological replicates and three technical replicates. After 24 hours of incubation, 10% v/v alamarBlue (Thermo Fisher, cat.no. DAL1025) was added to one of the plates, and fluorescence was measured (excitation 530nm, emission 590nm) after 2 hours continued incubation in 41°C, 5% CO₂. In the alamarBlue assay, an oxidation-reduction (REDOX) reaction of the resazurin, the active ingredient of alamarBlue will take place, thus undergoing a colorimetric change which indicate a response to cellular metabolic reduction. Hence, metabolically active cells convert resazurin to resorufin, a red-fluorescent indicator (Figure 4).

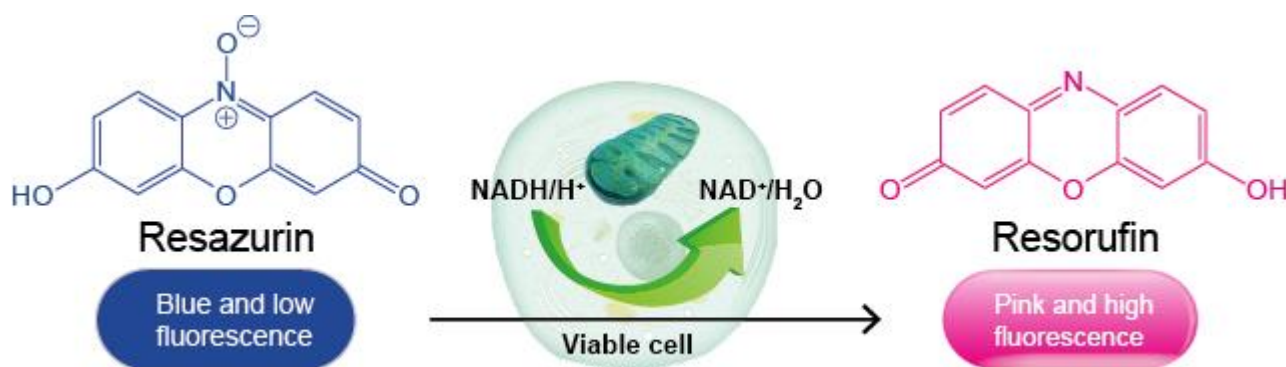


Figure 4. REDOX reaction induced by mitochondria from ABP Biosciences:

<https://www.abpbio.com/product/cell-quant-amarblue-cell-viability-reagent/>

The second plate was kept in 41°C, 5% CO₂ for a total of 48 hours and then used for flow cytometric analysis of T cell activation (Table 6, panel 1).

Table 2. Plant extracts used in the PBMC assays with or without ConA stimulation

Stimulation	Extract	Final extract concentration (v/v)
RPMI or ConA	Aronia berry water extract (w)	1 %
	Garlic water extract (w)	0.15%
	No plant control	0 %
	Garlic ethanol extract (e)	1.5%
	70% ethanol control (e)	1.5%

2.5 Plant effect on HD11 cell line stimulation

The supernatant was removed from the culture bottle whereafter cells were washed with 5ml of PBS. Cells were detached using trypsin/ethylenediaminetetraacetic acid (EDTA) (Lonza, cat. no. BE17-161F) and washed with 5ml of RPMI by centrifugation at 150xg for 5 minutes. Next, the supernatant was removed, and the cells were resuspended in CM without FBS and PEST. The cells were placed in 96-wells plates using 80.000 cells/well. A pre-experiment determined that 80.000 cells per well provided close to 100 % confluence after 18 hours of incubation and hence this cell concentration was used though-out the experiment. After 5 hours of rest in 41°C, 5% CO₂, the cells were

stimulated with LPS (0.5 µg/ml) or *A. galli* antigen (54 µg/ml or 108µg/ml) combined with different plant extracts. The *A. galli* antigen was prepared by from worms isolated from the intestine of infected commercial chickens. The worms were washed briefly in 70% ethanol and stored in PBS, pH 7.4) at -20°C until further processing. The worms were shredded with scissors in PBS and mixed thoroughly on a whirl mixer. Subsequently the extract was centrifuged at 1700×g (10 minutes, 4°C) and the supernatant was filtered through a 5-µm filter followed by a 0.45-µm filter. Finally, the filtrate was centrifuged at 12,000×g (10 minutes, 4°C) and treated for 30seconds with ultrasound. The experiment setup is shown in Table 3. 18 hours later, 100 µl of supernatant was removed and kept at -20°C for nitric oxide (NO) analysis. To the cells remaining in the plate, 10% v/v alamarBlue was added and fluorescence was measured (excitation 530nm, emission 590nm) after 2 hour of continued incubation in 41°C, 5% CO₂.

Table 3. Plant extracts used in the HD11 assays with or without LPS and/or A. galli antigen stimulation

Stimulation	Extract	Final extract concentration (v/v)
RPMI, LPS, <i>A. galli</i> , LPS & <i>A. galli</i>	Aronia berry water extract (w)	1 %
	Garlic water extract (w)	0.15%
	No plant control	0 %
	Garlic ethanol extract (e)	1.5%
	70% ethanol control (e)	1.5%

2.6 Nitric oxide assay

Nitric Oxide (NO) production by activated HD11 cells was measured by the Griess Assay. Dilution of the standard stock in PBS was added to the 96-wells plate as standard series. The samples were diluted in PBS, and 100 µl of each sample was added to each

well. Griess reagent (50 µl of 1% sulphanilamide and 50 µl of 0.1% naphthalene diamine – both in a phosphorus acid solution (H₃PO₄), Sigma-Aldrich cat. no. G4410) was added to the plate and incubated for 10 minutes at room temperature (RT) in order to let the Griess reagent turn purple upon reaction with nitrite ions in the cell culture supernatant. The optical density (OD) at 540 nm of each well was measured to determine the nitrite concentration of each sample according to the standard curve.

2.7 Animal material for *in vivo* study

Ninety-six 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 1 week pre infection, the hens were 18 weeks old. All hens received worm treatment via the drinking water with Fenbendazol (Panacur Aquasol, 2 mg fenbendazole pr. kg bodyweight pr. day) during the first 5 days to make sure none of the hens had worm infection at the beginning of the *in vivo* experiment. The hens were moved the experimental facilities after the deworming.

2.8 Animal trial study design

The study design consisted of 4 treatments and 4 repetitions/treatment (Table 4). After deworming, the hens were distributed randomly in 16 pens with 6 hens/pen which were distributed equally into two rooms. Each pen was equipped with a ladder and two nest boxes. Wood shavings were used as litter material. The temperature in the facilities was 21°C, and the light-dark cycle was 14 hours of light and 10 hours of darkness.

Table 4: Animal study design overview

Treatment #	Feed stuff	Number of pens	Number of hens per pen
1	A - Control	4	6
2	B – 1% Garlic	4	6
3	C – 1% Aronia	4	6
4	D – 0.5% Garlic and 0.5% Aronia	4	6
Total		16	96

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 from the Danish Animal Experiments Inspectorate (license no. 2017-15-0201-01211).

2.9 Infection material

Harvesting of *A. galli* eggs was performed in July and August 2021 at the Institute of Animal Science, Aarhus University. The *A. galli* eggs were harvested at least 30 days pre infection. 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 harvested by two methods: 1) Eggs were extracted from female worms under a microscope, and the eggs were embryonated in 0.1 N H₂SO₄ at RT for 30 days as previously described (Permin et al., 1997). Afterwards they were stored at 4°C until inoculation. 2) Female worms were cultured for 3 days in RPMI 1640 at 37°C (with 100 units/ml penicillin, 100U/ml of streptomycin, 250 ng/ml Amphotericin B). The media was changed every 24 hours, and eggs oviposited into the media were collected

by centrifugation(260xg for 7 minutes) of spent media and concentrated eggs were embryonated in 0.2 M H₂SO₄ at RT for 30 days.

When fully formed nematodes larvae were visible within the shell the eggs were judged to be infective(stage L3). Five days before inoculation, the number of infective eggs were counted as infective eggs per ml. On the day of inoculation, a suspension of 0.2 M H₂SO₄ with an amount of 1000 infective eggs (eggs isolated from both methods and mixed 1:1) was given orally.

2.10 Feed

During the deworming all hens were fed diet A. All diets were similar regarding their nutritive values. The compositions of the experimental diets used after deworming are shown in Table 5. Water and feed were provided *ad libitum*.

Table 5: Feed composition per consistently 100g of diets used during the animal experiment.

Nutrient	A - Control	B - Garlic	C - Aronia	D - Garlic + aronia
Wheat	43.93	42.93	42.93	42.93
Oat	8.00	8.00	8.00	8.00
Triticale	4.00	4.00	4.00	4.00
Sunflower cake	8.00	8.00	8.00	8.00
Soybeans (toasted)	9.00	9.00	9.00	9.00
Fishmeal (standard)	3.00	3.00	3.00	3.00
Calcium carbonate	4.00	4.00	4.00	4.00
Oyster shells	4.25	4.25	4.25	4.25
Rapeseed oil	0.20	0.20	0.20	0.20
Rapeseed cake (9%)	6.52	6.52	6.52	6.52
Soybean cake (toasted)	8.00	8.00	8.00	8.00
Monocalcium phosphate	0.30	0.30	0.30	0.30
Vitamin /Mineral DLG kylling slut	0.40	0.40	0.40	0.40
Salt	0.18	0.18	0.18	0.18
Sodium bicarbonate	0.18	0.18	0.18	0.18
Choline chloride	0.04	0.04	0.04	0.04
Garlic	-	1.00	-	0.50
Aronia	-	-	1.00	0.50
Total	100	100	100	100

2.11 Experiment samples outline

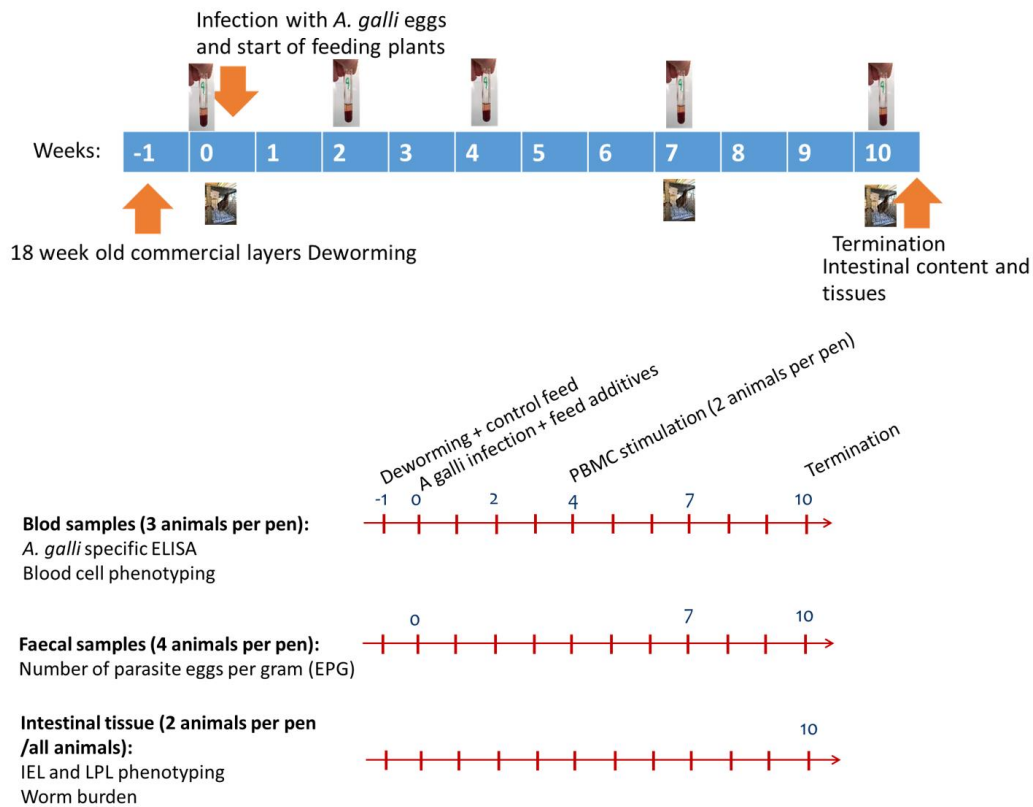


Figure 5. Sample overview of the 10 week *A. galli* experiment

a) Egg production

Eggs were collected every day in the pens. Once a week, the number of eggs was counted, and the weight of the eggs was measured. The egg production and the average weight of eggs were calculated with the following formula:

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

$$\text{Average Weight of eggs (gram)} = \text{Weight of total eggs in pens} / \text{Total number of eggs in the pen}$$

b) Faeces

Faeces samples were collected from four animals in each pen in weeks 0, 7 and 10 post infection(p.i) using the same animals all weeks. The samples were used to count the number of parasite eggs per gram faeces (EPG)

c) Blood

Blood samples were collected from the jugular vein of three fixed animals in each pen in weeks 0, 2, 4, 7, and 10 post infection. The sample was divided into a 2 ml-BD Vacutainer® tube coated with K3-EDTA (BD Bioscience, cat. no.367836) as well as into a tube without anticoagulant. The EDTA stabilized blood was used within a few hours for the whole blood leucocyte flow cytometry assay (Table 6, panel 2). Blood samples in serum tubes were placed at RT for a minimum 2 hours after sampling and stored at 4°C over night. The samples were placed at RT for 10 minutes before centrifuged at 1850xg for 10 minutes at 20°C. Serum was transferred to Eppendorf tubes and stored at -20°C until further use. The Serum was collected for Enzyme-linked immune sorbent assay (ELISA) to test the *A. galli* specific IgY titers.

d) Intestinal Tissue

At the end of the experiment, 2*6 cm of jejunum were cut right above the site of the MD from the same animals used for blood sampling at week 4. Then, the tissues were flushed with PBS and transferred to a tube containing PBS with 1% PEST. Samples were stored on ice until preparation of single cell suspensions for flow cytometry.

