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A multidisciplinary approach to gut health in animal production

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"Se volete trovare qualcosa, non c'è niente di meglio che cercare."

J.R.R. Tolkien

ABSTRACT

Gut health is defined as the absence/avoidance/prevention of gastrointestinal diseases in order to maintain animal welfare, health and performances. In poultry farming, the maintenance of an appropriate gut health has become a major issue since 2006, when the European Union bans the use of antimicrobial growth promoters in animal feed.

The aim of this PhD project was to assess whether insect live larvae (*Hermetia illucens* -HI and *Tenebrio molitor-TM*) or functional feed (Alphasoy Gold-ASG) can have beneficial effects on gut health of poultry using a multidisciplinary approach including histopathological, histochemical and biomolecular techniques. For this purpose, three experimental trials were performed. In Trial 1 and 2, a total of 180 chickens and 126 Muscovy ducks were randomly allotted to three dietary treatments: control, HI and TM. In both trials all the animals were fed a commercial diet and HI and TM groups received 5% of the expected daily feed intake of HI and TM larvae, respectively. In trial 3, a total of 576 as-hatched male broiler chickens were randomly allotted to 3 dietary treatments to explore the effects of ASG obtained by thermomechanical co-processing of soybean meal and hydrolyzed yeast (β-glucan) on gut health during pre-starter and starter phase: T1 (20% and 10% of ASG), T2 (5% of ASG in both phases) and a control group.

At slaughter, samples of duodenum, jejunum, ileum, spleen, liver, thymus and bursa of Fabricius were collected for histomorphometric analysis. Mucin composition was evaluated in the small intestine through histochemistry while MUC-2, selected cytokine and tight junction expression was evaluated by rt-qPCR. Cecal microbiota was also analyzed (Trial 1 and 2).

Considering trial 1 and 2, HI and TM did not impair intestinal morphometry, mucin composition and MUC-2 transcription levels ($P>0.05$), maintaining the integrity of the mucosal barrier. On the contrary, TM group showed lower pro-inflammatory cytokine IL-2 ($P= 0.044$, Trial 1) and higher cytokine IL-6 transcription levels ($P=0.009$, Trial 2), suggesting that the lower chitin content recorded in TM can positively modulate the local immune response towards an "anti-inflammatory pattern". In caeca, HI and TM slightly increased short-chain fatty acids producing bacteria, which provide an important energy source for the enterocytes.

Regarding trial 3, ASG did not impair the intestinal barrier function, maintaining the proximo-distal decreasing gradient of the morphometric indices and the integrity of the tight junctions at day 3 and 10 ($P>0.05$). Moreover, MUC-2 expression was reduced in T1 group at day 3, suggesting that ASG can reduce the intestinal inflammation and the need of mucous synthesis from goblet cells. This was further confirmed by the reduction of pro-inflammatory cytokines (IL-2, TNF-α and INF-γ) in the duodenum of T1 and T2 chicks at day 3.

In all the three trials, insect live larvae and ASG did not influence the severity of the observed histopathological lesions, thus suggesting that they did not negatively affect animal's health.

In conclusion, the present PhD project provides new insights about the use of insect live larvae and functional feed in poultry nutrition. The results herein obtained suggests that both chitin and β-glucans can act as probiotic/prebiotic in poultry farming, showing positive effects on gut health immune response and gut microbiota, without impairing gut morphometry, mucin and tight junctions.

RIASSUNTO

La salute intestinale è definita come l'assenza o prevenzione di malattie gastrointestinali che consente di garantire il benessere, la salute generale e le performances produttive dell'animale. Nel settore avicolo, il mantenimento di un'appropriata salute intestinale è diventato una sfida a partire dal 2006, quando l'Unione Europea ha vietato l'uso degli antibiotici come promotori di crescita. L'obiettivo di questo dottorato è stato valutare se le larve vive di insetto (*Hermetia illucens* -HI e *Tenebrio molitor-TM*) e l'alimento funzionale AlphaSoy Gold (ASG) possano favorire la salute intestinale di diverse specie avicole attraverso un approccio multidisciplinare che prevede l'utilizzo di tecniche istomorfometriche, istochimiche e biomolecolari. A tal fine sono state effettuate tre prove zootecniche. Nella Prova 1 e 2, 180 polli broiler e 126 anatre mute sono stati divisi casualmente in tre gruppi sperimentali: controllo, HI e TM. Tutti gli animali hanno ricevuto una dieta commerciale e ai gruppi HI e TM è stata aggiunta una dose di larve vive pari al 5% del tasso medio di ingestione giornaliera. Nella Prova 3, 576 pulcini maschi sono stati divisi in tre gruppi sperimentali per valutare gli effetti di ASG sulla salute intestinale durante la fase pre-starter e starter del ciclo produttivo: controllo, T1 (20% e10% di ASG) e T2 (5% di ASG in entrambe le fasi). ASG è ottenuto tramite trattamento termico della farina di estrazione di soia e di lieviti idrolizzati, ricchi di β-glucani. Alla macellazione, campioni di duodeno, digiuno, ileo, milza, fegato, timo e Borsa di Fabrizio sono stati prelevati e sottoposti ad analisi istomorfometriche. La composizione delle mucine intestinali è stata valutata mediante istochimica mentre l'espressione del gene MUC-2, di citochine selezionate e giunzioni strette è stata effettuata con rt-qPCR. Inoltre, si è valutato anche il microbiota cecale (Prova 1 e 2).

Nella Prova 1 e 2, la somministrazione di larve vive non ha influenzato negativamente né la morfometria intestinale né la composizione delle mucine o l'espressione del gene MUC-2, suggerendo il mantenimento dell'integrità della barriera intestinale. Al contrario, il gruppo TM ha mostrato una diminuzione dell'interleuchina pro-infiammatoria IL-2 ($P=0.044$, Prova 1) e un aumento dell'interleuchina IL-6 ($P=0.009$, Prova 2), permettendo di ipotizzare che il minor contenuto in chitina delle larve di TM possa positivamente modulare l'immunità locale, riducendo la risposta infiammatoria. Infine, le larve vive hanno provocato un leggero aumento di generi batterici minori produttori di acidi grassi a corta catena, importante fonte di energia per gli enterociti.

Nella prova 3, ASG non ha danneggiato la barriera intestinale, mantenendo il fisiologico gradiente prossimo-distale degli indici morfometrici e l'integrità delle giunzioni strette sia a 3 che a 10 giorni ($P>0.05$). Inoltre, il gruppo T1 mostrava una minor espressione del gene MUC-2 al giorno 3, suggerendo che ASG può ridurre l'infiammazione intestinale e la necessità di sintetizzare muco da parte delle cellule di Goblet. Tale risultato è stato confermato dalla ridotta espressione delle citochine pro-infiammatorie (IL-2, TNF-α e INF-γ) nel duodeno dei pulcini trattati al giorno 3.

In tutte e tre le prove, sia le larve vive d'insetto che ASG non hanno influenzato la gravità delle lesioni istologiche, confermando che essi non hanno effetti negativi per la salute animale.

In conclusione, questo progetto ha fornito nuovi interessanti dati sul potenziale utilizzo di larve vive di insetto e alimenti funzionali nell'alimentazione avicola. I risultati ottenuti suggeriscono che sia la chitina che i β-glucani possono avere un'azione probiotica/prebiotica positiva per la salute intestinale, migliorando la risposta immunitaria locale e il microbiota senza danneggiare la barriera intestinale.

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Abbreviations

AB: Alcian Blue

AGP: antimicrobial growth promoter

ASG: AlphaSoy™ Gold

C: control

Cd: crypt depth

CL: claudin

CP: crude protein

Cq: quantification cycle

D: diet

DM: dry matter

DU: duodenum

EU: European Union

F: forward

FOS: fructo-oligosaccharide

GADPH: glyceraldehyde-3-phospate

GALT: gut associated lymphoid tissue

GC: goblet cell

GIT: gastrointestinal tract

HI*: Hermetia illucens*

HID: high iron diamine

I: intestine

IL: interleukin

ILE: ileum

INF: interferon

IR: interquartile range

JE: jejunum

LAB: lactic acid bacteria

MOS: mannan-oligosaccharide

MUC: mucin

NGS: next-generation sequencing

OCL: occludin

OTU: operational taxonomic unit

PAMP: pathogen-associated molecular pattern

PAPs: processed animal proteins

PAS: periodic acid Schiff

R: reverse

rt-qPCR: real-time quantitative PCR

SCFAs: short chain fatty acids

SD: standard deviation

T: treated group

Th: T helper

TJ: tight junction

TM: *Tenebrio molitor*

TNF: tumour necrosis factor

Vh: villus height

VSA: villus surface area

Vw: villus width

ZO: *zonula occludens*

1.INTRODUCTION

Poultry farming will have to face the challenge to increase animal-based protein production for human consumption due to the world population growth[1]. In fact, according to the Food and Agriculture Organization of the United Nations, poultry meat production will reach 136 million tons in 2022 with a yearly growth rate of 2.6%, becoming the main contributor to the meat industry[2]. However, this increasing demand has put the farmers under continuous pressure to produce meat in the shortest period of time with maximum output [3]. For this reason, genetic selection, feed optimization, preventive medicines and better health management practices have been adopted in poultry industry [4]. Thus, antibiotics have been used in poultry feed at subtherapeutic doses as antimicrobial growth promoters (AGP) to increase growth rates and enhancing feed conversion efficiency [5]. Moreover, AGP also reduce the gastrointestinal colonization of pathogens and the incidence of dysbiosis, intestinal leakage, inflammation and bacterial gastrointestinal disease (e.g., necrotic enteritis) with an additional benefit for animal's growth performances [6]. Nevertheless, the use of AGP can contribute to the well-known antimicrobial resistance (AMR).

Antimicrobial resistance is defined as "the resistance of microbes to antiviral, anti-parasitic, antifungal or antibacterial drugs" and the World Health Organization has included it among the top 10 global public health threats facing humanity [7]. This is particularly alarming from a One Health perspective as human medicine and veterinary medicine often share the same antibiotics to threat infections [8]. For all the reasons stated above, serious attempts have been made to reduce the use of antibiotics in animal production, leading to the ban of AGP in food producing animals in the European Union (EU) and in the USA in 2006 and 2017, respectively [9].

After the ban on AGP, gastrointestinal diseases are becoming a major issue in poultry farming, reducing the growth performances of the birds and highlighting an urgent need to search effective alternative that could support gut health [6]. Particularly, the enhancement of gut health is crucial as the gastrointestinal tract (GIT) play a key role in productivity, pathogen entrance and disease prevention, determining feed intake and efficient absorption of nutrients [10]. Subsequently, prebiotics and probiotics have been presented as valuable alternatives to antibiotics, especially probiotics and prebiotics [11, 12].

2.THE USE OF ANTIBIOTIC GROWTH PROMOTERS IN POULTRY FARMS

AGP are defined as low, subtherapeutic doses of antibiotics added to feed [13]. Since 1940s they have been used as feed additives in poultry as birds fed diets supplemented with *Streptomyces aureofaciens* and containing chlortetracycline residues showed enhanced growth and feed efficiency [14].

Since then, numerous hypotheses have been proposed on the mechanisms of action of AGPs and their ability to enhance animal's growth performances. The majority of these are related to the antibacterial effects of antibiotics on the gut microbiota [15]. Firstly, it has been hypothesized that AGPs can reduce the total microbial density in the GIT, promoting a more favourable microbial balance and reducing sub-clinical infections. Secondly, AGPs can also have an indirect effect on the microflora, reducing competition for nutrients and the luminal concentration of microbial metabolites that depress growth [15]. Thirdly, AGPs provoke a reduction in the production of luminal short chain fatty acids (SCFAs) derived from microbial fermentation, which are responsible for a loss of mucosal cell proliferation, reduction in gut size, thinner intestinal villi and total gut wall. This has been proposed as the potential mechanism responsible for the enhanced nutrient digestibility observed with AGPs [16]. More recently, it has also been suggested that AGPs inhibit the production and release of catabolic mediators by intestinal inflammatory cells, acting as growth permitters [17].

However, soon after the discovery of AGPs ability to enhance growth performances, concerns were expressed about a potential relationship between AGP and the development of antimicrobial resistance [15].

For this reason, the first nation to eliminate the use of AGP was Sweden in 1986 [18]. A few years later, in 1993 glycopeptide-resistant enterococci were isolated from food-producing animals in England [19]. This finding was unexpected because the use of most of the glycopeptides was not approved in animals to treat infections. However, one of them, avoparcin, was in use as an AGP [18]. Although no connection was established between resistant bacteria in food-producing animals and infection in humans, avoparcin was banned in Denmark in 1995 and then in all the EU member states in 1997 [20]. Since then, different AGP were progressively banned. In January 1998 virginiamycin was banned in Denmark and in February 1998 all AGP were voluntary banned in Danish cattle and chicken farms [20]. In July and September 1999, tylosin, spiramycin, bacitracin, and virginiamycin were officially banned by the EU Commission to preserve their efficacy human medicine () [20]. Thus, in Denmark the use of antimicrobials was restricted to therapeutic purpose, by prescription only, since January 2000. Finally, the raising concern and the increasing number of multi-resistant bacteria in animal and human population led to the withdraw of the approval for AGP use in food-producing animals in the European Union since January 1, 2006 [13]. Apart from Europe, also the USA, Canada, Mexico, Japan, Hong Kong, China and India have limited the use of

antimicrobials in feed. Thus, in 2017, the USA Food and Drug Administration stated that antibiotics can no longer be used as AGP across the USA [21].

As a consequence, the poultry sector is facing a great challenge to maintain productive performance of birds and to find valuable alternatives to replace AGP in feed [21].

3. INNOVATIVE ALTERNATIVES TO ANTIBIOTICS

In the last decades, considerable efforts are being expended on the development of antibiotic alternatives that will have the same benefits without the associated threats to human health [22]. Since 2006, numerous feed additives have been proposed as valuable alternatives to AGPs for poultry industry. Particularly, research focus on those compounds that can have antimicrobial effects and modulate gut health via several mechanisms: alteration of intestinal pH; maintenance of the protective mucous layer; promotion of beneficial intestinal bacteria, and/or inhibition of pathogens; increase of volatile SCFAs; enhance of nutrient uptake; and improvement of the local and humoral immune response [23]. In this context, functional feed seems the most promising alternatives [24, 25]. Functional feed can be defined as "dietary ingredients that, besides providing nutrients and energy, beneficially modulate one or more targeted functions in the body, by enhancing a certain physiological response and/or by reducing the risk of disease" [26].

Recent researches have focused on the potential use of prebiotics and probiotics as functional feed to replace AGPs and positively influence bird's gut health and performances [27].

3.1 Prebiotics

Prebiotics are defined as "non-digestible feed ingredients that beneficially affect the host by selectively stimulating the growth or activity of one or a limited number of bacteria in the gut" or "selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon the host health" [28, 29]. Important characteristics for a good prebiotic are acid stability in the stomach, resistance to GIT enzymes, lack of absorption in the upper GIT and being a substrate for the growth of gut probiotic bacteria [30]. Thanks to their ability to pass through the proximal part of the GIT and reach intact the distal portion, a variety of non-starch polysaccharides or oligosaccharides can act as prebiotics, including mannan-oligosaccharide (MOS), fructo-oligosaccharide (FOS), β-glucan and inulin [31]. Particularly, MOS and ß-glucan derived from the outer cell wall of the yeast *Saccharomyces cerevisiae* while FOS and inulin are extracted from plants [32].

Prebiotics have been shown to improve health by selective enrichment of gut microbiota and anti-obesity, anti-neoplastic, anti-allergic, hypo-cholesterolemic and immunomodulatory effects [30]. However, the majority of these beneficial effects seem to be mediated predominantly through the modulation of the intestinal microbiota. In fact, the usual targets for prebiotics are the lactic acid bacterial (LAB) genera: *Bifidobacterium* and *Lactobacillus*. The higher of these bacterial species in the gut results in competitive exclusion of pathogens and in bacteriocins production, which can reduce the growth of pathogens [33]. As these LAB are employed as probiotics, functional feed generally combined prebiotics and probiotics to mutually enhance their efficacy. Moreover, prebiotics are fermented by the gut microflora leading to the production of SCFA, an important energy sources for enterocytes and, thus, for the maintenance of the integrity of the gut lining [24]. Also, SCFA showed anti-inflammatory properties, reducing the production of pro-inflammatory cytokines (IL-4, TNF- α, IL-2) and, thus, positively modulating the intensity of the inflammatory response [28].

To date, both MOS and FOS have been tested in broiler nutrition showing higher body weight and feed conversion ratio along with higher intestinal villi, improved immune-competence, altered jejunal gene expression and improved microbiota composition [32, 34]. Recently, also ß-glucan has arisen as a valuable prebiotic and its properties will be discussed in the next subsection.

3.1.1 ß-glucan

β-glucans are polymers of glucose contained in the cell wall of bacteria, fungi, and yeast, or cereal grains (e.g., oats and barley). Their structure varies depending on their origin and the type of linkages present on the glucose polymers, which also influence their effects [35]. Particularly, β-glucans from yeast and fungal sources are characterised by 1,3-linked glucopyranosyl residues with small numbers of 1,6-linked branches, showing immunostimulant properties and increase the host resistance against pathogens [36]. In contrast, β-glucans derived from cereals, present a different structural composition, (β (1→3) (1→4)-β-glucans), producing an increase in digesta viscosity and having a detrimental effect on poultry health and performance [37].

These compounds are attracting significant attention as dietary immunomodulators and indirect growth promoters, being able to increase immunocompetence in food animals [38]. In fact, they can use various mechanisms to improve poultry growth performances. Firstly, they can bind to various cell surface receptors activating T-helper cells, cytotoxic macrophages, and natural killer cells, as well as inducing the proliferation and differentiation of T-cells. This results in an increased immune response in which different pro-inflammatory cytokines (IL-1, IL-2, INF-γ, and TNF-α) may act as the key signalling molecules [39]. This immune cell activation is also reflected in the increase of the relative weight of spleen recorded in chickens fed β-glucans [40]. Secondly, they can enhance gut morphology and alleviating the intestinal mucosal barrier impairment in *Salmonella typhimurium/E.coli* experimentally-infected chickens [36, 41]. Thirdly, β-glucan supplementation can reduce the severity of intestinal inflammation during Eimeria/coccidia infection in poultry [42] . Finally, β-glucan supplementation seems to increase the expression of jejunal tight junction proteins, protecting the deeper layers of the intestinal tract from the invasion of pathogens [36].

3.2 Probiotics

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host" or as "live microbial feed additives which beneficially affect the host animal via enhancing the balance in the gut and consequently improving feed efficiency, nutrient absorption, growth rate, and economic aspects of poultry" [14, 43]. A good probiotic should be non-pathogenic and non-toxic, acid tolerant and capable of adhering to gut epithelium, as well as producing antimicrobial substances. Moreover, it should not be absorbed in the upper gastrointestinal tract to exhibit beneficial effects [43]. Following these requirements, live bacterial species belonging to the genera *Lactobacillus*, *Streptococcus*, *Lactococcus*, and *Bifidobacterium* or yeast such as *Saccharomyces cerevisiae* and *Saccharomyces boulardii* were mainly used as probiotics. Probiotics can be composed by one or more bacterial strains and they can be administered alone or combined with other additives in feed or water [3].

Although the modes of action of probiotics are not yet completely understood, the gut microbiota and immune regulation seem to be the two most important mechanisms through which they exert their beneficial effects on chicken's gut health and growth performances [44]. On one hand, probiotics help to establish an unfavourable gut environment for the colonization of pathogens through the production of lactic acid, SCFA, and the reduction of the intestinal pH. Also, they compete for nutrients with undesired bacteria (e.g., *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli*, *Clostridium perfringens)* and they produce and secrete antibacterial substances such as bacteriocins (*Lactobacillus*, *Bacillus* spp.), organic acids, and hydrogen peroxide. Moreover, they inhibit bacterial adherence and translocation through competitive exclusion [32]. On the other, probiotics can enhance both the innate and acquired immune responses through the activation of macrophages and intraepithelial lymphocytes and the release of cytokine or immunoglobulins (IgA), respectively [45].