2.12 Faecal shedding of *A. galli* eggs (EPG) and worm burden

The number of *A. galli* EPG was counted by the McMaster counting technique (Henriksen & Aagaard,1976). Briefly, 2-3 g of faeces sample was diluted with 14ml of water and filtered with two layers of gauze. Ten ml of each diluted faeces sample was centrifuged at 300xg for 5 minutes. After removing the supernatant, flotation fluid (375g/l glucose monohydrate and 250 g/l sodium chloride in distilled water) was added to the sediment until the total volume of the solution reached 4ml. Subsequently, the samples were transferred to McMaster counting chambers and left for 3-5 minutes before eggs were counted under a microscope. The slides had two chambers which are T1 and T2. EPG was calculated with the following formula:

$$EPG(\text{eggs/gram})=(T1+T2)*20$$

All the birds were sacrificed at the end of the experiment, and adult *A. galli* worms were collected from their intestine and counted, determining each individual bird's worm burden. Furthermore, the worms from the birds used for blood sampling were sexed to calculate the sex ratio between male and female worms.

2.13 *A. galli* specific IgY in serum

A. galli antigen was prepared as described above and this crude extract served as the coating antigen at the concentration of 5 µg/ml in coating buffer (0.15 M Na₂CO₃ and 0.35 M NaHCO₃). 100 ml of coating *A. galli* solution was added to the 96-well microtiter plates and incubated at 4°C. The next day, plates were washed three times with 300ml wash buffer (PBS with 0,1 % bovine serum albumin (BSA, Roche, cat. no. 10735086001)). Subsequently, PBS with 0.5% BSA was used to block the residual protein-binding sites. After 45 minutes of incubation on a shaking table at RT and washing with wash buffer, 100 µl controls and serum samples were added. After two hours, plates were washed with wash buffer, and 100 µl Horseradish peroxidase-conjugated polyclonal goat anti-chicken IgG (IgY)-Fc antibodies (Bio-Rad, cat no. AAI29P) were added as detection antibody and incubated for one hour. Finally, the plates were washed three times and 3,5,3',5'-tetramethylbenzidine (TMB, Invitrogen, cat. no. SB02) was used for visualization of antibody binding. 15 minutes later, 1 M H₂SO₄ was added to stop the reaction. The *A. galli* specific IgY concentration was determined by spectrometric values measured at absorbance 450nm with absorbance 650nm as a reference wavelength. A dilution series of a highly positive sample was used as standard, and the highest titre was set at the relative value 2. A high titre sample and a negative sample were included on all plates in order to correct for inter-plate variation.

2.14 PBMC mitogen and antigen re-stimulation

At week 4 p.i, the peripheral blood sample was taken from two animals from each pen. PBMC was isolated with the Ficoll method mentioned above. The PBMC (1×10^6

cells/well) was stimulated with ConA (5 µg/ml) or *A. galli* antigen (1.08 mg/ml) or left as medium controls. Cells were incubated at 41°C, 5% CO₂ for 72 hours before flow cytometry analysis of T cell activation (Table 6, panel 3).

2.15 Isolation of intra-epithelial lymphocytes (IEL) and lamina propria leukocytes (LPL)

The intestinal samples were incubated with PBS-Dithiothreitol (PBS/DTT; PBS w/o Ca and Mg, 2mM DTT) for 5 min at 37°C in shaking incubator (150 rpm). The suspension was filtered by a coarse metal sieve and the supernatant was discarded. The tissue was then transferred back to the bottle, rinsed with PBS and re-filtered through the metal sieve. Next, the tissue was incubated with 10 ml 2 mM EDTA in RPMI for 30 minutes at 37°C in a shaking incubator (150 RPM) after which, cells were passed through a 70 µm cell strainer and the IEL fraction was harvested. The EDTA isolation procedure was repeated to obtain a second IEL fraction. The remaining tissue was transferred back to the tube and rinsed with PBS to remove the EDTA residues and next incubated with 20 ml RPMI-collagenase (25 mg/mL Collagenase type 1 (Gibco,cat.no.17018-029) in R10 medium (RMPI 1640, 10% FCS and 1% PEST)) for 1 hour at 37°C. After the incubation, the supernatant was harvested as the LPL fraction. Finally, the IEL fractions were mixed in one tube. Both IEL and LPL were centrifuged (300 x g for 10 min.) and washed with PBS. Afterwards, the cells were resuspended in R10 medium and the number of cells was counted before flow cytometry analysis of relative subset frequencies (Table 6, panel 4).

2.16 Flow cytometric analyses

a) Plant effect on PBMC mitogen stimulation

After 48 hours of stimulation, 2 mM EDTA was added to detach activated T cells, which tend to stick to plastic. Subsequently, each sample was incubated with 5µl of antibody master mix for 15 minutes at RT (Table 6 panel 1). The cells were washed twice with 100 µl FACS buffer centrifugation at 600 x g for 5 minutes. Finally, the supernatant

was removed, and the cells were suspended in 200 µl of Fluorescence-activated cell sorting (FACS) buffer (0.2% BSA, 0.2% sodium azide, 0.05% normal horse serum in PBS), and 50 µl acquired by the flow cytometer.

b) Whole blood leucocyte counts

50 µl of EDTA stabilized blood from 4 animals in each pen were diluted 25 times with FACS buffer. From each animal, 50 µl of diluted blood were added to the wells in a 96-well plate and incubated with the antibody master mix (Table 6, panel 2) at 4°C for 20 min 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. Finally, 40 µl of each sample was acquired on the flow cytometer, and the number of leucocytes was calculated according to 123count eBeads™ manufacturer's instructions.

c) PBMC mitogen and antigen re-stimulation

The procedure was the same as mitogen stimulation under b), but labelling was performed using a different antibody master mix (Table 6, panel 3)

d) IEL and LPL phenotyping

100 µl of IEL or LPL were added to wells in 96 well plates. Afterwards, the plates were centrifuged at 600 x g for 5 minutes, and the supernatant was removed. The cells were then incubated with 100µl of antibody master mix (Tabel 6, panel 4) for 15 minutes in darkness at RT. After the incubation, 100 µl of FACS buffer was added and centrifuged 600 x g for 5 minutes to wash the cells. Next, 100 µl secondary antibody or streptavidin was added in Brilliant stain Buffer (BD Biosciences, cat. no. 563794) (diluted 1:1 with FACS buffer) (final dilution of BV659 and BV786 reagent 1:1000)) and incubated for 15minutes in RT. Washing was repeated three times with FACS buffer before resuspending the cells in 100 µl of FACS buffer, 50 µl was acquired on the flow cytometer.

Table 6. Monoclonal antibodies used for immunolabelling, and combinations (panels) used in the different experimental sets

Abbreviation	Clone	Specificity	Fluorochrome	Panels			
				1 ^f	2	3 ^g	4 ^h
CD8a-FITC ^a	3-298	Chicken CD8 α	Fluorescein	X			X
						X	
TCR1-PE ^a	TCR-1	Chicken TCR $\gamma\delta$	R-phycoerythrin	X	X		
CD25-A647 ^b	13504	Chicken IL-2R α (CD25)	Alexa fluor [®] 647	X			
CD4-pBlue ^a	CT-4	Chicken CD4	Pacific Blue [™]	X			X
CD41/61-FITC ^b	11C3	Chicken integrin CD41/61	Fluorescein		X		
CD45 ^b -PerCP/ Cy5.5 ^d	UM16-6	Chicken CD45	Peridinin chlorophyll- cyanine 5.5		X		
CD3-A700 ^a	CT3	Chicken CD3	Alexa fluor [®] 700		X		X
Kul-01-A647 ^a	KUL01	Chicken monocyte/macrophage	Alexa fluor [®] 647		X		
Bu-1-pBlue ^a	AV20	Chicken Bu-1	Pacific Blue [™]		X		
TCR1-FITC ^a	TCR-1	Chicken TCR $\gamma\delta$	Fluorescein			X	
MHCII-PE ^a	2G11	Chicken MHC class II β -chain	R-phycoerythrin			X	
CD25 ^b - PerCP/Cy5.5 ^d	AV142	Chicken IL-2R α (CD25)	Peridinin chlorophyll- cyanine 5.5			X	X
CD4-A700 ^a	CT-4	Chicken CD4	Alexa fluor [®] 700			X	
Bu-1-PE ^a	AV20	Chicken Bu-1	R-phycoerythrin				X
TCR1 ^a -APC/Cy7 ^d	TCR-1	Chicken TCR $\gamma\delta$	Allophycocyanin- cyanin 7				X
CD45 ^b -BV650 ^c	UM16-6	Chicken CD45	BD Horizon [™] BV650				X
MHCII ^a -BV786 ^c	2G11	Chicken MHC class II β -chain	BD Horizon [™] BV786				X

X = used in panel

^a Purchased from Southern Biotech (www.southernbiotech.com).

^b Purchased from BIO-RAD AbD Serotec (www.abdserotec.com).

^c Isotype specific secondary, BV650 rat anti-mouse IgG2a (BD Biosciences cat.no. 744532).

^d Fluorochrome conjugation by Lightning-Link[®] kits from Abcam (www.abcam.com).

^e MHC-II antibody was biotinylated – staining obtained by use of BV786 labelled streptavidin (BD Biosciences, cat. no. 563858).

^f Panel combined with Sytox[™] Blue (Invitrogen cat.no. S11348) for dead cell exclusion.

^g Panel combined LIVE/DEAD[™] fixable Near-IR dead cell stain kit (Invitrogen, cat. no. L10119) for dead cell exclusion.

^h Panel combined with BD Horizon[™] Fixable Viability Stain 510 (BD Biosciences, cat. no. 564406) for dead cell exclusion.

All flow cytometric analyses were conducted on a BD FACSCelesta™ flow cytometer and analyses of acquired samples were performed in the FACS Diva software. 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.

2.17 Statistical analysis

All statistical analysis and visualization were performed in the R studio. Shapiro-Wilk's test was used to test for normality. Bartlett's test was applied to test the homogeneity of variance across groups. If the data within the group was normally distributed and the variance across groups was the same, ANOVA was applied to test the significant difference between the group followed by a Tukey HSD if the ANOVA was significant. If the data was normally distributed, but the variance was not homogeneous, Welch one-way test were applied followed by a pairwise t test if significant. If the data was not normally distributed, the Kruskal Wallis test were applied followed by a Dunn test if significant. After the statistical analysis, the visualization of the results was achieved by the ggplot2 package (Wickham, 2009) or the ggbubr package (Kassambara, 2017).

3. Result

3.1 *In vitro* study

3.1.2 Plant effects on PBMC with or without mitogen (ConA) stimulation

In vitro studies were performed on PBMC isolated from hens of the chicken line L133 to determine the effect of the plant extracts on the cells by using the alamarBlue assay to assess mitochondrial activity or by flow cytometric analysis to assess T cell activation. The lectin ConA is known to bind to a mannose moiety of cell surface glycoproteins like the T cell receptors and hence inducing polyclonal activation and proliferation of the cells(Weiss and Imboden, 1987). In addition to plant effects on mitogen stimulation, also plant effects on resting cells in culture medium alone were investigated.

3.1.3 Plant effects measured by alamarBlue™ fluorescence (PBMC)

The oxidation-reduction sensitive dye alamarBlue, a fluorometric indicator of metabolic activity, was used as a tool for examining mitochondrial activity. The effects of the plants on alamarBlue fluorescence were measured after 24 hours of incubation of PBMC with 5µg/ml ConA or kept in media alone (Figure 6). The early time point was chosen in order to ensure that the measured output reflected increased mitochondrial activity rather than cell proliferation i.e. at 24 hours no cell division had occurred and hence the number of cells were identical in all wells. In addition, the ConA concentration was chosen from a preliminary experiment to secure minimum impact on cell viability (data not shown).