Overall, probiotics has been administered to poultry as potential alternatives to antibiotics in order to increase poultry growth performances, egg quality, meat safety and improve immunity and birds' health status [14]. To date, the majority of studies performed in poultry have used *Lactobacillus*, *Bacillus* or *Enterococcus* [43]. However, thanks to their content in chitin also insect can act as probiotic when administered at low doses [25].

4. INSECTS IN POULTRY FARMING

At first, insects have been identified as a valuable and alternative protein source for animal feeds in many countries around the world. In fact, they present a lower environmental impact and they are able to convert organic side streams into high-value protein products, representing a great example of circular economy [46]. Later on, it has also been demonstrated that insect can be successfully used as environmental enrichment in poultry farming, helping them in performing locomotor activities, increasing leg strength and bone density and reducing the incidence of leg deformations and lameness [47]. Finally, at low doses insects can be also used as novel prebiotics to improve gut health, thanks to their chitin content [48].

4.1 Current legislation for insect inclusion in animal feed

The European approach to the use of insects in animal feed has been greatly affected by the Bovine Spongiform Encephalopathy outbreak, which forced the European Union to ban the use of processed animal proteins (PAPs) in animal feed with the Reg (EU) 999/2001. In this regulation, proteins derived from insects are considered PAPs and their use as feed material for other farmed animals was prohibited.

After a few years, Reg 56/2013 amended the prohibition on the use of PAPs from any source, except ruminants, in aquaculture. However, insect regulation as feed raw material was still absent (Reg (UE) 68/2013).

Five years after, Reg (EU) 2017/893 authorized the feeding of insect PAPs in aquaculture only and in Annex II there were listed the insect species that could be used to produce these PAPs. So far, only the house cricket (*Acheta domesticus*), banded cricket (*Gryllodes sigillatus*), field cricket (*Gryllus assimilis*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), black soldier fly (*Hermetia illucens*), and the common house fly (*Musca domestica*) are permitted. The regulation also specified the substrates allowed to rear insects. Particularly, the substrate can contain products of non-animal origin or the following animal products: fishmeal, blood, hydrolysed proteins, gelatine, collagen from non-ruminants, di- and tri-calcium, eggs and egg's products, milk, milk based or milk derived products and colostrum, honey and rendered fats. No contact with other animal products, manure, catering waste or any other waste is allowed (Reg (EU) 2017/893).

In 2017, Reg (EU) 2017/1017 amended Reg (EU) No. 68/2013 and permitted the use of treated or untreated live terrestrial invertebrates and dead terrestrial invertebrates as feed materials, but not as PAPs (Reg (EC) No. 1069/2009). Thereby, terrestrial invertebrates are considered appropriate feed ingredients in all their life stages but all the species that can potentially represent a threat for n plant, animal, or human health are not permitted. In August 2021, the Reg (EU) 1372/2021 amended the prohibition to feed non-ruminant farmed animals, other than fur animals, with animals derived PAPs contained in the Annex IV to Reg (EC) No 999/2001 as regards. This authorised the use of insect PAPs also in poultry and pig feed, representing a relevant milestone for the European insect sector [48]. Among the seven authorized insect species, yellow mealworm (*Tenebrio molitor* -TM) and black soldier fly (*Hermetia illucens* - HI) seem to be the most promising ones to use as feed, environmental enrichment and probiotic in poultry [25, 49, 50].

In the following paragraphs an overview on these two insect species will be provided.

4.1.1 Hermetia illucens

Hermetia illucens (Diptera*: Stratiomyidae*) is commonly named black soldier fly and it is distributed worldwide prominently in the equatorial tropics [51]. Life cycle of HI is characterized by four phases: eggs (4 days), larva (18 days), pre-pupa (14 days) and adults (9 days). Thus, in optimal environmental conditions (29-31°C and 30% of relative humidity) the larvae become mature in 45 days, even though it can require up to 4 months in case of insufficient feed availability [52]. The larvae can measure up to 27 mm in length and 6 mm in width and its weight can reach 220 mg, having a dull, whitish colour [53] (Figure 1A). The larvae feed intake is comprised between 25 and 500 mg of fresh matter/larva/day and the rearing substrate can contain a wide range of organic products (e.g., rotting fruits and vegetables, coffee bean pulp, distiller grains, fish offal or animal manure) [54–56]. At the end of the larval stage, the prepupae migrates to find a dry and suitable pupation site. The adult flies are black, wasp-like and they measure around 15–20 mm in length (Figure 1B). They are neither pest nor disease vector as they do not feed themselves and they survive on the fat stored in their larval stage [52].

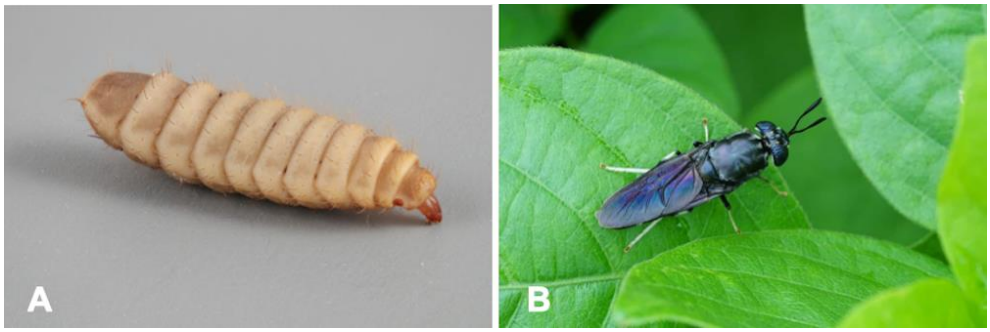

Figure 1. A) *Hermetia illucens* larvae (source: https://bit.ly/3wDZkY6); B) *Hermetia illucens* adult (source: https://bit.ly/36tU4LZ).

This has converted HI in an ideal candidate for mass rearing since the 1990s as it is able to adapt to a wide range of challenging environmental conditions, such as drought, food shortage or oxygen deficiency and it is not linked with microbiological risks for human and animal's health [55].
Black soldier fly larvae can be administered live, chopped, or dried and ground to birds and they are a high-value protein and fat source [57]. Fresh larvae showed a quite high dry matter (DM) content, between 35.0 and 45.0%, which makes them easier and less costly to dehydrate than other fresh by-products [58]. They contain about 40.0–44.0% crude protein (CP) and 11.0-28.0% DM of ash [59]. The larvae are also rich in calcium (5.0–8.0% DM) and phosphorus (0.6–1.5% DM) [60–62].

Their fat content and their fatty acid composition is extremely variable, depending on the diet: 15.0–25.0% for larvae fed on poultry manure [61], 28.0% on swine manure [63], 35.0% on cattle manure [60] and 42.0–49.0% on oil-rich food waste [57].

Regarding chitin content, it is similar in larvae, pre-pupae and pupae stages being around 9-10% of DM. However, it decreases in adult flies (5.0-8.0%) [64].

4.1.2 Tenebrio molitor

Tenebrio molitor (Coleoptera: *Tenebrionidae*), commonly named yellow mealworm, is indigenous to Europe and is now distributed worldwide [57]. Its life cycle can last from 280 to 630 days, depending on the environmental conditions and it can be divided into four stages as previously described for HI: eggs (10-12 days at 25-27°C), larvae (3-4 months at 18°C), pupae (7-9 days) and adults (2-3 months) [65]. The mature larva is light yellow-brown coloured, it can measure 20–32 mm and weights 130–160 mg (Figure 2A). In the adult stage, the mealworm is a black beetle of 12–15 mm [65] (Figure 2B).

Mealworms are omnivorous and they can be feed on vegetables or animal products (e.g., meat and feathers) [66]. Their rearing substrate are typically composed by cereal bran or flour (wheat, oats, maize) and supplemented with protein sources such as soybean flour, skimmed milk powder or yeast. Fresh fruits and vegetables can be also use to feed mealworms [54, 67]. The rearing substrate must be balanced to contain about 20% protein on DM basis [68].

Figure 2. A) *Tenebrio molitor* larvae (source: https://bit.ly/3IJE7hD); B) *Tenebrio molitor* adult (source: https://bit.ly/36tSiub).

Yellow mealworms require an easy breeding system as a stable protein content can be obtained, independently from the rearing substrate [69]. For this reason, they have been chosen for industrial manufacturing of pets, zoo animals, fish and poultry feed [70, 71].

Mealworms are typically fed as live larvae, but canned and dried larvae are also commercially available [57]. Fresh larvae contain about 60% of water, which makes them difficult to preserve. The CP content of TM larvae is 52.4% on average and it ranges from 47.0% to 60.2%, being greater than that of conventional soybean meal (49.4%) and lower than that of fishmeal (67.5%) [72]. They are also relatively low in ash (<5.0 % DM) and they are low in Ca and Ca:P ratio [73]. Thus, the exclusive feeding of mealworms can cause Ca deficiency or symptomatic metabolic bone disease and a calcium supplementation is recommended [71]. Fat content can vary between 31.0 and 43.0%

and they are richer in essential polyunsaturated fatty acids such as omega-3 (47.3 g/100g) and omega-6 (31.6 g/100 g) compared to HI [74].

As already described for HI, chitin content is similar in TM larvae and pupae but lower in adults. In fact, TM larvae and pupae contained 6.41 % of chitin on average ranging from 4.9 % and 13.0% on DM (13.0 g/100.0 g of chitin) while adults contained 0.01 g/100 g [74, 75]. Also, TM larvae contain 11.6 mg/g of chitosan, which allow them to be used as prebiotics [74].