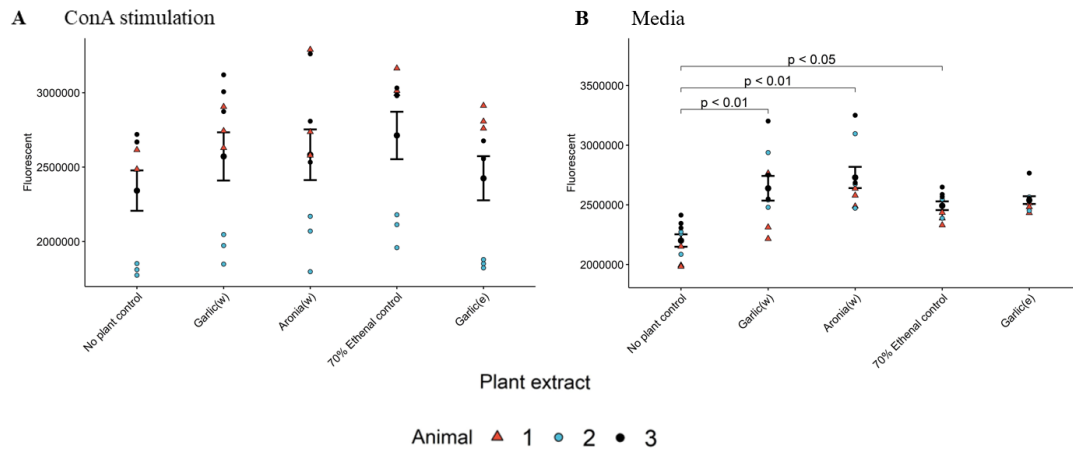


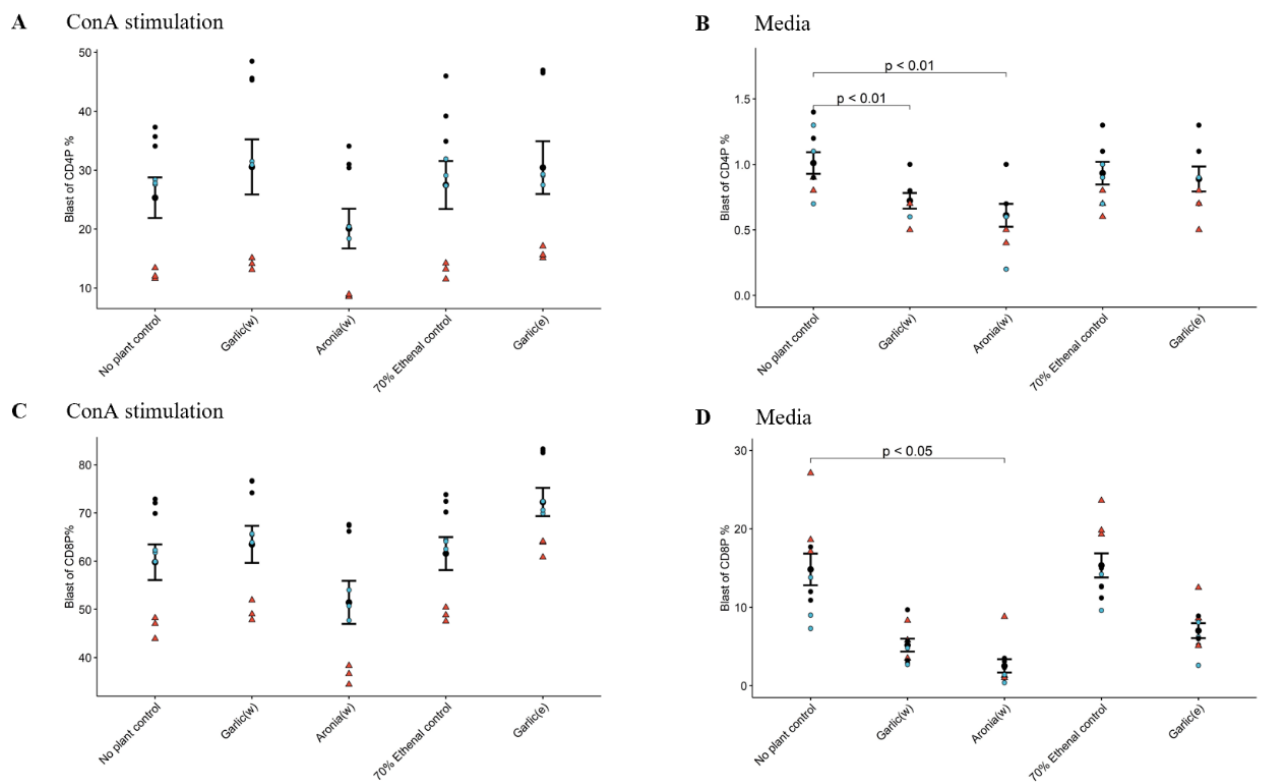
Figure 6: Effect of plants on PBMC mitochondrial activity measured using an alamar Blue assay. Plant extracts were either ethanol (e) or water based (w). Results are shown as mean fluorescence \pm SE, and individual values (three technical replicates from three animals) are shown as well. A) PBMC mitochondrial activity after 24 hours with mitogen stimulation ($5\mu\text{g/ml}$ ConA). B) PBMC mitochondrial activity after 24 hours in culture medium alone (no mitogen stimulation).

When stimulated with ConA, garlic(w) and aronia(w) tended to increase the fluorescence signal of alamarBlue as compared to the respective no plant control. garlic(e) tended to decrease the fluorescence signal as compared to the respective ethanol control. However, the differences were not statistically significant (Figure 6A). In media alone without mitogen stimulation, garlic(w) and aronia(w) significantly increased ($p < 0.01$) the fluorescence signal as compared to the no plant control, indicating a positive effect on mitochondrial activity. Adding 70% ethanol also increased the fluorescent signal significantly ($p < 0.05$) as compared to the no plant control but garlic(e) showed no effect as compared to the respective ethanol control (Figure 6B).

3.1.4 Plant effects measured by flow cytometric analysis of T cell activation (PBMC)

Subsets of T cells from PBMC samples were analyzed using flow cytometry (gating

strategy in Appendix Fig. 18). The measured outputs indicative of T cell activation were surface expression of the activation marker CD25 and lymphoblastogenesis i.e. increased volume by nucleus/cytoplasm growth and mRNA/protein synthesis reflected by increased size (FSC) and granularity (SSC) of the cells. No major plant effects were seen on $\gamma\delta$ T cells (TCR1+) (data not shown). Hence the analysis focused on TCR1-cells indicative of conventional T cells with the $\alpha\beta$ version of the TCR. Frequencies of cells with lymphoblast properties (Blast) or expression of CD25 were determined within the CD4+ and CD8+ population after incubating cells for 48 hours with or without 5 μ g/ml ConA (Figure 7).



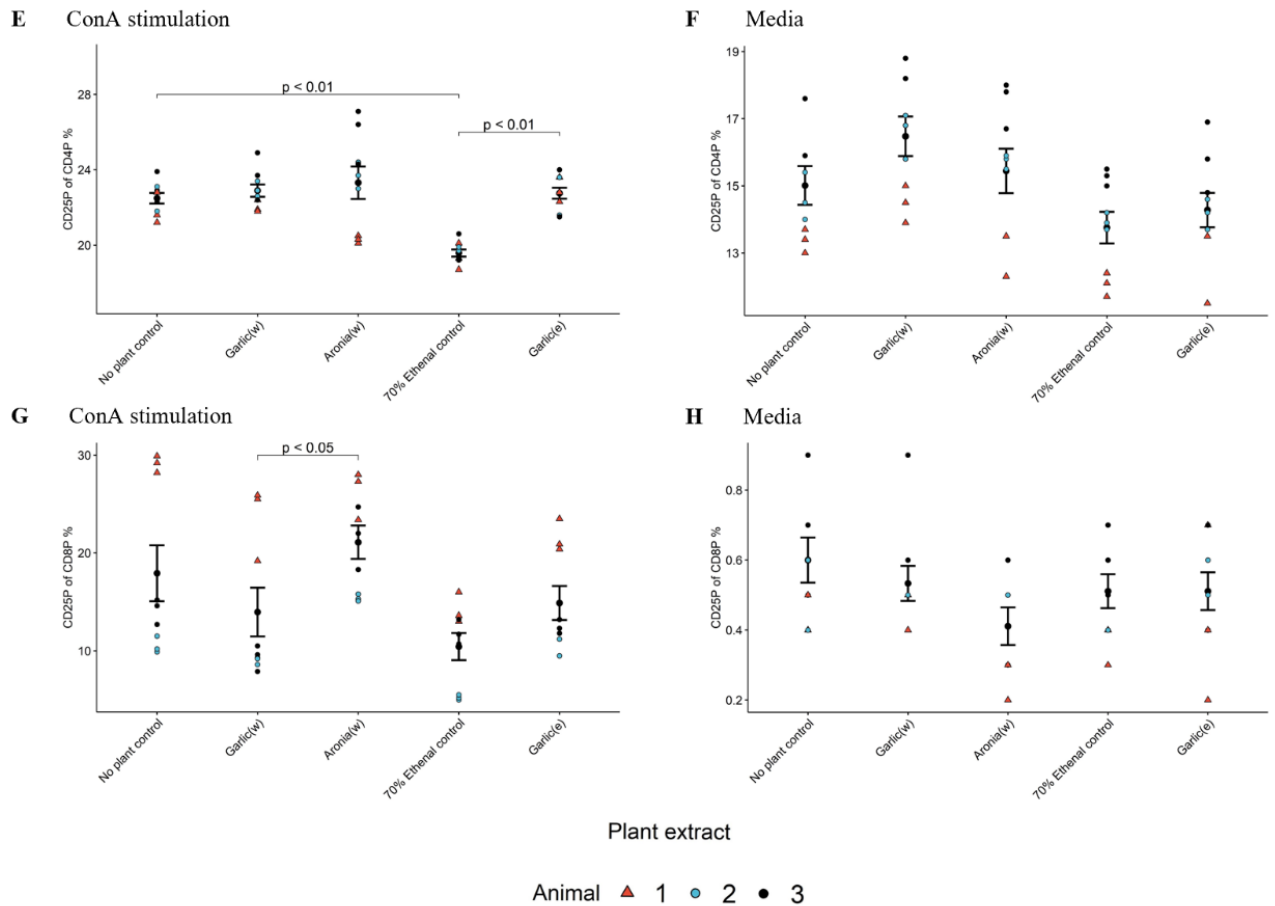


Figure 7. Flow cytometric analysis of plant effects on T cell subsets with or without 5µg/ml ConA stimulation for 48 hours. Plant extracts were either ethanol (e) or water based (w). Results are shown as mean percentage of parent population ± SE and individual values (three technical replicates from three animals) are shown as well. A) Blast of CD4+ T cells (%) with ConA stimulation, B) Blast of CD4+ T cells (%) in media alone, C) Blast of CD8+ T cells (%) with ConA stimulation, D) Blast of CD8+ T cells (%) in media alone, E) CD25+ T cells of CD4+ T cells (%) with ConA stimulation F) CD25+ T cells of CD4+ T cells (%) in media alone, G) CD25+ T cells of CD8+ T cells (%) with ConA stimulation, H) CD25+ T cells of CD8+ T cells (%) in media alone.

For the CD4+ T cells, garlic(w) extract tended to increase the percentage of blasting cells when stimulated with ConA, however the increase was not statistically significant (Figure 7A). In media alone, the percentage of the spontaneous CD4+ T cell blasts in

garlic(w) and aronia(w) were significantly lower ($p < 0.01$) than in the respective no plant control group (Figure 7B).

Regarding the CD8+T cell stimulated with ConA, garlic(w) and garlic(e) showed tendencies to increase the amount of blasting cells, and aronia(w) seemed to reduce the amount of blasting cells (Figure 7C). However, these differences were not statistically significant. In media alone, all the plant extracts seemingly showed a reducing effect on the percentage of spontaneous CD8+ T cell blasts, however only aronia(w) showed a statistically significant decrease ($p < 0.05$) when compared to the no plant control (Figure 7D).

When stimulated with ConA, 70% ethanol significantly reduced ($p < 0.01$) the percentage of CD4+CD25+ T cells compared to the no plant control. The garlic(e) group had a significantly higher percentage ($p < 0.01$) of CD4+CD25+ T cells than the respective 70% ethanol control (Figure 7E). In media alone, all the plant extracts showed an increasing trend in the percentage of CD4+CD25+ T cells compared to the respective controls, but without statistical significance (Figure 7F).

In terms of CD8+T cells in samples stimulated with ConA, aronia(w) showed significantly ($p < 0.05$) more CD25+ cells than garlic(w). However, no plant extracts showed statistically significant differences compared to their respective control (Figure 7G). In media alone, the frequencies of CD8+CD25+ T cells were very low and no statistically significant differences were found (Figure 7H).

3.1.5 Plant effects on HD11 cells with or without LPS and *A. galli* stimulation

In vitro studies were performed on the chicken macrophage cell line HD11 to determine the effect of the plant extracts by using the alamarBlue assay to assess mitochondrial activity or the NO assay to assess cell activation. LPS from the outer membrane of the Gram-negative cell wall, a potent TLR-4 agonist known activate cells of the monocyte/macrophage lineage was used and also *A. galli* crude antigen containing a heterologous suspension of various TLR agonists (helminth PAMPS) but potentially also helminth derived immunosuppressive molecules. In addition to plant effects on

LPS and *A. galli* antigen stimulation, also plant effects on resting cells in culture medium alone were investigated.

3.1.6 Plant effects measured by alamarBlue™ fluorescence (HD11)

The effects of the plants on alamarBlue fluorescence were measured after 18 hours incubation of HD11 cells with TLR agonists or in media alone (Figure 8).

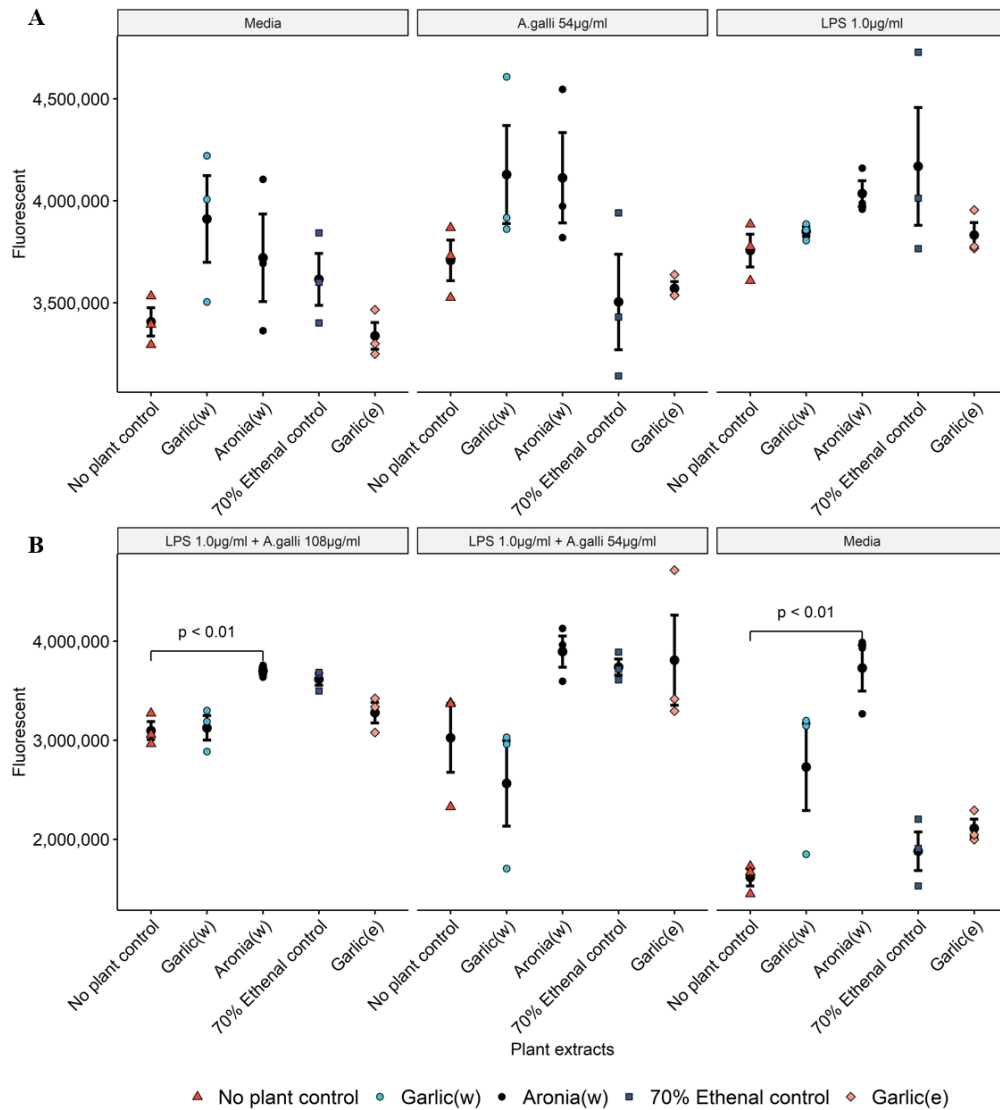


Figure 8: Effect of plant extracts on the mitochondrial activity of HD11 cells stimulated with LPS/*A. galli* antigen for 18 hours. A) HD11 cells stimulated with 54µg/ml *A. galli*, 1.0µg/ml LPS or left in media alone. B) HD11 cells stimulated with 54µg/ml *A. galli* plus 1.0µg/ml LPS or 108µg/ml *A. galli* plus 1.0µg/ml LPS or left in media alone. Plant extracts were either ethanol (e) or water based (w). All results are shown as mean

fluorescence from three technical replicates ± SE.

In the first experiment (Figure 8A), garlic(w) and aronia(w) tended to increase the fluorescent signal in media alone or when stimulated with 54µg/ml *A. galli* or LPS. However, none of the differences were statistically significant. Nevertheless, in the second experiment, aronia(w) significantly increased ($p < 0.01$) the fluorescent signal when the cells were stimulated with 108µg/ml *A. galli* together with 1.0µg/ml LPS or even left in media alone, indicating a positive effect of aronia berry on mitochondrial activity (Figure 8B).

3.1.7 Plant effects measured by Nitric oxide assay (HD11)

A genuine feature of immune cells and other cells involved in an immune reaction is to generate nitric oxide (NO), which is involved in a broad spectrum of processes in the immune system (Bogdan, 2001). Hence, measuring the NO production may indicate cellular activation.

NO production was measured as nitrite development after Griess reaction in supernatants sampled from HD11 cells incubated with different plant extracts and TLR agonists for 18 hours or left in media alone (Figure 9).

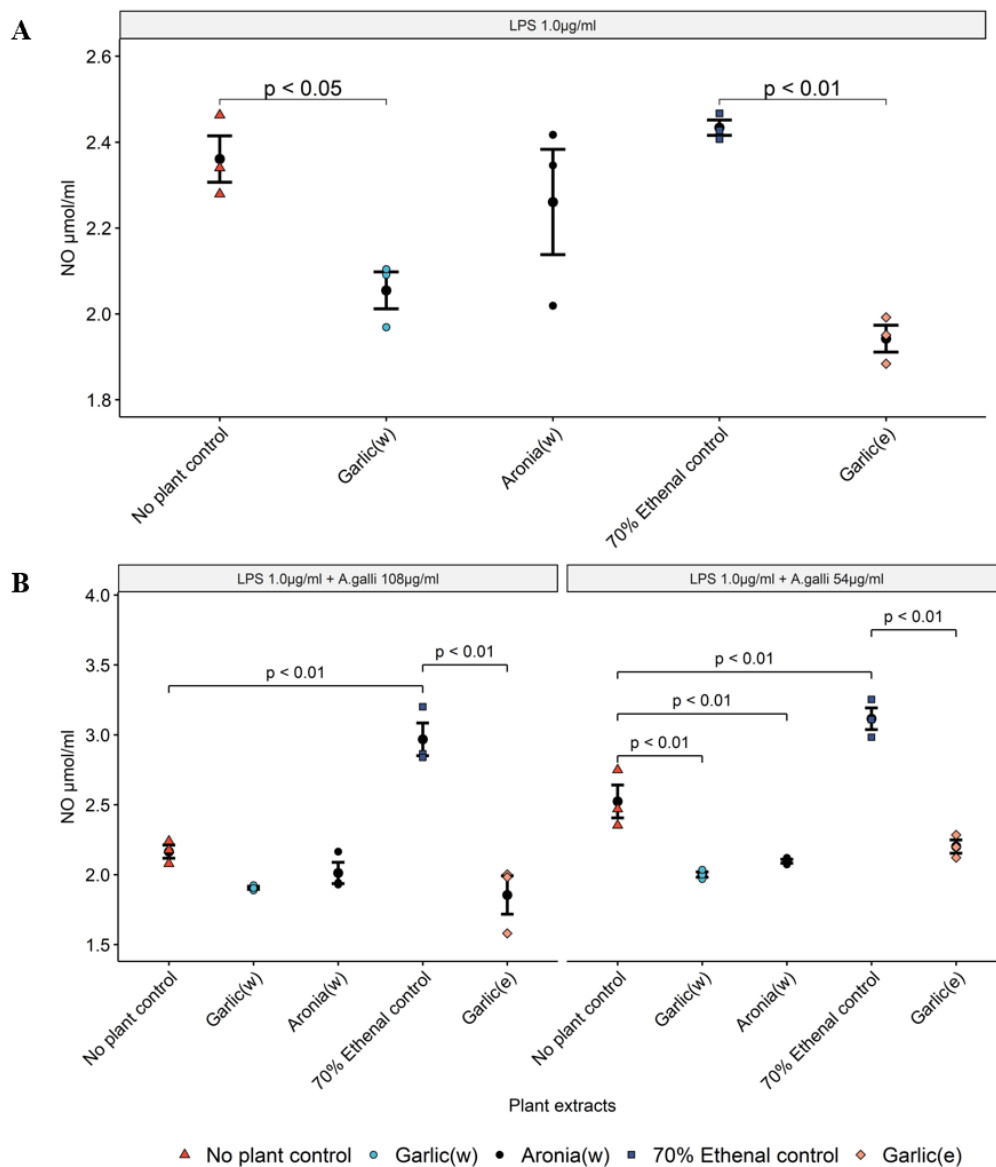


Figure 9: Effect of plants on NO production of HD11 cells stimulated with LPS/*A. galli* antigen for 18 hours. A) HD11 cells stimulated with 1.0µg/ml LPS. B) HD11 cells stimulated with 54µg/ml *A. galli* together with 1.0µg/ml LPS or 108µg/ml *A. galli* together with 1.0µg/ml LPS. Plant extracts were either ethanol (e) or water based (w). All results are shown as mean values from three technical replicates ± SE.

Cells stimulated with *A. galli* or in media alone did not produce any NO (data not shown). When stimulated with 1.0µg/ml LPS, garlic(w) and garlic(e) significantly reduced the NO production from the cells (Figure 9A) compared to the no plant control

($p < 0.01$) and the ethanol control ($p < 0.05$), respectively. When LPS and *A. galli* were combined, 70% ethanol promoted ($p < 0.01$) NO production, and garlic(e) reduced ($p < 0.01$) NO production as compared to the ethanol control (Figure 9B). When the cells were stimulated with 54 μ g/ml *A. galli* together with 1.0 μ g/ml LPS, garlic(w) ($p < 0.01$) and aronia(w) ($p < 0.01$) significantly reduced the NO production as compared to the respective no plant control.

3.2 *In vivo* experiment

3.2.1 egg production

Chickens were 19 week of age when they received the experimental *A. galli* infection. Chicken eggs were collected and weighed once a week and hence the first egg count was at the age of 20 weeks but representing the number of eggs laid from week 19 to 20. Registration of eggs continued until the age of 29 weeks (Figure 10).

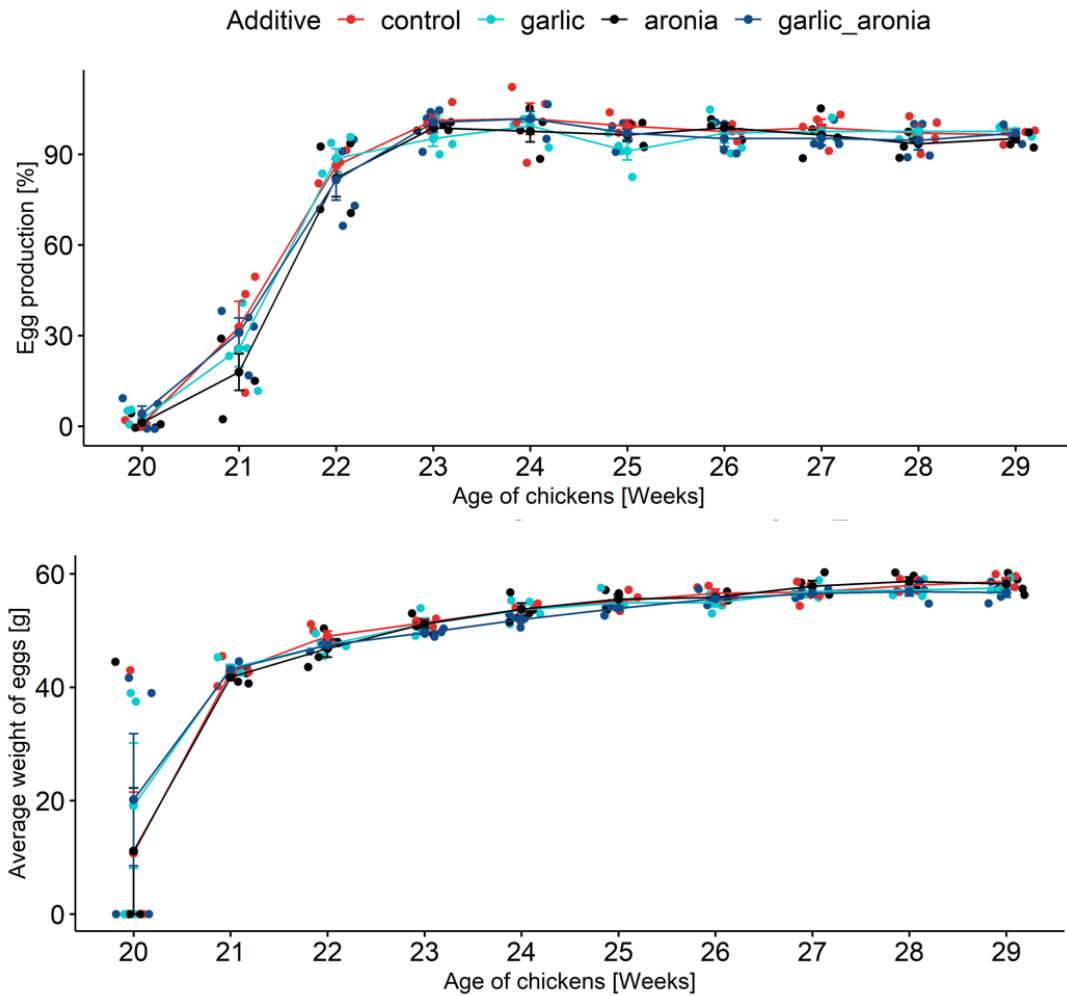


Figure.10: Egg production (%) and average weight of eggs (g) when the experimental chickens were of age 20-29 weeks. Mean values from four pens per treatment is shown \pm SE. Individual pen counts are also shown colour coded according to the four feed additive groups as indicated.

The egg production in percentage increased during the first 3 weeks, as did the average weight of the eggs. However, there is no statistically significant difference between the egg production of hens fed with different kinds of plants on the different timepoints, nor the egg weight, hence no adverse effects of the plant feed additives were observed in relation to performance of the animals.

3.2.2 A. galli burden

a) EPG

As an indication of the worm infection pressure, *A. galli* eggs were counted per gram feces (EPG) collected from individual birds in weeks 0, 7 and 10 p.i. (Figure 10).

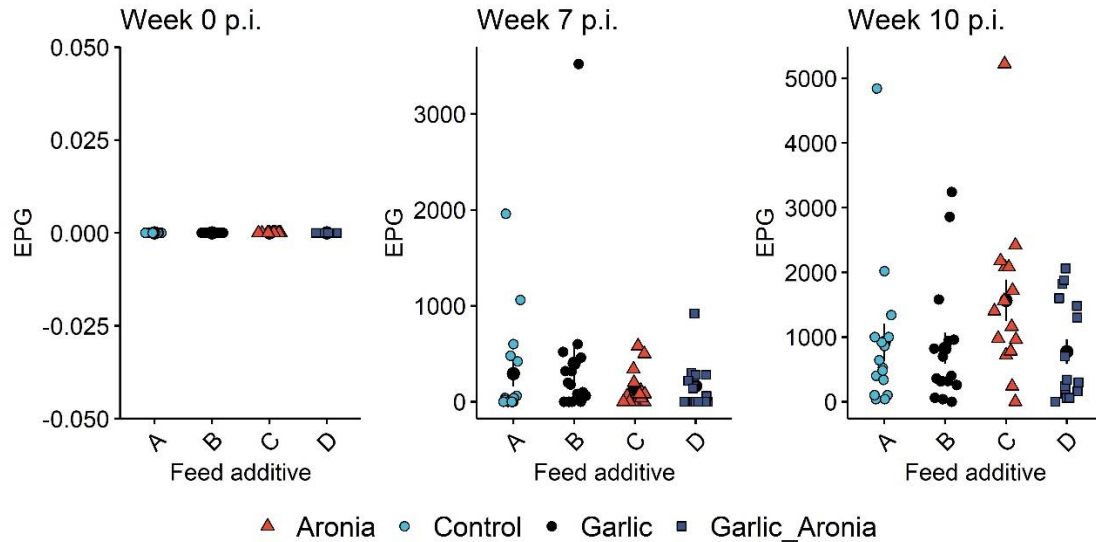


Figure.11: EPG on weeks 0, 7 and 10 p.i. for the four different treatment groups (A=Control, B=Garlic, C=Aronia, D=Garlic and Aronia). Mean values are shown \pm SE. Individual counts are also shown colour coded according to the four feed additive groups as indicated.

There were no *A. galli* egg in the feces of the hens after deworming in week 0 p.i. In weeks 7 and 10 p.i., there was no significant difference in EPG between the hens fed with different plants. However, there was a large variation in EPG within each group especially at week 10 p.i.

b) Worm burden

The number of worms in the hens' intestines was counted at the end of the experiment 10 weeks p.i. (Figure 12). Most of the worms were collected in the jejunum, and a small number of worms were found in the ileum.