4.2 The role of chitin

Chitin is a linear β-1, 4-linked polymer of N-acetyl-D-glucosamine and it is the second most abundant polysaccharide in nature, functioning as a major structural polymer in many organisms [76]. Chitin has been found in organisms inhabiting both terrestrial and underwater habitats such as crustaceans, molluscs and insects [64, 77, 78]. Three polymorphs- α, β, and γ- can be distinguished based on the orientation and packing of the chitin molecular chains [79]. Mammals and birds are not able to synthesize chitin but they possess two active chitinases in their genomes: chitotriosidase and acidic chitinase. These chitinases belong to the family of glycoside hydrolases, which are able to hydrolyse the β-1, 4 glycoside bonds of chitin [80, 81]. Particularly, acidic chitinase is highly expressed in poultry stomach and it can digest chitin in their GIT [76]. Chitin can be deacetylated by chitinase in chitosan, a polymer formed by a combination of N-acetyl-d-glucosamine and β-(1,4)-linked-d-glucosamine [82]. Unlike chitin, chitosan cannot be degraded by chitinase and following ingestion it will arrive in its intact form in the intestine [83].

As a consequence, thanks to their content in chitin and chitosan, insects have attracted considerable interest because of their potential capacity in reducing gastrointestinal diseases, through the enhancement of local immunity, the promotion of beneficial bacteria and the inhibition of pathogenic ones (e.g., *Salmonella spp*. and *E. coli*) [56]. In fact, it has been shown that chitin metabolization by chitinases, can activate innate immune responses, and, eventually, induce adaptive t-helper 2 (Th2). The Th2 cytokines (IL-4; IL-6 and IL-10) can stimulate chitinase production, generating additional chitin fragmentation and reinforcing these responses [84, 85]. To date, chitin and chitosan activation of the innate immune response seem to be size-dependent. Firstly, large chitin polymers are biologically inert (>70 μm). Secondly, intermediately sized fragments (40–70 μm) can act as pathogen-associated molecular patterns and induce inflammation. Thirdly, smaller fragments (< 40 μm) stimulate macrophage IL-10 production, an anti-inflammatory cytokine [86]. Thus, the immune modulatory properties of chitin seem to be based on a self-limiting mechanisms as large chitin polymers are inert, smaller fragments induce inflammation, and even smaller fragments show an anti-inflammatory activity mediated by the release of anti-inflammatory cytokine IL-10. It could be hypothesized that when chitin-containing pathogens are sense by the host immune system, they activate the innate response and stimulate the release of chitinases, chitin fragmentation. As a consequence of chitinase action, intermediate fragments are produced and they activate the pattern recognition receptors inducing the local inflammation. This would continue until the immune system control the pathogens and smaller chitin fragments are generated. These small fragments would induce the release of anti-inflammatory molecules like IL-10 that will send negative feedback, lowering the local inflammatory response [86].

Apart from these potential benefits that can be applied for modulating gut health, chitin content of feed should be monitored as it can negatively influence protein digestibility. To date, it has been suggested that dietary inclusion of chitin higher than 5.0 g/kg decreased efficiency of nitrogen utilization, increasing the viscosity of the ingesta, decreasing the motility of the GIT and, as a consequence, depressing growth performances [87].

5. GUT HEALTH: DEFINITION AND BIOMARKERS

In recent decades, the term "gut health" has been frequently used in human and veterinary medicine [88–90]. A consensus on the definition of "gut health" has not yet been reached due to the complexity of the digestive, immunological, neurological and endocrine functions of the GIT [91]. Gut health is generally defined as "the absence, prevention or avoidance of intestinal disease so that the animal is able to perform its physiological functions in order to withstand exogenous and endogenous stressors" [89]. However, a broader definition of gut health should encompass an adequate digestion process, a proper structure of gastrointestinal barrier, the absence of gastrointestinal disease, a stable intestinal microbiota, and an effective immune response [88].

Gut health depends on a delicate balance between the host, the intestinal environment and the feed [91]. It can be considered a synonymous of animal's health in food-producing animals, being of vital importance for animal's growth performances [89]. In fact, digestion and nutrient absorption are strictly linked to the maintenance of gut heath and any impairment of the GIT functionality can impair feed conversion ratio, increasing the susceptibility to disease and provokes higher production costs [89].

The maintenance of an optimal gut health relies on an adequate gut morphology, gut mucin expression, local immune response and gut microbiota, which are the components of the so called "intestinal immunity" [92]. Firstly, gut morphometry and tight junctions are crucial for evaluating the presence of any intestinal mucosa damage, representing the "physical mucosal barrier" that separates the lumen from the sterile underlying tissue, avoiding bacterial translocation and assuring adequate nutrient absorption [93]. Secondly, the mucus layer covers the GIT mucosa and it represents the "chemical barrier", regulating contact between the commensal bacteria and the epithelium [92]. Thirdly, the gut immune system forms the "immunological barrier" where mucosal and submucosal components as epithelial cells, dendritic cells, macrophages, B and T cells, differentiate or become activated, resulting in chemokines, cytokines or IgA secretion [92]. Finally, gut microbiota constitutes the "microbiological barrier" which provides resistance against pathogens colonization thanks to the production of metabolites that modulate immune signalling and promote immune homeostasis [94]. Therefore, a multidisciplinary approach that encompasses all the four above-mention parameters seems to be crucial for the evaluation of animal's gut health. After a brief description of the anatomy and histology of the chicken intestine, each parameter will be discussed in the following paragraphs.

5.1 Anatomy of the chicken's gut

In most birds, the intestine is shorter in relative terms than in domestic mammals and it is divided in small intestine and large intestine (Figure 3). Among avian species, grain- and grass-feeders showed a longer intestine compared to carnivores. Moreover, the digestive tract of the modern chicken has had to adapt to abrupt changes due to intensive breeding for growth rate for broiler chickens, especially in the pre-starter and starter phase [95].

The small intestine is composed by duodenum, jejunum and ileum. The function of the small intestinal mucosa, supported by the activity of the muscular layers, is digestion of nutrients and absorption of the resulting breakdown products [96] (Figure 3).

The duodenum begins from the pylorus of the ventriculus, it is U-shaped and it can be divided in a descending portion and an ascending component. The pancreas is located between these two segments. In contrast to mammals, three pancreatic ducts and two bile ducts open in the ascending duodenum [97].

The duodenal-jejunal flexure mark the transition between the duodenum and the jejunum and it is placed ventrally and cranially to the cranial mesenteric artery. The jejunum and ileum are arranged in loops and they are placed in the right caudal quadrant of the celomatic cavity. The Meckel's diverticulum, an embryonic remnant of the yolk stalk, divides the jejunum from the ileum, even though no discernible morphological differences can be detected among the two intestinal segments [97].

The large intestine comprises the caecum and the rectum (Figure 3). Ongoing digestion of residual foodstuffs, particularly hydrolysis of cellulose, is taken over by carbohydrate- and protein-cleaving intestinal bacteria and protozoa in the caecum. Other important functions of the large intestinal mucosa include absorption of water and electrolytes, in exchange for calcium and bicarbonate, associated thickening of the intestinal contents and secretion of mucus from goblet cells for lubrication of the faeces. Absorption of nutrients and vitamins also takes place in the large intestine [96].

In contrast to mammals, two large caeca can be observed in domestic poultry. They are connected to the end of the ileum through a ileo-caecal ligament. The caecal body has a thin wall and it is ampulliform, while the apex may be pointed or vesicular. Each caecum shows a lymphatic structure at its base, sometimes called the "caecal tonsil". The caeca continued in the rectum, which is the final, straight segment of the intestine that opens in the cloaca [97].

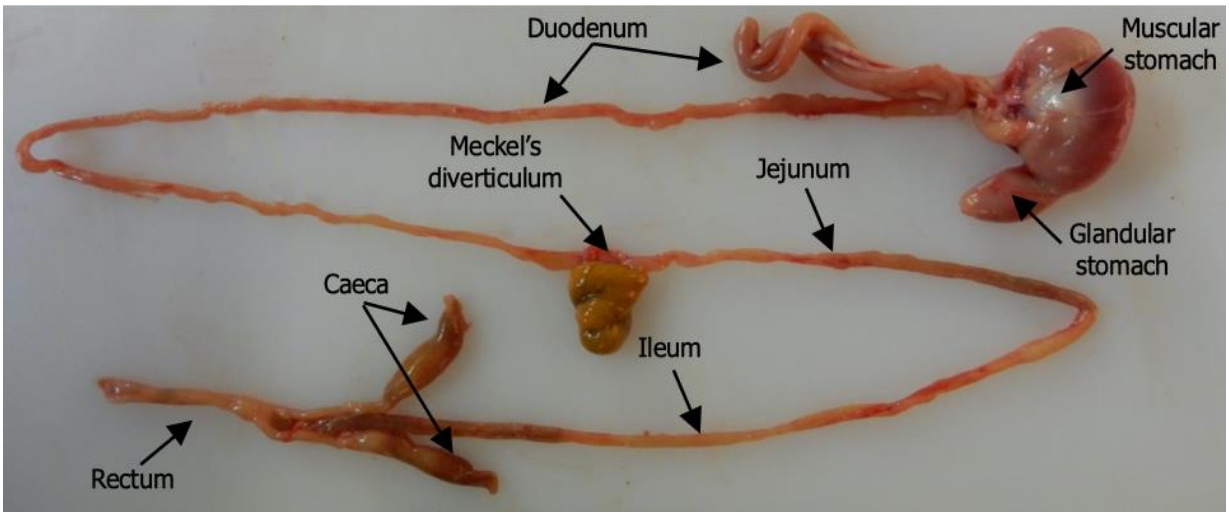

Figure 3. Gross anatomy of the chicken gastrointestinal tract. Small intestine: duodedum, jejunum and ileum; large intestine: caeca and rectum.

5.2. Histology of chicken's gut

The intestine is a multi-layered tube containing a mucosal layer, submucosal layer, circular muscle layer, longitudinal muscle layer and serosal layer [98] (Figure 4).