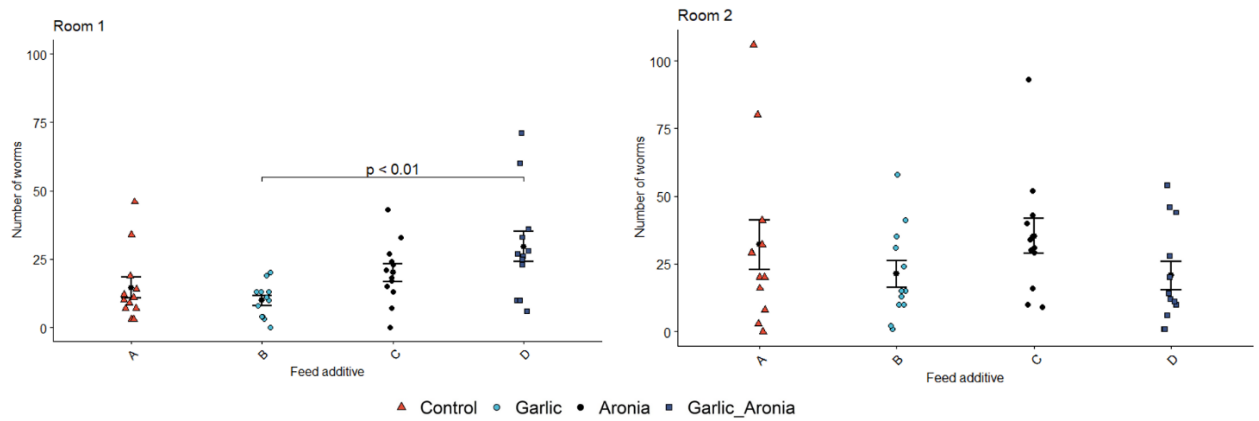


Figure.12: Plant effect on the worm burden of the hens in two separate experimental rooms. (A=Control, B=Garlic, C=Aronia, D=Garlic and Aronia). Mean values are shown \pm SE. Worm counts from individual chickens are also shown colour coded according to the four feed additive groups as indicated.

Overall, the different plants had no significant effect on the number of worms, but garlic did tend to reduce the worm burden in both experimental rooms of the chicken facility (Figure.12). However, in room one, the hens fed with aronia berry and garlic had a significantly ($p < 0.01$) higher worm burden than those only fed with garlic. None of the plant groups showed statistically significant difference compared to the control group.

c) Ration of female/male worms

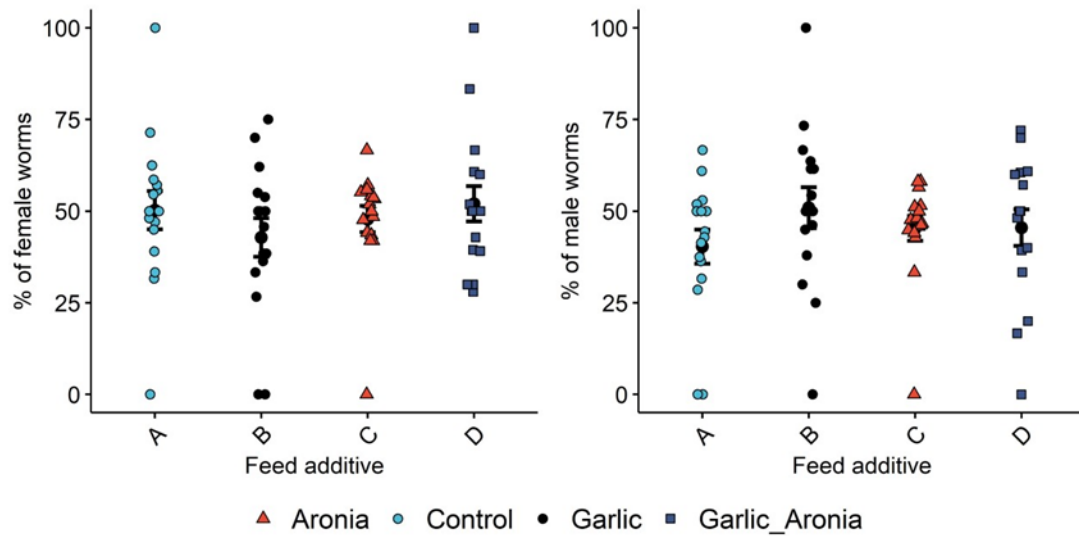


Figure.13: Plant effect on the % of female and male worms of total worm count from hens in two separate experimental rooms. (A=Control, B=Garlic, C=Aronia, D=Garlic and Aronia). Mean values are shown \pm SE. The percentage from individual chickens are also shown color coded according to the four feed additive groups as indicated.

Generally, the different plants show no significant different on the ration of female worm, but garlic have a tendency to reduce the ration of female worm in total worm counts (Figure 13).

d) *A. galli* specific serum IgY

A. galli specific IgY levels in serum from the hens were measured by ELISA at weeks 0, 2, 7 and 10 p.i. (Figure 14).

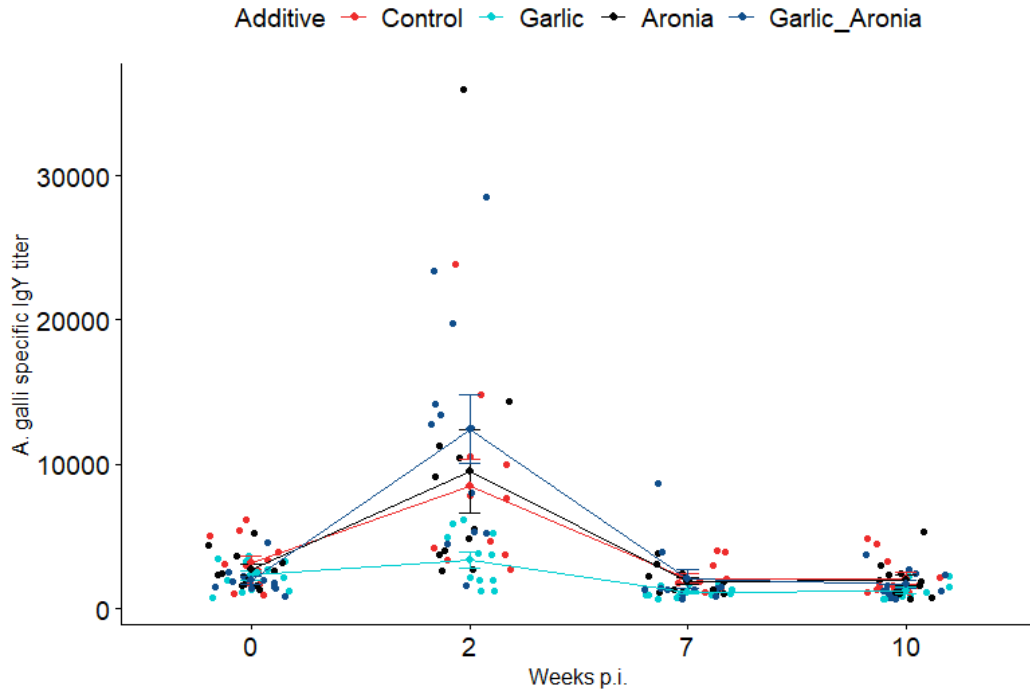


Figure 14: Plant effect on *A. galli* specific serum IgY level on weeks 0, 2, 7 and 10 p.i.. Mean values are shown \pm SE. Titers from individual chickens are also shown colour coded according to the four feed additive groups as indicated.

The serum IgY level increased at week 2 p.i. but dropped back to the original level at weeks 7 and 10 p.i. Interestingly, the hens fed with garlic combined with aronia had the highest IgY levels numerically, and the hens fed with garlic had the lowest at 2 weeks p.i. However, there was no statistically significant difference in *A. galli* specific IgY level between the different groups at any time point.

3.2.3 Immunocompetence

a) Whole blood leukocyte counts

Subsets of leukocytes in peripheral blood of the hens fed with different plants were quantified by flow cytometry in weeks 0, 2, 7 and 10 p.i. (gating strategy in Appendix, Fig. 18). The subsets quantified were thrombocytes, monocytes, heterophils, B cells, $\gamma\delta$ T cells (TCR1+), $\alpha\beta$ T cells (TCR1-), eosinophils/basophils/NK cells and finally the heterophil-lymphocyte ratio was calculated (Figure 15).

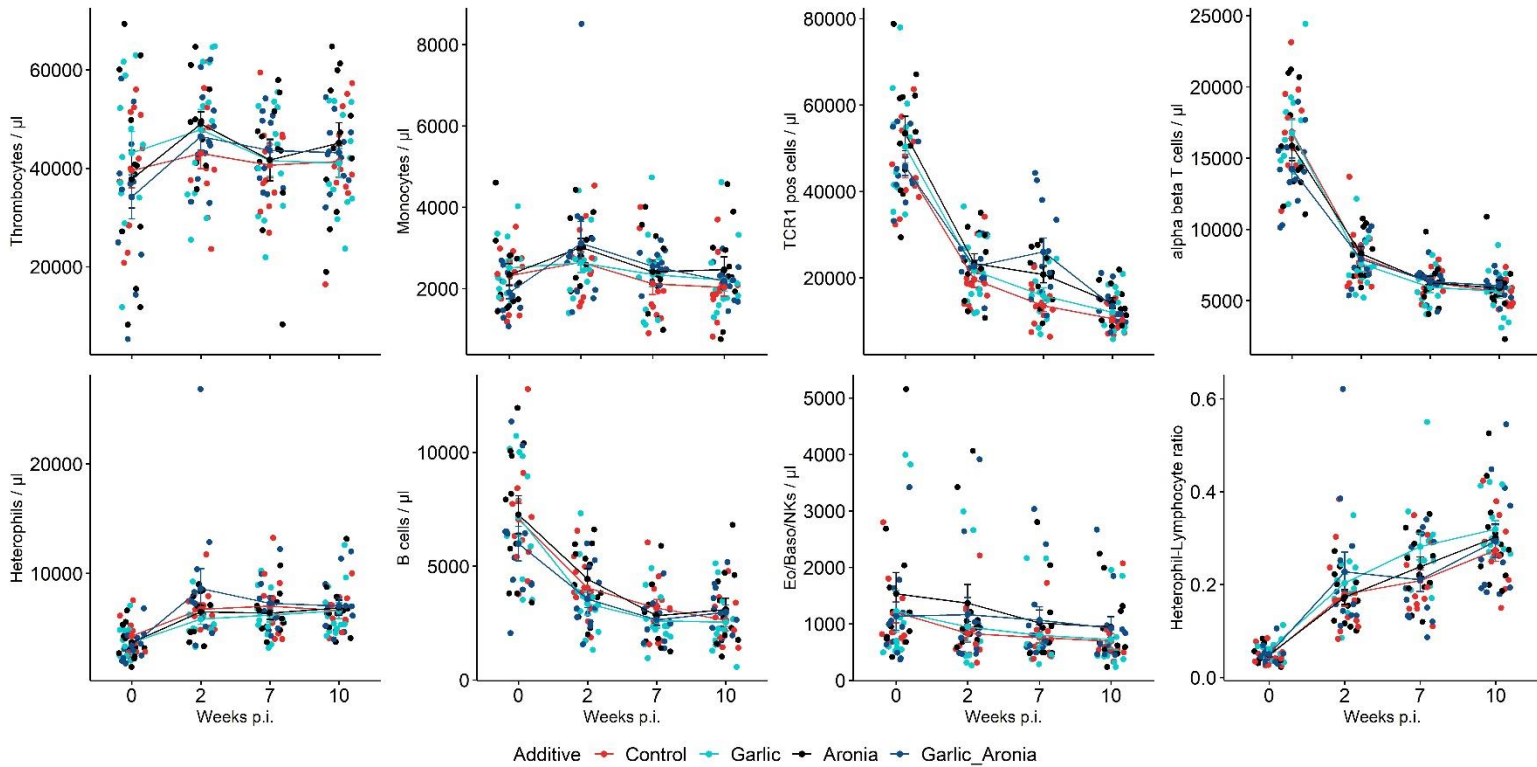


Figure 15: Whole blood leukocyte counts on weeks 0, 2, 7 and 10 p.i. Mean values are shown \pm SE. Individual counts are also shown colour coded according to the four feed additive groups as indicated.

The numbers of B cells, TCR1+ cells and $\alpha\beta$ T cells decreased during the *A. galli* infection period; whereas, the heterophil-lymphocytes ratio increased. There were no significant difference for the different subsets of cells between the hens fed with plants and the control group.

b) PBMC Mitogen stimulation

To assess how the different plants in the diets affected the immune function of the hens, PBMC were isolated from blood at week 4 p.i. The PBMC were stimulated with 5 μ g/ml ConA or 108 μ g/ml *A. galli*, and the T cell response was analyzed by flow cytometry after 72 hours of incubation (Figure 16 and gating strategy in Appendix, Fig.20)

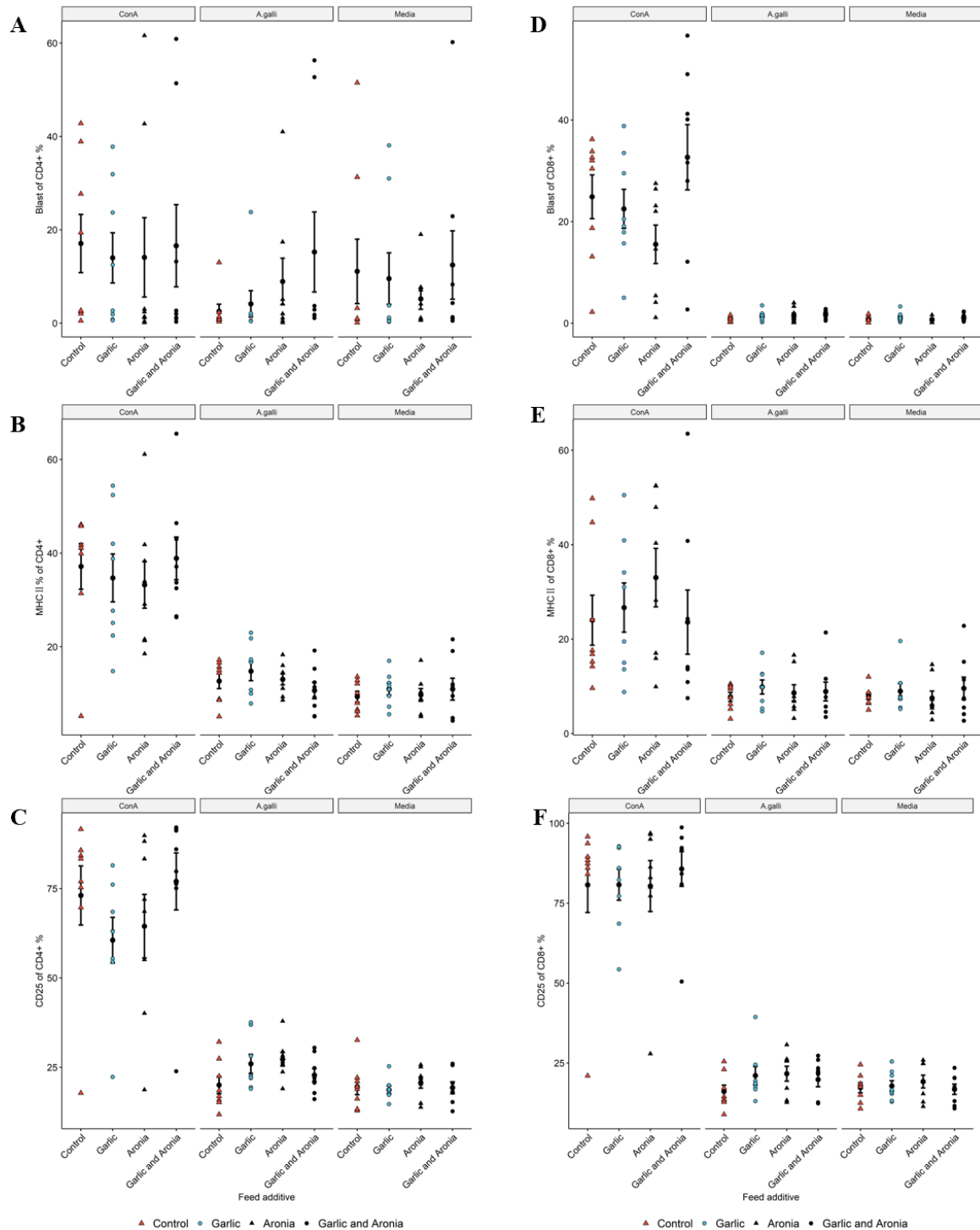


Figure 16: Flow cytometric analysis of the T cell response of PBMC samples from week 4 p.i. stimulated with 5µg/ml ConA or 108µg/ml *A. galli* antigen. The following frequencies are shown: A) Blast of CD4+ T cells (%) B) MHCII+ of CD4+ T cells (%) C) CD25+ of CD4+ T cells (%) D) Blast of CD8+ T cells E) MHCII+ of CD8+ T cells (%) F) CD25+ of CD8+ T cells (%). Mean values are shown ± SE. Percentages from individual chickens are also shown colour coded according to the four feed additive groups as indicated.

In ConA stimulated PBMC samples from the garlic and aronia group tended have a lower percentage of CD4 cells expressing CD25 (activated Th cells) (Figure 16C) and lower percentage of CD8+ lymphoblasts (activated Tc cells) (Figure 16D) as compared to PBMC samples from the control and aronia /garlic groups, indicating that these animals were less prone to respond to the mitogen *in vitro*. *A. galli* in general showed a limited effect on stimulating CD4+ T cells and CD8+ T cells indicating that only few *A. galli* specific T cells were present in the infected animals at this early time point, 4 weeks p.i. However, *A. galli* antigen-stimulated PBMC from the hens fed with garlic or aronia diets tended to have higher percentages of CD25 expressing CD4+ T cells (Figure 16C) and CD8+ T cells (Figure 16F), indicating that these animals potentially produced a slightly higher number of circulating *A. galli* specific T cells in response to the infection. Due to large individual differences between animals within each treatment group, none of the mentioned differences between groups were statistically significant.

C) IEL and LPL phenotyping

At slaughter, week 10 p.i., lymphocytes were isolated from the intestinal tissues from the hens (IEL and LPL) and analyzed by flow cytometry (gating strategy in Appendix, Fig.21). Five subsets of lymphocytes were identified namely: TCR1+CD8- ($\gamma\delta$ T cells), TCR1+CD8+ (subset of $\gamma\delta$ T cells), TCR1-CD4-CD8+ (Tc cells), TCR1-CD4+CD8+ (Double positive cells) and TCR1-CD4+CD8- (Th cells). No differences were found between animal treatment groups within the TCR1+CD8+ and TCR1-CD4-CD8+

population (data not shown). Frequencies of activated (CD25+) T cells for the remaining three subsets are shown in Figure 17.

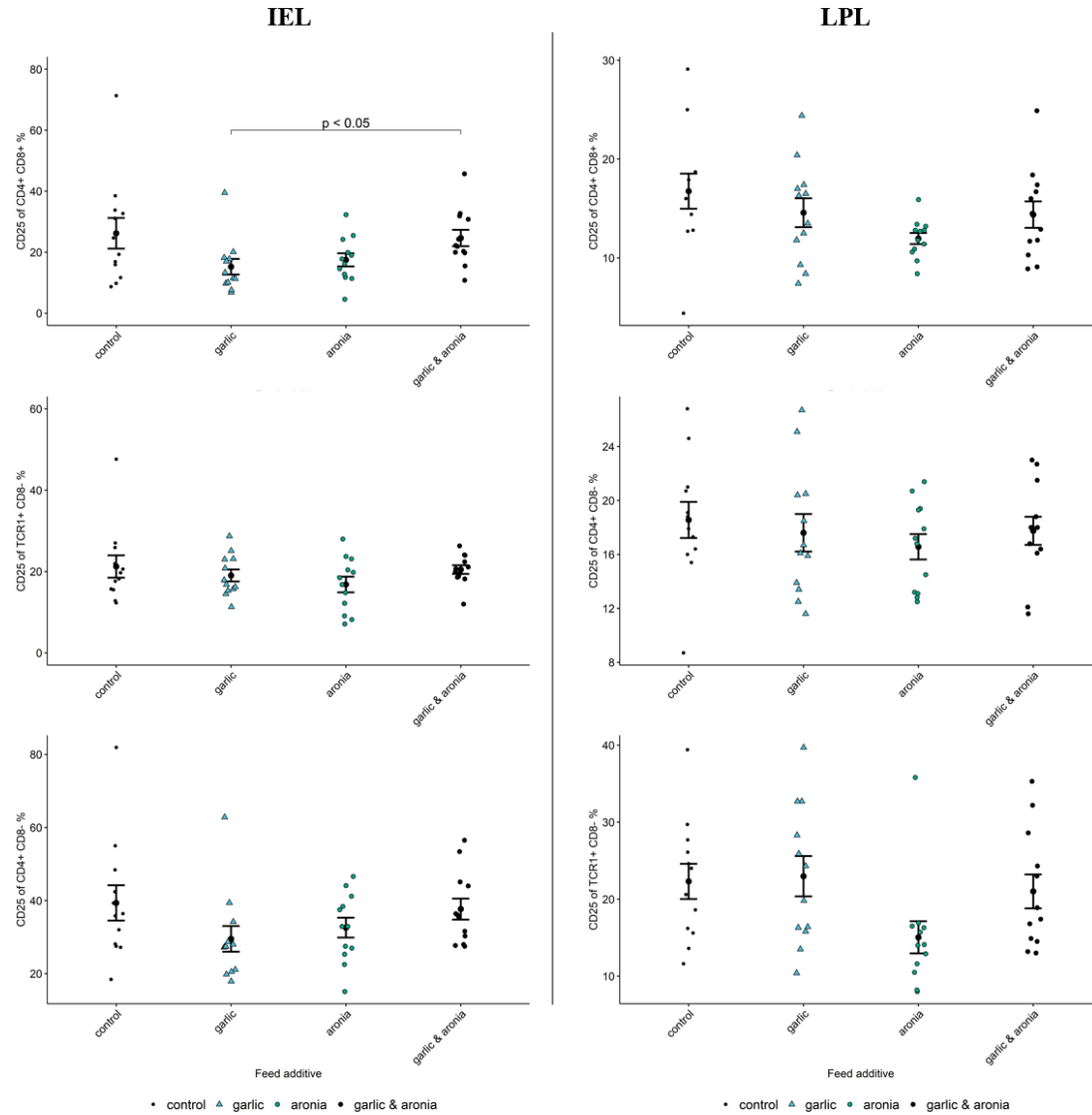


Figure.17: Immunoprofiling of lymphocytes isolated from the intestinal tissue at week 10 p.i. Subset of lymphocytes in the intestinal epithelium (IEL) are shown to the left, whereas subsets of lymphocytes in the lamina propria (LPL) are shown to the right. The percentage of activated cells (CD25+) within the following lymphocyte subsets are shown: Double-positive T cells(CD4+CD8+), gamma/delta T cells (TCR1+CD8-) and T helper cells(CD4+CD8-). Mean values are shown \pm SE. Individual observations are also shown colour coded according to the four feed additive groups as indicated.

The garlic diet tended to decrease the percentage of CD25⁺ T cells of all measured T cell subsets in the IEL sample fraction indicating that fewer activated cells were present in the animals fed this diet (Figure 17, left). The decreased CD25⁺ frequency was only statistically significant with the CD4⁺CD8⁺ IEL subset and only when comparing animals fed garlic to those a combination of garlic and aronia compared to hens fed only garlic ($p < 0.05$). Besides, hens fed the aronia diet tended to have a lower percentage of CD25⁺ cells within all measured T cell subsets in the LPL fraction (Figure 16, right), however, there were no statistically significant differences.

4. Discussion

This study points to the anthelmintic and immunomodulatory effects of garlic and aronia berry: When plant extracts were incubated with ConA stimulated PBMC, both water and ethanol extract of garlic tended to increase the proliferation of CD4⁺T cells (Th) and CD8⁺ T cells (Tc). The garlic ethanol extract specifically was shown to significantly promote the activation of CD4⁺ T cells. When a chicken macrophage cell line was incubated with plant extracts, garlic and aronia berry generally showed anti-inflammatory effects by significantly reducing macrophage production of nitric oxide. *In vivo*, however, the current feed inclusion levels of garlic and aronia berry showed no or very limited effect on the burden of adult *A. galli* worms in experimentally infected hens. However, there was a numeric garlic-induced reduction of worm burden in one experimental room and potential impact of garlic was also shown on the larvae stage of *A. galli* as IgY titers remained very low in the histotrophic phase of the worm development around 2 weeks p.i. where IgY titers peaked in control animals. Finally, a potential impact of garlic and aronia berry was shown of the phenotype and activation state of intestinal immune cells of importance for helminth control and gut health in general.

4.1 Plant immunomodulatory effects *in vitro*

The alamarBlue results indicate that garlic(w) and aronia(w) can boost the mitochondrial activity of PBMC in cell culture media (Figure 6B). However, the flow cytometry result showed that the spontaneous proliferation (blast transformation) of CD4⁺ T cells and CD8⁺ T cells in culture medium alone are reduced as compared to the no-plant control (Figure 7B,D). It is indeed possible for increased mitochondria activity and reduced/absence of proliferation to occur simultaneously. One situation could be when the cellular activation leads to e.g. cytokine production influenced by mitochondrial activity (Salvioli et al., 2006). Mitochondria are located near the endoplasmic reticulum (ER), supplying the energy for protein and lipid synthesis (Grimm, 2012). The activation of different pathways in mitochondria can e.g. enhance or suppress the pro-inflammatory cytokine secretion in T cells in mammals (Fischer et al., 2007, Dostert et al., 2008). Furthermore, the metabolic response in mitochondria can influence the differentiation of immune cells. For instance, Fatty acid β -oxidation (FAO) can selectively control the direction of the differentiation of naïve murine CD4⁺ T cells (Michalek et al., 2011).

During ConA stimulation, cells with garlic(w) extract tended to have higher mitochondrial activity (Figure 6A), also increasing the proliferation of CD4⁺ T cells and CD8⁺ T cells (Figure 7A, C) and for cells with garlic(e), there was a presence of higher numbers of activated CD4⁺ T cells (CD25⁺, Figure 7E). There is also evidence that mitochondrial activity is associated with the pro-inflammatory effect of T cells. For instance, serine hydroxymethyltransferase 2 (SHMT2) catalyzes glycine synthesis from serine in mitochondria that can support CD4⁺ T cell activation in mice (Ron-Harel et al., 2016). Also, the junction signal of mitochondrial and ER is essential in CD8⁺ memory T cells in mice (Bantug et al., 2018).

In general poultry production circumstances, without infection, garlic is expected to reduce the inflammatory response, maintaining the homeostasis, reducing oxidative stress as described in numerous studies. In presence of an acute infection, garlic would rather support a pro-inflammatory response to eradicate the pathogen which in

agreement with the different results we get in media versus ConA stimulation in the current study.

In HD11 macrophages, aronia(w) and garlic(w) tended to boost the mitochondrial activity when cells were activated with *A. galli* antigen (Figure 8A). In the presence of both LPS and *A. galli* antigen, garlic(w) showed no significant effect on mitochondrial activity whereas aronia(w) significantly increased it (Figure 8B). Interestingly both plants showed tendencies to reduce NO production of LPS stimulated macrophages with or without *A. galli* antigen (Figure 9). Reduction of NO production may seem to be a detour away from parasite expulsion but clearly the LPS interferes with the *A. galli* response as also earlier shown *in vivo* (Permin et al., 2006). Furthermore, there is also evidence that PBMC are stimulated with LPS and *Ascaris* combined, their cytokine production are influenced (Jakobsen et al., 2019) The high mitochondrial activity of aronia(w) in *A. galli* stimulated macrophages may suggest that a pathway alternative to the one leading to NO production was activated in the cells. A possibility could also be that the reduction observed for NO may be caused by the polarization of macrophages into a Th2 response (He and Carter, 2015), which helps parasite expulsion more efficiently (Perrigoue et al., 2008). Thus, potentially the secretion of Th2 related cytokine could be investigated to verify the anthelmintic effect of aronia berry.