Regarding the small intestine, the mucosa consists of an inner epithelium, a middle lamina propria, and an outer *muscularis mucosae*. The lumen is lined by a simple columnar epithelium containing numerous goblet cells interspersed among columnar absorptive cells. In addition, the entire intestinal mucosa is covered with intestinal villi, finger-like evaginations of the lamina propria lined with simple epithelium, that contribute significantly to the total mucosal surface area. The intestinal glands (crypts of Lieberkühn) open between the bases of the villi and penetrate the mucosa as far as the lamina muscularis. Goblet cells are interposed between enterocytes and they are monocellular glands that produce mucus rich in glycoproteins and glycolipids. Their number increases from the duodenum towards the ileum [99]. The lamina propria mucosae consists of loose connective tissue and it forms the core of the intestinal villi. Much of the lamina propria is occupied by the intestinal glands. Between the glands, blood and lymph vessels, nerve fibres, myofibroblasts and smooth muscle cells are present. Each villus contains a dense and substantial capillary network that serves primarily to support the secretory activity of epithelial cells and to transport products of digestion, water, electrolytes and vitamins. Smooth muscle cells originating from the lamina muscularis mucosae pass through the lamina propria along the longitudinal axis of the villus. These cells undergo rhythmic contraction, resulting in shortening and lengthening of the villus. Through these contractions, the cells act as a pumping station that aids in transport of blood and lymph into deeper vessels. The lamina propria contains dense aggregates of lymphatic tissue rich in T and B lymphocytes, plasma cells, monocytes, macrophages, mast cells, neutrophils and eosinophils. The lymphatic tissue of the intestinal mucosa is collectively termed GALT (gut-associated lymphoid tissue). An inner circular and outer longitudinal layers of smooth muscle are the main components of the lamina muscularis [99].

Regarding the large intestine, in contrast to mammals, birds presented intestinal villi in all the segments. Throughout the large intestine, elongated, relatively straight and unbranched intestinal glands extend into the lamina propria. The surface enterocytes continue into the intestinal glands as a simple columnar glandular epithelium. Distally these epithelial cells become less frequent and are replaced by an increasing number of goblet cells. Goblet cells are the dominant cell type in the intestinal glands of the middle and distal segments of the large intestine [99].

Both in the small and large intestine, the tunica submucosa is extremely thin in poultry. It is a collagen fibre-rich connective tissue containing vessels and neural networks (submucosal nerve plexus) and solitary lymphatic nodules [99]. An inner circular and outer longitudinal smooth muscle

layers form the tunica muscularis. The myenteric plexus in contained in the connective tissue between the two muscle layers [99, 100].

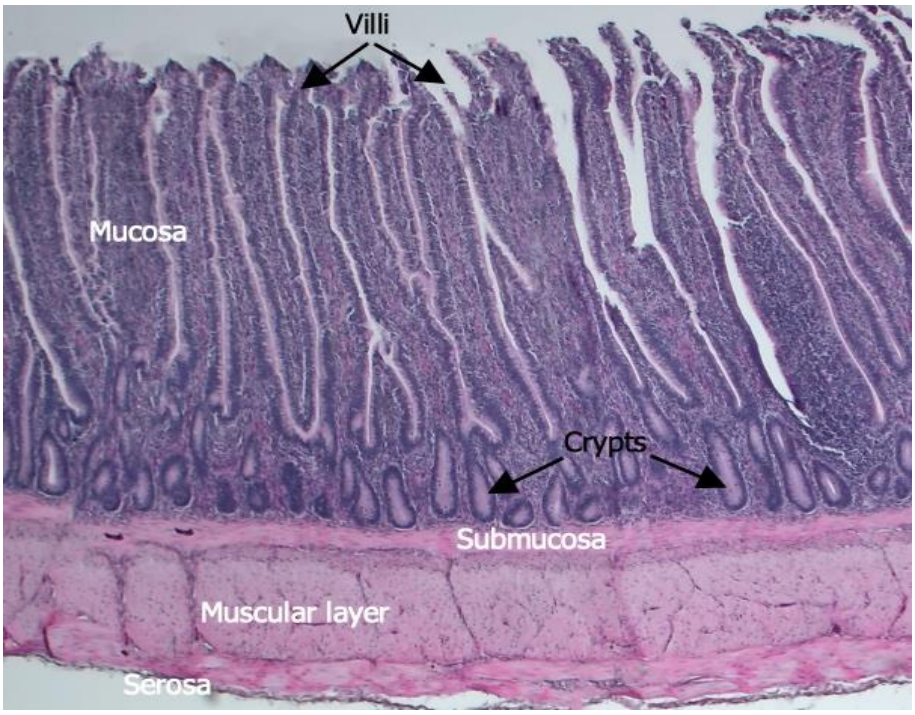

Figure 4. Histological picture of the chicken's jejunum.

5.3 Gut mucosal morphometry

The small intestine mucosa presented intestinal villi, whose are covered with a simple columnar epithelium composed by absorptive, goblet and entero-endocrine cells [100]. The pool of crypt-based stem cells continuously renewed these epithelial cells, which migrate up to the villus tip where they enter a programmed cell death and exfoliate into the lumen. Cells differentiation take place during migration, being the enterocytes near the villus tips the most important for nutrient absorption. As a consequence, when an increase loss of villous epithelial cells occurs, a decreased villus length is the most common findings in intestinal health problems. Moreover, crypt stem cells increase their proliferation in order to compensate this loss, resulting in increased crypt depth [6, 93]. Therefore, simple measurements of villus height (Vh), villus width (Vw) and crypt depth (Cd) are considered the gold standard for the evaluation of gut health in animals [6]. The villus height to crypt depth ratio (Vh/Cd) is also evaluated, being an indicator of the enterocyte's maturity and functionality [101]. The physiological gut development is characterized by long, slender villi and shallow crypts. Indeed, these asset guarantees a higher villus surface area and a satisfactory digestive enzymatic activity thanks to the presence of longer villi which lead to a higher transport of nutrients [101]. Moreover, when the survival of villi is prolonged, shallower crypts are observed, with a reduced energy expenditure for enterocytes renewal and a maximized nutrient utilization for growth [102]. Also, it is well known that the increase of Vh and the Vh/Cd ratio increases the mucosal surface and the total area of nutrient absorption, which can be calculated as follow [103]: $(2\pi)(\frac{Vw}{2})(Vh)$. On the contrary, lower and thickened villi and greater crypt depth lead to poor digestion and absorption of nutrients and, as a consequence, poor growth performances of the animals [104]. Considering the muscular layer, it controls the motility of the gut and the progression of a bolus and its thickness is also an important feature to consider as it can affect the absorption processes [105].

Previous studies have provided reference values for Vh, Cd and Vh/Cd for duodenum, jejunum and ileum in broiler chickens at 23 days of age, being approximately 1400, 900 and 700 μm for villus height, 190, 170 and 160 μm for crypt depth, and 8, 6 and 5 for villus height to crypt depth ratio, respectively [105]. These values highlighted also another physiological characteristic of the morphometric indices: they showed a proximo-distal decreasing gradient from duodenum to ileum in accordance with the different intensity of nutrient absorption processes that take place in each segment [101].

Morphometric indices can be influenced by dietary modifications. Particularly, TM and HI insect meals seem to have a dose-dependent effect. On one hand, low levels of insect meal inclusion in substitution of soybean meal (<10%) can improve gut morphology of broilers chickens [106–111].

On the other, insect meal inclusion levels higher than 10% reduce villus height of chickens, showing a negative modulation of gut morphometry [106–109]. In ducks, the use of increasing HI meal inclusion levels (3,6,9%) did not affect gut morphometric indices [112]. However, no studies are available on the effect of insect live larvae neither in chickens nor in ducks.

Also, B-glucans have been tested in broilers chicken reporting beneficial effects on gut morphometry in the finisher phase [113, 114].

5.4 Tight junctions

Tight junctions (TJ) are multi-protein complexes that seal epithelial cells together and dynamically regulate paracellular transport of molecules (e.g., water, nutrients, electrolytes) from the intestinal lumen through the epithelium into the bloodstream [115]. They are required for the to guarantee a proper nutrient absorption and utilization, leading to optimal health and performances of the bird [116].

Structurally, TJ are composed of transmembrane and membrane-associated proteins that are assembled together to form stable complexes to determine function [117]. Within this network of proteins, pores facilitate transport of water, ions, and small nutrients [118]. Among transmembrane proteins, claudins (CL) and occludin (OCL) are suggested to form a physical barrier. Claudin family (Claudin 1, 3, 5, and 15) has been observed in the chicken intestinal epithelium and it modulates paracellular transport in the gut epithelium but it also has an important role in regulating the cellular signalling [119]. Occludin consists of four transmembrane domains and they are capable of altering epithelial permeability through their shifting among various paracellular locations. In fact, barrier function loss is frequently associated to the movement of occludin from the tight junction and it can be triggered by multiple stimuli [117].

On the contrary, *Zonula occludens* (ZO-1, ZO-2 and ZO-3) are membrane -associated proteins, linking the TJ complex to the actin cytoskeleton [118]. Particularly, ZO-1 coordinates TJ formation and cell polarization, establishing links between transmembrane TJ proteins and cytoskeleton components [120].

In chicken, the TJ are already established and functional at hatch but their permeability and expression can be influenced by different factors [115]. On one hand, enteric pathogens such as *Escherichia coli, Campylobacter jejuni, Clostridium difficile, Clostridium perfringens, Salmonella thyphimurium* or their toxins can alter the TJ complexes or reduce their expression producing the well-known "leaky gut syndrome", which is characterized by decreased absorption of nutrients, increased secretory loss of ions and water, diarrhoea and increased dysbiosis which may induce inflammation [6]. On the other, different food substances can positively regulate TJ permeability [29]. In fact, some amino acids (e.g., arginine), some vitamins (e.g., vitamin D), SCFAs (e.g., acetate, propionate, butyrate) and middle-chain fatty acids (e.g., capric acid and lauric acid), polyphenols (e.g., resveratrol) and β-glucans can improve intestinal barrier functions enhancing the expression of TJ proteins [36, 121–124]. However, no studies are available evaluating neither the effect of β-glucans on TJ in new-born chicken in basal conditions nor of chitin in adult birds.