Immunocompetence is the ability of the immune system to distinguish “non-self” and “self”, protecting the host from infectious agents existing in the environment. Immunocompetence can also refer to the healing ability of injury and trauma (Calder, 2007). Regarding *A. galli* infection, particular in mature stages of the parasite, the worm may be too robust to be eradicated by the immune system of the chicken. Increased inflammatory responses may hence cause more harmful consequence to the chicken than the *A. galli* infection itself. Thus, a better strategy may be to alleviate the damage caused by the infection. The NO result indicates that when stimulated with higher doses of *A. galli* antigen, the anti-inflammatory effect of garlic(w) and aronia(w) were no longer significant. Besides, when stimulated with ConA or media, the cells showed different responses towards garlic(w). Thus, *in vitro* studies of the interaction of plant

compounds and *A. galli* are helpful to better understand the regulation of the immune system by plants in the presence of infection.

4.2 Plant antiparasitic and immunomodulatory effects *in vivo*

In the current *in vivo* study, it was difficult to see if the different plants had significant effect on the EPG (Figure 11) and the worm burden (Figure 12) compared to the control group due to large individual variation even between animals within the same treatment group. In one of the rooms, the animals fed with garlic had a significantly lower worm burden than those fed with aronia berry and garlic combined. However, there was no significant difference between these two groups in the other room. Although the animals were of the same breed, and the environment in each room were identical, significant variation may still occur due to a variety of reasons. Ad libitum feeding applied in current experiment may cause differences in feed intake, which may explain the variation in antiparasitic activity. Also, despite being of the same breed individual genetic differences are inevitable. Furthermore, the garlic and aronia berry powder must go through the chicken's beak, crop, proventriculus, and gizzard before reaching the intestine (Bell, 2002), which may cause the loss and changes of plant compounds. In a simulated gastrointestinal digestion experiment, the total organosulfur compounds and (poly)phenols in fresh garlic decreased significantly before the intestinal digestion. Of the effective compounds, for instance the alliin, only 14.2% reached in the intestine (Moreno-Ortega et al., 2020). Dose differences may have large influence on the efficacy of the plants against *A. galli*. Evidence shows that the lower concentration of garlic extract is the longer time it took to kill *A. galli* (Yuniarti et al., 2017). In addition, the dose of plant intake may also influence the immune response considerably. A study by Liu et al. reported that feeding male rats with 50 mg/kg of garlic oil can increase IFN- γ secretion. However, feeding with 200 mg/kg of garlic oil upregulated IL-4 and IL-10 expression, suppressing IFN- γ expression and inducing a Th1 response to polarize into a Th2 response (Liu et al., 2009)

In the histotrophic phase of the worm development around two weeks, the IgY titers in

the animal feed with garlic numerically remained very low, but IgY titers peaked in control animals (Figure 14). Meanwhile, there was a numeric garlic-induced reduction of worm burden in one experimental room, which implied that the reduction of IgY level might partially result from the combination of immunomodulatory effect and reduction of worm burden.

In the current study, when the PBMCs was stimulated with *A. galli* antigen at week 4 p.i., the results indicated that garlic or aronia berry tended to promote the proliferation of CD4⁺ T cells and increase the relative frequencies of activated CD4⁺ T cells (Th) and CD8⁺ T cells (Tc) (Figure 16). Our results show that garlic and aronia berry may potentially also facilitate the *A. galli* specific of Th cells and Tc cells, responding with more robust helper functions to produce antibodies and intense cytotoxic effects as seen in other species (Berard and Tough, 2002). When cells were stimulated with ConA, garlic and aronia berry showed a reduced tendency of the proliferation and the activated frequency of Th cells and Tc cells (Figure 16). The apparent difference in effect between ConA and *A. galli* antigen stimulation may be caused by interactions, or simply be because of the effect on memory T cell re-activation pathways. In humans, both Th cells and Tc cells express CD45RO when they become memory T cells (Yamazaki et al., 1993). CD45 is also expressed in chickens (Dóra et al., 2017). This marker is possibly worth studying to investigate the effect of the plants on restimulation of memory T cells.

IEL and LPL results indicated no statistically significant immunomodulatory effect of different plants compared to the control group (Figure 17). However, a tendency for garlic to reduce frequencies of CD25⁺ CD4⁺CD8⁺ T cells was observed. This is a very interesting population which is associated with the gut microbial status and their short chain fatty acid (SCFA) production. The IEL and LPL samples were taken at the end of the experiment, while the adult worm burden in the animals assumingly was at its highest this experiment. It is well known that parasitic worm can excrete a variety of immunomodulatory proteins (Hewitson et al., 2009), and the dominant effect of these on the immune cells may overshadow the immunomodulatory effect of the plants at this

time points. One limitation of this study was that a control group without *A. galli* infection was absent. Excluding a potential *A. galli* interference, would have allowed us to directly assess the immunomodulatory effect of plants on chicken intestinal cells.

5. Future perspectives

Using medicinal plants to prevent and treat helminth infection originates from ethnomedicine practices (Athanasiadou et al., 2007). It is usually based on long-term experience and practice (Xutian et al., 2009). Nowadays, advanced research methods have allowed us to scientifically confirm the immunomodulatory and antiparasitic effects of plants.

The experiment in the current study provides a new interpretation of the interaction between medicinal plants, immune cells, and *A. galli*. The mitochondrial activity of chicken PBMC was shown to be impacted by plant compounds through mitogen-induced proliferation of T cells, implying that the plants may also promote e.g. cytokine secretion in PBMC. Thus, investigating the functional activity of the immune cells in depth may help us to understand the details of the immunomodulatory effect of garlic and aronia berry. Besides NO production, substitute pathways such as the Th2 responses are also beneficial to worm expulsion, and the plants may indeed have an impact on innate cytokine responses instead of NO responses. Thus, investigating Th2-related cytokines could help us gain a better insight into the anthelmintic effect of garlic and aronia berry.

The individual variation in the *in vivo* study reveals that ensuring appropriate dosage and identifying the compounds which finally reach the intestine is the main challenge of an *in vivo* study but also for applying the feed intervention in commercial poultry production. Incubating the immune cells and plants in a simulated gastrointestinal digestion experiment may act to stimulate immune responses more in correspondence with the *in vivo* situation. In a poultry production setting, developing plant preparation techniques to reduce their degradation and alteration during digestion may facilitate the full use of plants as valuable feed additives used for prevention of parasite infections

and for support of general gut health.

6. Conclusion

Overall, *in vitro*, garlic showed diverse immunomodulatory effects on different immune cells, promoting T cell proliferation and activation, reducing macrophage NO production. Aronia berry showed an immunomodulatory effect by significantly reducing NO production from macrophages. *In vivo*, the current feed inclusion levels of plants showed no or very limited effect on the burden of adult *A. galli* worms in experimentally infected hens. However, *A. galli*-specific IgY titers remained very low in the histotrophic phase of the worm development around two weeks, coinciding with a numeric reduction of worm burden of animals fed with garlic in one experimental rooms, suggesting a potential antiparasitic effect of garlic. Furthermore, the garlic and aronia berry may potentially facilitate the development of *A. galli*-specific Th cells and Tc cells. The obtained result also implied that garlic and aronia berry have potential impacts on phenotype and activation state of intestinal immune cells, which are essential for helminth control and gut health in general. Further studies of the potentially correlated immune response, such as Th2-related cytokine production and *A. galli*-specific T cell activation, should be conducted to better understand the immunomodulatory effects.

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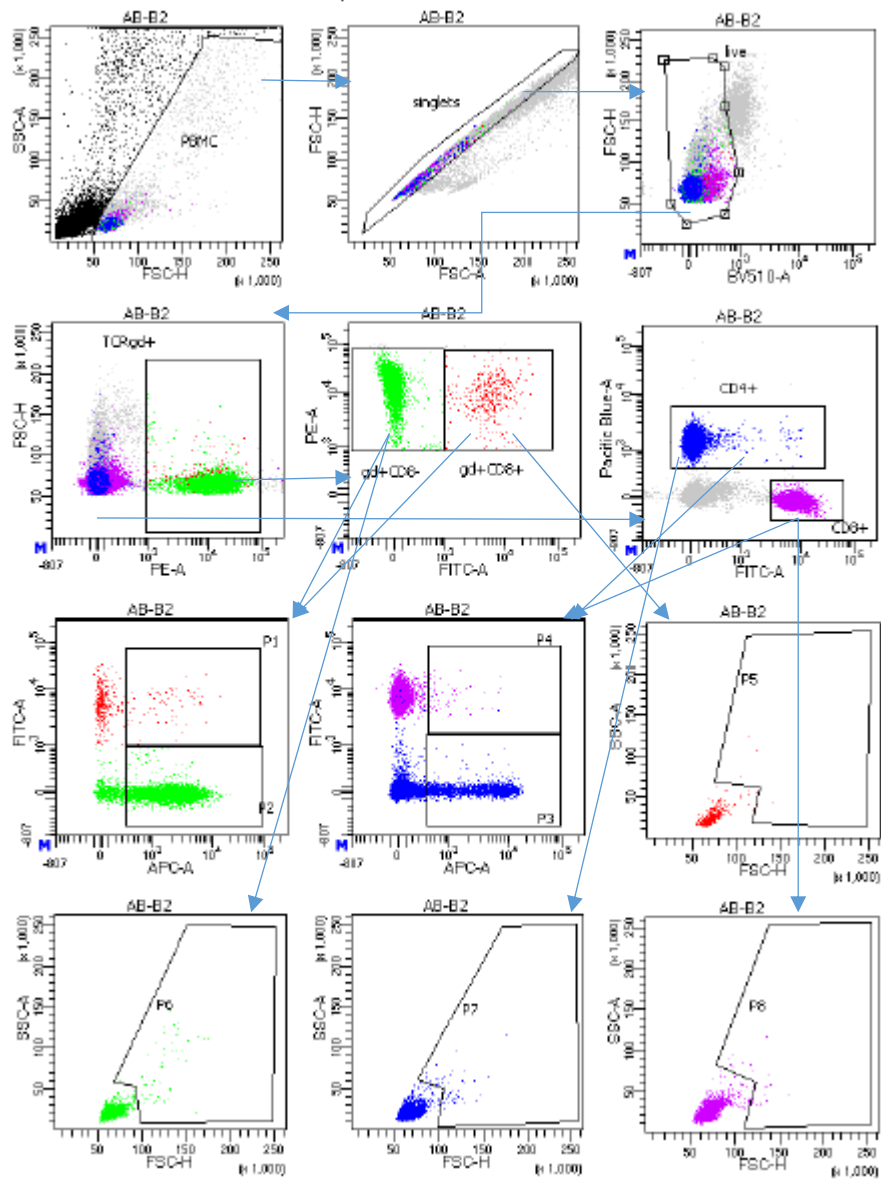
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Appendix

Appendix 1. Gating strategies for flow cytometry analyses

Figure 17. PBMC *in vitro* activation

A.



B.

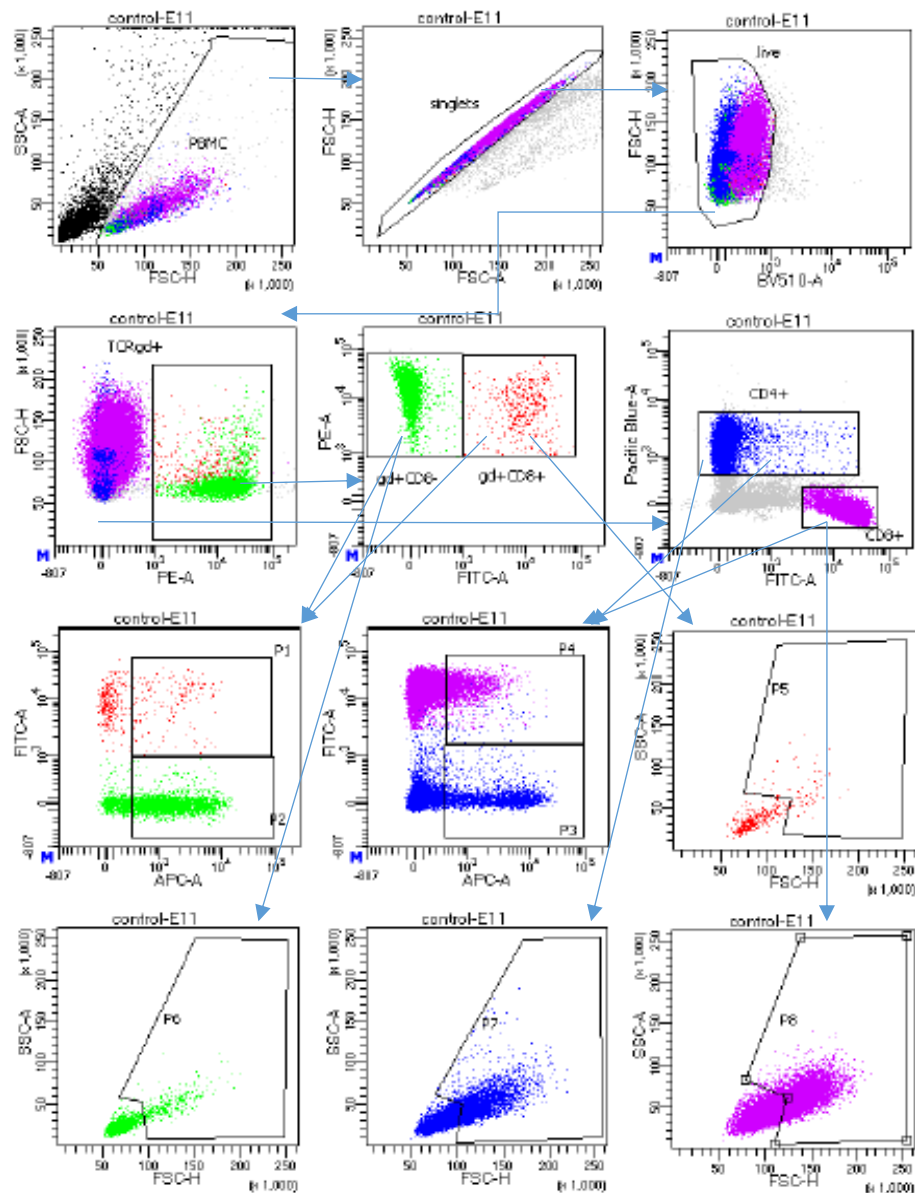


Figure 17. Gating strategy for flow cytometric analysis of *in vitro* plant effects on PBMC as medium control (A) or ConA stimulated (B). The gating strategy used was: SSC-H/FSC-H defined PBMC sized cells, singlet, live cells, TCR+ or TCR- cells. Within the TCR1+ population CD8+ and CD8- cells were defined and in the TCR1- population CD4+ and CD8+ cells were defined. This particular chicken line has very few CD4+CD8+ cells and hence they were included in the CD4+ gate. Finally CD25 (APC vs FITC plot) is shown for all four cell subsets; TCR1+CD8- (green), TCR1+CD8+ (red), TCR1-CD4+ (blue), TCR1-CD8+ (purple). Finally, lymphoblasts (defined by increased FSC and SSC properties) are shown for the same four subsets. Staining of PBMCs from a representative animal is shown as an example.