5.5 Mucin composition

The gastrointestinal tract is covered by a mucus layer which represents an important barrier against pathogens. The top layer of the mucus gel surface is composed primarily by mucins (MUC) [125]. Mucins are highly-glycosylated glycoproteins rich in proline, threonine and/or serine sequences, which contributes to their physical and biological properties [126]. These glycoproteins are responsible for the competitive exclusion of bacteria through adherence via heterogenous oligosaccharide chains, avoiding the contact between the noxious agents and the underlying epithelial cells [127]. However, simultaneously, mucin high carbohydrate content creates a favourable environment for the proliferation of specific microflora [128]. Thus, the chemical composition of mucus is essential for the establishment of the mucosal barrier [129]. Mucins are encoded by mucin genes, which are generally named as MUC and a number that reflects the order in which the mucin gene was discovered. In chicken, the MUC2 is the main gene related to mucin synthesis in the small and large intestine [125]. Mucins are secreted by goblet cells (GC), which are located in the crypts of the small and large intestine. Their maturation occurs during the migration from the crypt to the villus tip, where they are rapidly substitute by newly formed GC after 2-3 day in chickens [130]. Immature GC are located at the crypt base, being large, pyramidal shaped and containing less mucin granules. Then, matured GC become cup-like shaped containing more mucin granules at the apical portion of the cells. Due to the protective function of mucins, GC density increased proximo-distally from duodenum to ileum. In fact, duodenum is the most important segment for digestion and absorption of nutrients and, as a consequence it employs most of the available energy in the proliferation of absorptive cells over secreting cells, explaining the presence of less but bigger GC cells in this tract [126]. Thus, the lower GC density and mucus secretion in the duodenum should enhance its absorptive capacity, accommodating it to the short digesta retention time. Furthermore, the increase of the microbiota diversity and activity, along with the digesta retention time, in the distal small intestine can motivate the higher GC density in the ileum [126].
Histochemically, mucins can be separated into neutral and acidic mucins, with the latter being further divided into sulfated and sialylated mucin types [129]. This is due to the chemical nature of the oligosaccharide sugar moieties that allows specific histological techniques to detect the acidity or neutrality of mucins thanks to the presence of these sugar groups [129]. The physiological role of each mucin types and the related protective properties are not yet well understood. However, it has been proven that, many bacteria in the gut are able to produce glycosidases or proteases, which can degrade mucus. The terminal ends of O-glycan with O-acetylated sialic acid (sialomucins) or sulfated group (sulfomucins) seems to be able to protect the mucus layer from the degradation provoked by these bacterial enzymes as well as other proteolytic host enzymes [126].

Mucus production is not only linked with bacteria enzymatic digestion but it is also strictly linked with the digesta and gut. Therefore, dietary modification (e.g., feed restriction, protein sources and level, carbohydrate sources, feed form) that can affect diet digestibility and gut microbiota composition (e.g., symbiosis and dysbiosis) could potentially also influence the intestinal GC as well as mucins production and dynamic. Regarding insect meals, previous studies have observed that inclusion up to 7.5% of TM meal in free-range chickens did not alter mucin composition [106]. On the contrary, inclusion up to 10% of TM and HI meals in broiler chickens increased mucin content in the small intestine [131, 132]. However, higher inclusion of TM and HI meals (≥ 15%) decreased significantly mucin expression in the small intestine [131, 132]. No studies are available about the use of insect live larvae. Also, supplementation with β-glucans seems to enhance goblet cell number and to upregulate mucin-2 production [37].

5.6 Gut local immuno-response

The GIT is constantly exposed to infectious and non-infectious stressors, which convert it in the active immune organ with the highest number of resident immune cells in the host. The mucosal immune system can be separated into two branches: the innate immune response and the acquired/adaptive immune response [133].

On one hand, the innate immune response is mediated by the epithelial cells residing in the intestinal mucosa and by granulocytes, monocytes and macrophages recruited from the blood [134]. Thus, innate immune cells recognize microbes through pattern recognition receptors, which recognize pathogen-associated molecular patterns (PAMP) from microbial cells, such as lipoproteins, carbohydrates and nucleic acids [134].

On the other, the acquired immunity depends on the recognition of antigens by B and T lymphocytes [135]. Following the activation of adaptive immunity, antibodies are produced by B cells, infected host cells are killed by cytotoxic T cells, and cytokines are produced by T helper (Th) cells. In poultry, as in mammals, there are 2 types of effector Th cells, Th1 and Th2 which can be distinguished based on the cytokines they produce and the response they induce on the immune system. For example, Th1 cells typically produce cytokines such as IFN-γ, TNF- α and IL-2 which play an important role in the activation of the cell-mediated immune response, whereas Th2 cells produce cytokines such as IL-4, IL-5, and IL-10 that play important roles in the activation of the acquired B-cells response [136].

Chitin/chitosan are potential targets for the bird immune system since they lack these biopolymers naturally. Particularly, chitin is sensed by the innate immune system as a PAMP and it activates various molecular signalling cascades that alter the cytokine profiles and cellular phenotype [137]. It has been reported that chitin can mainly regulate Th2 immune responses, considering its ability to stimulate the production of type 1 cytokines, which are involved in the inhibition of Th2 inflammation [86]. For example, Reese et al. [138] reported that chitin induce the accumulation of IL-4-expressing innate immune cells in tissues. Also, bigger chitin fragments of 40 to 70 μm size can enhance T cell functions, neutral killer cell activity, and IFN-γ production [139]. On the contrary, smaller fragments induce IL-10 release [86].

Similarly, also β-glucans as components of fungi membranes can be recognized by avian innate immune cells. Through phagocytosis process, macrophages and dendritic cells fragmentize the β-glucans and secreted TNF-α, IL-2, IL-10, which activates adaptive immune response [37].

Particularly, IFN-γ, TNF- α and IL-2 are defined as Th1 pro-inflammatory cytokines as they trigger inflammation [139]. Precisely, TNF-α is produced by macrophages/monocytes during acute inflammation and its release activate a diverse range of cell signalling events, leading to necrosis or apoptosis [140]. The INF-γ is the central effector of cell mediated immunity and it can amplify

antigen presentation through antigen presenting cells, increases the production of Reactive Oxygen Species /Reactive Nitrogen Intermediates, and induces anti-viral responses [141]. Finally, IL-2 stimulates T-cell proliferation and differentiation, increases cytokine synthesis, and promotes regulatory T cell development [142]. On the contrary, IL-4, and IL-10 are anti-inflammatory Th2 cytokines. Particularly, IL-4 promotes Th-2 lymphocyte development and it inhibits the synthesis of lipopolysaccharide-induced proinflammatory cytokines [143]. Also, IL-6 inhibits TNF- α and IL-1 production by macrophages and IL-10 inhibits monocyte/macrophage/neutrophil cytokine production and Th1-type lymphocyte responses [143].

5.7 Microbiota

The GIT of the chicken harbours a diverse bacterial community known as "microbiota" in which each microorganism is adapted to its own ecological niche and synergistically lives with other bacterial species. Microbiota composition and diversity is able to influence nutrient absorption, feed digestibility, energy harvest and therefore productivity [94]. For these reasons, intestinal microbiota is a fundamental component in the maintenance of the host health through the modulation of several physiological functions including nutrition, metabolism, and immunity [144]. Firstly, the commensal bacteria of the digestive system contribute nutrients such as SCFAs, ammonium, amino acids and vitamins which play a key role in the metabolism of chickens [145]. In fact, most intestinal bacteria are capable of hydrolysing complex carbohydrates into primary sugars and then ferment them, producing SCFAs (e.g., acetate, propionate, and butyrate) [146]. In the ceca, SCFAs are absorbed through the epithelium by passive diffusion and they participate in different metabolic pathways [146]. In the gut, SCFAs are an important energy source for the gut wall, providing up to 50% of the daily energy requested by enterocytes and stimulating mucin production and release [146]. Intestinal bacteria can also contribute to the metabolism of nitrogen, permitting its absorption and use for amino acids synthesis [144]. Moreover, the same intestinal bacteria-particularly lactic acid bacteria- can themselves be a source of vitamins [147]. Secondly, the microbiota contributes to maintain the functionality of the innate immune system of the host [148]. In the steady- state, PRRs expressed by dendritic cells or macrophages of the innate immune system can sense different metabolites and components of the commensal microbiota, regulating intestinal epithelial barrier function, cellular lifespan of phagocytes, and inducing secretion of antimicrobial peptides and IgA [92]. Regarding the acquired immune system, commensal bacteria stimulate the Th cells and control the quantity of mediators secreted by these cells, protecting the mucosa and modulating the immune response, by Levy et al. [148]. Thirdly, intestinal microbiota reduces the pathogen adhesion and colonization in the gut though competitive exclusion [144]. This can be the result of different mechanisms, such as the physical occupation of the attachment sites on the gut cells, the competition for the same nutrients in a given niche or the production of bacteriocins that either inhibit the growth of, or make the environment unsuitable for pathogens [89]. For all the reasons stated above, the modulation of microbiota can indirectly modulate also gut histomorphometry, gut local immune response and mucin production.

Different factors can influence the composition and function of the microbiota. Particularly, age of the birds, location in the GIT and diet seem to be the most influential ones [91]. The initial colonization of the gastrointestinal tract of birds starts at hatching. Thus, after this initial colonization the richness and complexity of the microbiota increase exponentially as the birds grow, until reaching a state of maturation and stabilizing around 3 weeks of life in broilers [149–151]. Also, the

abundance and diversity of the microbiota varies along the GIT and, predictably, the regions that have less tolerable conditions and faster passage of digesta have lower numbers of bacteria [91]. In chicken, the cecal microbiota is the most investigated intestinal microbiota as caeca account for the longest time of digesta transit and as a consequence the highest microbial cell densities (up to 1011 cells/g) and diversity [145]. Wei et al. [152] recorded 915 operational taxonomic units (OTUs)in the chicken's ceca, equivalent to species and classified in 13 phyla, of which Firmicutes (70.0%), Bacteroidetes (12.3%) and Proteobacteria (9.3%) accounted for >90% of all the sequences. Cyanobacteria, Spirochaetes, Synergisteles, Fusobacteria, Tenericutes, and Verrucomicrobia were found in small proportions. Overall, 117 genera were described, among which *Clostridium*, *Ruminococcus*, *Lactobacillus* and *Bacteroides* predominated [152]. Finally, microbiota composition is strictly linked with the nutrients contained in the diet provided to chickens thus, diet is the factor that has the major impact on microbiota growth and establishment. In particular, feed form (whole or milled grains, or pellets); the quantity of water-soluble non-starch polysaccharides; and the sources of fat, starch and proteins are the main characteristic that can influence microbiota [153]. Regarding protein sources, the inclusion of TM and HI meals in poultry diet has been proven to increase SCFAs producing bacteria relative abundance (e.g *Ruminococcus*, *Firmicutes*), especially at lower doses (e.g., 5%) [106, 131, 154]. On the contrary, inclusion of 10 and 15% of TM meals seems to negatively affect caecal microbiota, reducing the above-mentioned SCFAs bacteria [132, 155]. No previous studies are available on the effect of insect live larvae on chicken and duck microbiota.

Moreover, β-glucans, MOS and FOS as prebiotics have been demonstrated to be able to positively modulate gut microbiota acting as growth substrates for beneficial bacteria such as butyrate-producing Clostridia and Bifidobacteria, enriching the resident microbial groups [156]. Their administration in the first day's post-hatching seems particularly helpful in favouring the early intestinal colonization with a beneficial microbiota [157].

6. AIM OF THE STUDY

This PhD project aimed to assess whether insect live larvae or functional feed can positively modulate gut health in chickens and ducks through a multidisciplinary approach including histopathological, histochemical and biomolecular techniques. Three specific hypotheses have been formulated:

1) Insect and functional feed can enhance gut development.
2) Insect and functional feed can down-regulate pro-inflammatory cytokines production and reducing intestinal and systemic inflammation.
3) Insects can increase the SCFA-producing bacteria and reducing mucolytic ones, improving mucin composition.

For these purposes, four specific objectives were identified:

1) Evaluation of the effects of insect live larvae and functional feed on gut structure through histomorphometric measurements (Vh, Cd, Vh/Cd, Vw, muscular and mucosa thickness) and real time rt-qPCR for tight junction expression.
2) Evaluation of the effects of insect live larvae and functional feed on gut inflammation through histopathology and real time rt-qPCR for selected cytokines expression.
3) Evaluation of the effects of insect live larvae on mucin composition through histochemistry and real time rt-qPCR for MUC-2 expression.
4) Evaluation of the effects of insect live larvae on caecal microbiota composition through next generation sequencing (NGS)

The histomorphometric and histochemical analysis along with the histology and rt-qPCR were performed by the PhD student. The microbiota composition was evaluated using NGS with the help of Prof. Ferrocino (Department of Agricultural, Forestry and Food Sciences, University of Turin, Italy).

7. MATERIAL AND METHODS

The three experimental trials were conducted at the poultry facility of the University of Turin (Italy) in collaboration with Prof. Schiavone (Department of Veterinary Sciences, University of Turin).
The poultry house was 7 m wide × 50 m long × 7 m high, with an automatic ventilation system and equipped with a waterproof floor and walls. Each pen was 1.20 m wide × 2.20 m long and rice hulls was used as bedding. During the first 3 weeks, the birds were heated by infrared lamps to maintain the suitable temperature, according to the standard breeding practices [158]. Lighting schedule was 23 h light:1 h darkness until day 7 and then 18 h light:6 hours darkness was adopted until the slaughtering age. Environmental conditions (e.g., temperature and relative humidity) were set according to the Ross guidelines [159]. The animals and the environmental parameters were daily checked during the whole experimental period.

7.1 Birds and diet

7.1.1 Trial 1

The experimental protocol was approved by the Ethical Committee of the University of Turin (Italy) (ID: 814715). A total of 180 four-day-old male broiler chickens (Ross 308) were randomly allotted to three dietary treatments (6 replicates/treatment; 10 animals/replicate): i) C: control group receiving a commercial diet (Famaarco SPA, Cuneo, Italy); ii) HI: C + HI live larvae; iii) TM: C + TM live larvae. The commercial diet was based on soybean meal, corn and soybean oil added with a vitamin-mineral premix, synthetic amino acids, cocciodiostats and histomonostats (Famaarco SPA, Cuneo, Italy). The nutrient composition of the two diets (starter: 1-11 days and grower-finisher: 12-38 days) is reported in Table 1. Considering the current literature available on the dosage of insect live larvae in broiler chickens [49], live larvae were distributed in two plates once a day (11:00 am) based on 5% of the expected daily feed intake, using two different sizes of larvae for starter (length: 0.80 cm) and grower (length: 1.50 cm) periods. To avoid potential bias, two plates with control feed were also provided once a day (11:00 am) to the C animals. Every day, the larvae consumption was confirmed by stopwatch recording the time spent by birds eating HI and TM, starting from the moment the plates with the larvae were located in the box until the plates were empty. The chitin content of the whole larvae was analysed by Prof. Gasco and Dr. Caimi (Department of Agricultural, Forestry and Food Sciences, University of Turin, Italy) following the method described by Woods et al. [160]. The samples were totally defatted by solvent extraction, and subsequently subjected to demineralization and deproteinization. All the values are reported as means of quadruplicate analyses: HI contained 8.84% of chitin on dry matter and TM 5.13%. Thus, HI-fed birds received from 0.03% (day 4) to 0.26% of chitin (day 38) while the TM-fed birds received from 0.02% (day

4) to 0.15% of chitin (day 38) with the insect live larvae administration during the trial. At 39 days of age, 18 birds/treatment (3 birds/replicate) were electrically stunned and slaughtered.

Table 1. Nutrient composition of the commercial feed used in trial 1

	Starter period (4-11 days)	**Grower-Finisher period (12-38 days)**
Crude protein (%)	22.40	20.00
Ether extract (%)	4.90	5.90
Crude fibre (%)	2.75	2.65
Ash (%)	5.00	3.90
Methionine (%)	0.50	0.47
Lysine (%)	1.20	1.05
Calcium (%)	0.70	0.45
Phosphorus (%)	0.64	0.48
Sodium (%)	0.10	0.10

7.1.2 Trial 2

The experimental protocol was approved by the Ethical Committee of the University of Turin (Italy) (ID: 380576). A total of 126 3-days-old females Muscovy ducklings (Canedins R61 Barred blue, Grimaud Freres Selection, France) were randomly allotted to three dietary treatments (6 replicates/treatment, 7 birds/pen): i) C: a control group receiving a commercial diet (Borello Mangimi s.r.l, Bra, Cuneo, Italy); ii) HI: C + HI live larvae; iii) TM: C + TM live larvae. The commercial diet was based on corn, wheat, soybean meal, and soybean oil added with a vitamin-mineral premix (Borello Mangimi s.r.l, Bra, Cuneo, Italy). A 2-feeding phase program was applied: started diet (from 3 to 31 days old), and grower-finisher period (from 32 to 55 days old). The nutrient composition of the two diets is reported in Table 2. As reported for Trial 1, insect live larvae were provided daily at the same time (10.00 am) in a plate based on 5% of the expected daily feed intake and the larvae consumption was checked by stopwatch recording the time spent by birds eating them, starting from the moment the plates with the larvae were located in the box until the plates were empty. The chitin content of the whole larvae was 8.84% and 5.13% on dry matter for HI and TM, respectively. Thus, HI-fed birds received from 0.04% (day 4) to 0.25% of chitin (day 51) while the TM-fed birds received from 0.02% (day 4) to 0.014% of chitin (day 51) with the insect live larvae administration during the trial. On day 52, 12 ducks/treatment (2 birds/replicate), after a feed withdrawal of 12 hours, were slaughtered by electrical stunning and bleeding, according to the standard EU regulations.

Table 2. Nutrient composition of the commercial feed used in trial 2.

	Starter period (3-31 days)	**Grower-Finisher period (32-52 days)**
Crude protein (%)	19.55	17.25
Ether extract (%)	3.64	4.12
Crude fibre (%)	3.55	3.39
Ash (%)	2.96	2.67
Methionine (%)	0.53	0.43
Lysine (%)	1.00	0.92
Calcium (%)	1.14	1.19
Phosphorus (%)	1.11	0.95

7.1.3 Trial 3

The experimental protocol was approved by the Ethical Committee of the University of Turin (Italy) (ID: 0284800). A total of 576 as-hatched broiler chicks (Ross 308) from the same fairly young parent stock were randomly allotted to 3 dietary treatments (8 pens/treatment; 24 chickens/replicate): a control group (C) without AlphaSoy™ Gold (ASG, AB NEO, Denmark) and two treatment groups, T1 (20% and 10% of ASG in pre-starter and starter phase, respectively) and T2 (5% of ASG in both phases). For each dietary treatment, the diets (provided as crumbled feed) were divided into 2 feeding phases: pre-starter (days 0 to 3) and starter (days 4 to 10). All the diets were formulated to meet or exceed the NRC requirements [161], and adjusted according to the Aviagen broiler nutrition specifications [159] (Table 3). ASG is a functional, protein-rich feed resulted from a co-processing of high-protein, thermomechanical and enzyme-facilitated soybean meal and selected yeast fractions (minimum 10,000 mg/g β-1,3/1,6 glucans and 5,000 mg/g MOS in the final product) by enzymatic treatment and extrusion, designed to support the healthy development of the gastro-intestinal system and optimum microbiota balance during the early growth stage of the young animals (Alphasoy™ Gold, AB NEO, Denmark) (Table 4). Feed and water were provided *ad libitum*. On days 3 and 10, all the birds were individually weighed and 16 broilers/diet (2 birds/pen) were chosen to be slaughtered. On day 3, the chicks of the pen with the highest live weight were selected for slaughter in order to consider the animal with the best growth. On day 10, the birds that were closest to the average pen live weight were, instead, slaughtered in order to have a representative sample of the pen.