Figure 18. Leucocyte counts in peripheral blood

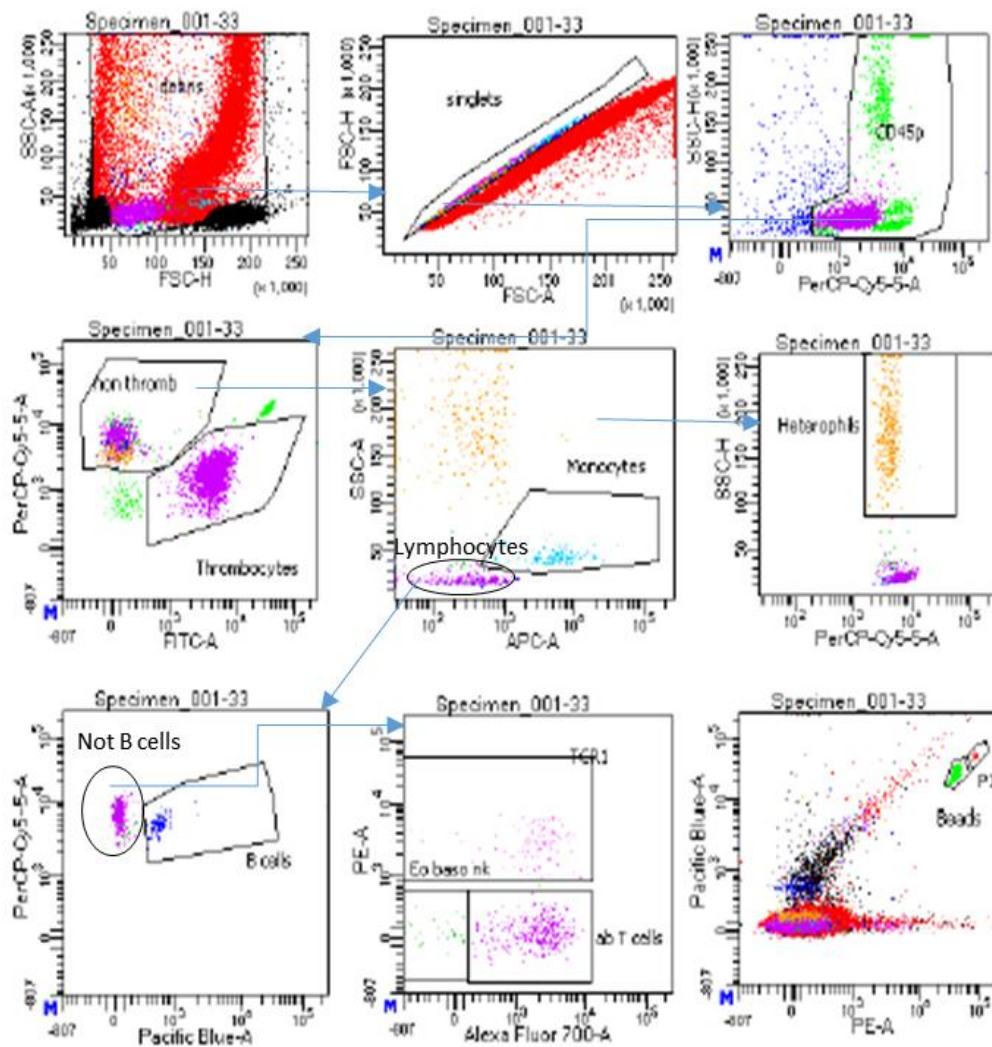


Figure 18. Gating strategies for flow cytometric analysis of absolute leukocyte counts in peripheral blood. First a gate was used to exclude debris, next gates were set on single cells and CD45+ cells. No viability marker was used as very few dead cells are present in fresh whole blood. Next thrombocytes (CD41/61+) were gated and the inverted gate of CD41/61-CD45+ cells was used as parent to gate for monocytes (Kul01+). Lymphocytes and heterophils were defined by their FSC/SSC profile in the CD45+CD41/61-Kul01- population. Within the lymphocyte gate, B cells were identified as BU-1+. And within the BU-1-lymphocyte population TCR1+ and TCR1- cells were gated being either TCRgd or TDRab T cells. PE versus Pacific Blue was used for gating of 123count eBeads™ counting beads. Staining of one representative animal from 10 weeks p.i. is shown as an example, but the gating strategy was identical for all weeks.

Figure 19. Mitogen and antigen re-stimulation of PBMC from animal experiment.

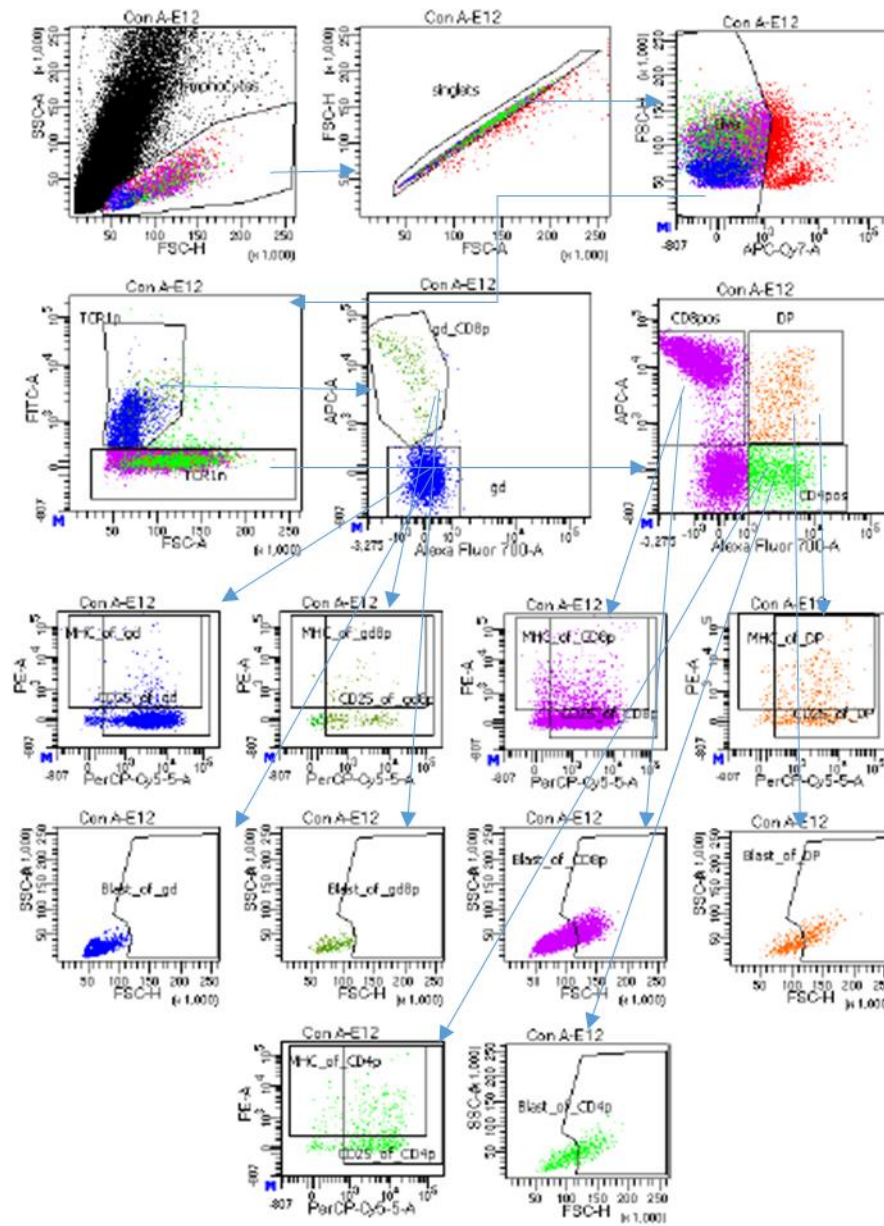


Figure 19. Gating strategy for flow cytometric analysis of mitogen and antigen stimulated PBMCs from in vivo experiment. The gating was FSC/SSC defined lymphocytes, singlets, live cells, TCR1+ and TCR1- cells. Within the TCR1+ population CD8+ and CD8- cell were gated, Within the TCR1- population CD4+CD8-, CD4+CD8+ (DP) and CD4-CD8+ cells were gated. Finally gates were set for CD25+, MHC-II+ and lymphoblasts (defined by increased FSC/SSC) within the five identified T cell populations, namely: TCR1+CD8- (blue), TCR1+CD8+ (dark green), TCR1-CD4-CD8+ (purple), TCR1-CD4+CD8+ (orange) and TCR1-CD4+CD8- (light green). Staining of ConA stimulated PBMCs from a representative animal is shown as an example. Gating was identical for stimulations with A. galli antigen and medium controls.

Figure 20. IEL and LPL phenotyping from animal experiment.

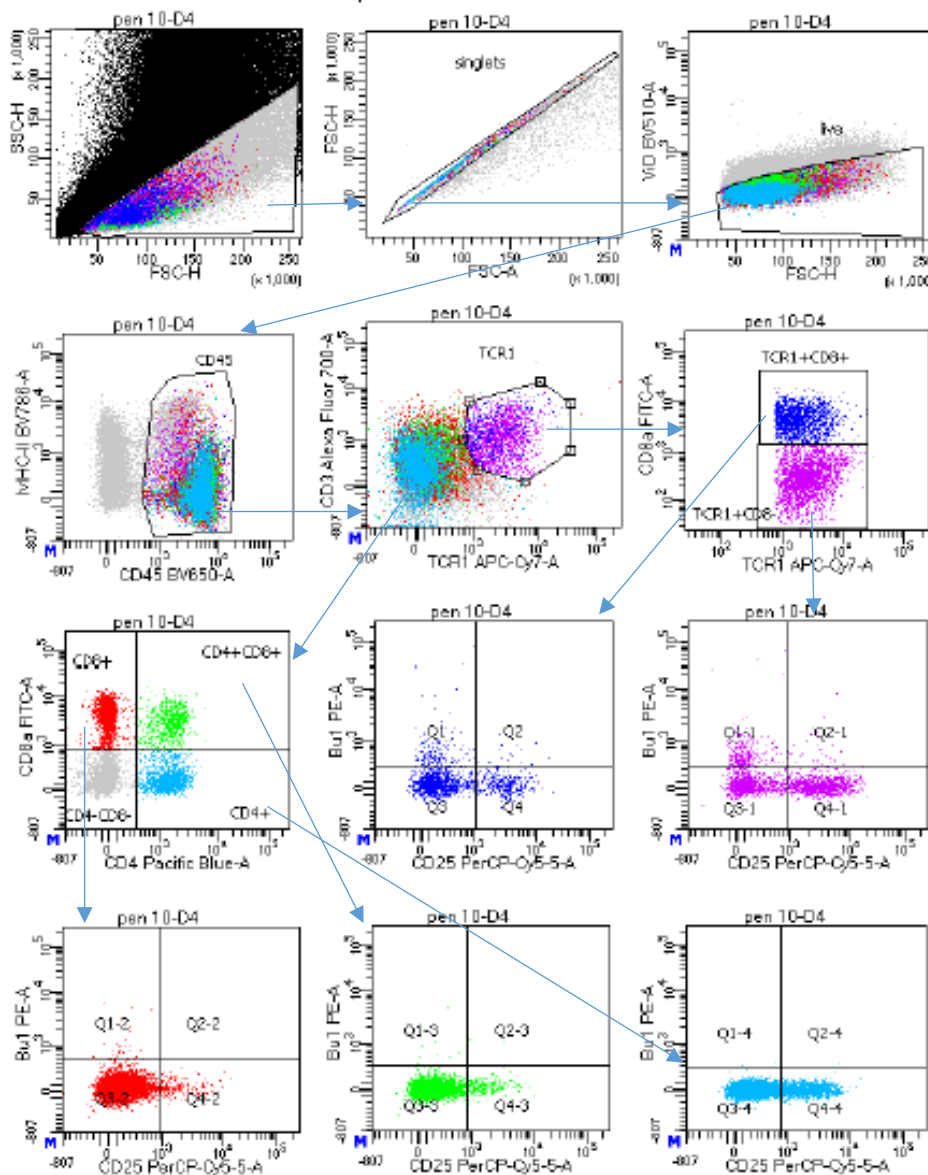


Figure 20. Gating strategy for flow cytometric analysis of IEL and LPL samples from animal experiment. The gating was FSC/SSC defined lymphocytes, singlets, live cells. Within the CD45+ population TCR1+ and TCR1- cells were defined. Within the TCR1+ population CD8+ and CD8- cell were gated, Within the TCR1- population CD4+CD8-, CD4+CD8+ (DP) and CD4-CD8+ cells were gated. Finally gates were set for CD25+, and Bu1+ cells within the five identified T cell populations, namely: TCR1+CD8- (purple), TCR1+CD8+ (blue), TCR1-CD4-CD8+ (red), TCR1-CD4+CD8+ (green) and TCR1-CD4+CD8- (light blue). Staining of and IEL sample from a representative animal is shown as an example. Gating was identical for also LPL samples.