Table 3. Feed ingredients and nutrient composition of the experimental diets used in trial 3

	Pre-starter period (0-3 days)			Starter period (4-10 days)		
Ingredients (% as fed)	**C**	**T1**	**T2**	**C**	**T1**	**T2**
Corn meal (CP 7.5%)	36.55	39.47	37.27	36.55	38.02	37.27
Soybean meal (CP 47.5%)	36.82	15.15	31.40	36.82	25.98	31.40
Wheat meal (CP 11.5%)	20.00	20.00	20.00	20.00	20.00	20.00
Soybean oil	3.32	2.04	3.00	3.32	2.68	3.00
Dicalcium phosphate	1.07	1.09	1.08	1.07	1.08	1.08
Calcium carbonate	0.55	0.55	0.56	0.55	0.55	0.56
Sodium chloride	0.21	0.21	0.21	0.21	0.21	0.21
Sodium formate	0.10	0.10	0.10	0.10	0.10	0.10
DL-methionine	0.35	0.35	0.35	0.35	0.35	0.35
L-lysine	0.25	0.27	0.26	0.25	0.26	0.25
Valine	0.02	0.01	0.02	0.02	0.01	0.02
Coccidiostats (Maxiban)	0.06	0.06	0.06	0.06	0.06	0.06
3-phytase	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin-mineral premix[a]	0.40	0.40	0.40	0.40	0.40	0.40
ASG	0.00	20.00	10.00	0.00	5.00	5.00
Nutrient composition (% as fed)						
Dry Matter	89.05	89.07	89.04	88.66	89.72	89.71
Crude protein	23.59	23.67	23.75	23.46	23.07	23.25
Ether Extract	5.21	4.96	4.87	4.91	4.57	4.93
Neutral Detergent Fibre	3.44	3.35	3.72	3.40	3.24	3.21
Acid Detergent Fibre	7.10	6.86	6.77	7.28	6.87	7.14
Ash	4.85	4.67	4.87	4.65	4.88	4.85
AMEn (kcal/kg)	3050.00	3050.00	3050.00	3050.00	3050.00	3050.00

AMen: apparent metabolisable energy; ASG: AlphaSoy™ Gold; C: control; CP: crude protein; T1 = 20% inclusion of functional protein in the pre-starter phase and 10% in the starter phase; T2 = 5% inclusion of functional protein in the pre-starter and starter phases. [a]Mineral–vitamin premix (per kg diet): vitamin A (retinyl acetate), 12,500 IU; vitamin D3 (cholecalciferol), 3, IU; vitamin E (DL-a-tocopheryl acetate), 40 mg; vitamin K (menadione sodium bisulphite), 2 mg; biotin, 0.20 mg; tiamin, 2 mg; riboflavin, 6 mg; pantothenate, 15.21 mg; niacin, 40 mg; choline, 750 mg; pyridoxin, 4 mg; folic acid, 0.75 mg; vitamin B12, 0.03 mg; Mn, 70 mg; Zn, 62.15 mg; Fe, 50 mg; Cu, 7 mg; I, 0.25 mg; Se, 0.25 mg.

Table 4. Nutrient content and digestibility of the co-processed yeast and soybean meal.

Proximate composition (% as is)	
DM	95.00
CP	52.00
EE	2.50
CF	3.70
Ash	6.70
AMEn, MJ/Kg	11.00
Macrominerals (g/kg as is)	
Calcium	3.00
Phosphorus	6.00
Sodium	0.40
Potassium	21.00
Chloride	0.10
Magnesium	3.70
Aminoacids (g/kg as is; true digestibility, %)	
Lysine	3.10 (2.90)
Methionine	0.70 (0.60)
Cystine	0.70 (0.60)
Threonine	2.00 (1.80)
Tryptophan	0.70 (0.60)
Isoleucine	2.50 (2.30)
Leucine	4.10 (3.80)
Valine	2.50 (2.30)
Phenylalanine	2.70 (2.50)
Histidine	1.40 (1.30)
Arginine	3.70 (3.50)
Glutamic acid	9.20 (8.40)
Tyrosine	1.80 (1.60)

AMen: apparent metabolisable energy; CF: crude fibre; CP: crude protein; DM: dry matter; EE: ether extract.

7.2 Histomorphometric evaluation

7.2.1 Trial 1 and 2

Samples of approximately 5 cm in length of duodenum (loop of the duodenum), jejunum (tract before Meckel's diverticulum) and ileum (the tract before the ileocolic junction) were excised and flushed with 0.9% saline to remove all the content. Also, samples of liver, spleen, thymus and Bursa of Fabricius were collected. All the samples were fixed in 10.00% buffered formalin solution for 7 days for histomorphometric analysis. In particular, the fixed tissues were routinely embedded in paraffin wax blocks, sectioned at 5 μm thickness, mounted on glass slides and stained with Haematoxylin and Eosin. One slide per intestinal segment was examined by light microscopy and each slide was captured with a Nikon DS-Fi1 digital camera (Nikon Corporation, Tokyo, Japan) coupled to a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germania) using a 2.5× objective lens. The NIS-Elements F software was used for image capturing and morphometric analysis was performed by Image®-Pro Plus software (6.0 version, Media Cybernetics, Maryland, USA). The evaluated morphometric indices were as follows: Vh (from the tip of the villus to the crypt), Cd (from the base of the villus to the submucosa), and Vh/Cd ratio [101] (Figure 5). These morphometric parameters were measured on 10 well-oriented and intact villi and 10 crypts chosen from duodenum, jejunum and/or ileum [104].

In addition, the following histopathological alterations were evaluated: white pulp hyperplasia in the spleen, cortical depletion in the thymus, follicular depletion in the bursa of Fabricius, and hepatocyte degeneration and lymphoid tissue activation in the liver. Regarding gut histopathological findings, inflammatory infiltrates and Gut-Associated Lymphoid Tissue (GALT) activation were separately assessed for mucosa and submucosa for each gut segment. The observed histopathological alterations were evaluated using a semiquantitative scoring system as follows: absent (score = 0), mild (score = 1), moderate (score = 2), and severe (score = 3) [162]. The total score of each gut segment was obtained by adding up the mucosa and submucosa scores.

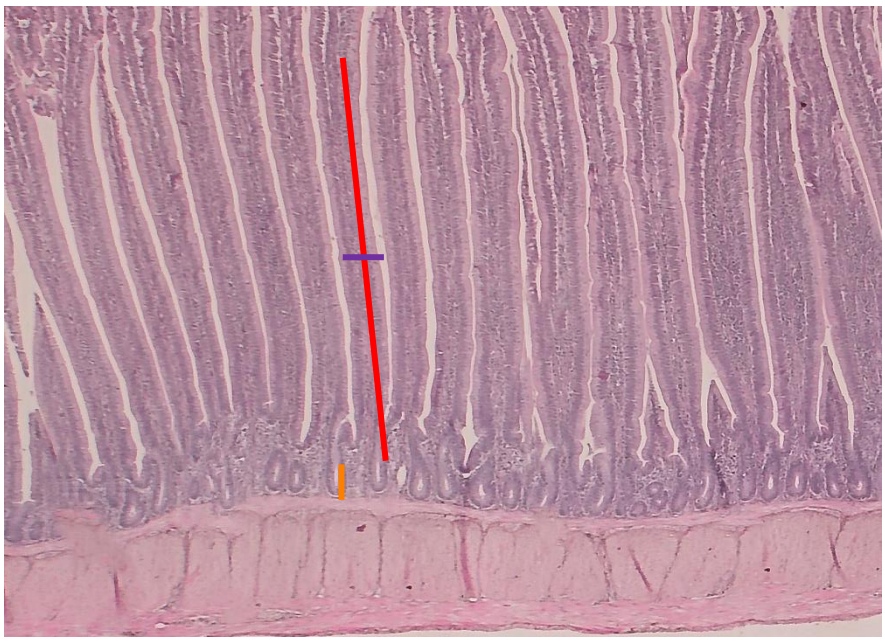

Figure 5. Morphometric indices evaluated in the gut (red line: villus height; orange line: crypt depth; purple line: villus width).

7.2.2 Trial 3

Samples of approximately 2 cm in length of duodenum (loop of the duodenum) and jejunum (tract before Meckel's diverticulum) were excised and flushed with 0.9% saline to remove all the content. Also, samples of liver and Bursa of Fabricius were collected. All the samples were fixed in 10.00% buffered formalin solution for histomorphometric analysis as already described for trial 1 and 2. One slide per each intestinal segment was examined by light microscopy and each slide was captured with a Nikon DS-Fi1 digital camera (Nikon Corporation, Tokyo, Japan) coupled to a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germania) using a 2.5× objective lens. The NIS-Elements F software was used for image capturing and morphometric analysis was performed by Image®-Pro Plus software (6.0 version, Media Cybernetics, Maryland, USA). The evaluated morphometric indices were Vh, Cd, villus width (Vw), mucosal and muscular thickness (Figure 6). Thus, the villus surface area (VSA) was calculated according to the following formula [103]:

$$(2\pi)(\frac{Vw}{2})(Vh)$$

Vh, Vw and Cd were measured on 10 well-oriented and intact villi and 10 crypts chosen from duodenum and jejunum while muscular and mucosal thickness was evaluated on three points of the gut mucosal and muscular layers per each captured field [104].

Histological changes were scored using a semi-quantitative scoring system as follows: absent (score 0), mild (score 1), moderate (score 2) and severe (score 3) [162]. The semi-quantitative scoring system was applied for the evaluation of follicular depletion in the bursa of Fabricius, hepatocyte degeneration and lymphoid tissue activation in the liver, inflammatory infiltrates and GALT activation in the mucosa and submucosa of each gut segment. Thus, the total score of each gut segment was obtained by adding up the mucosa and submucosa scores.

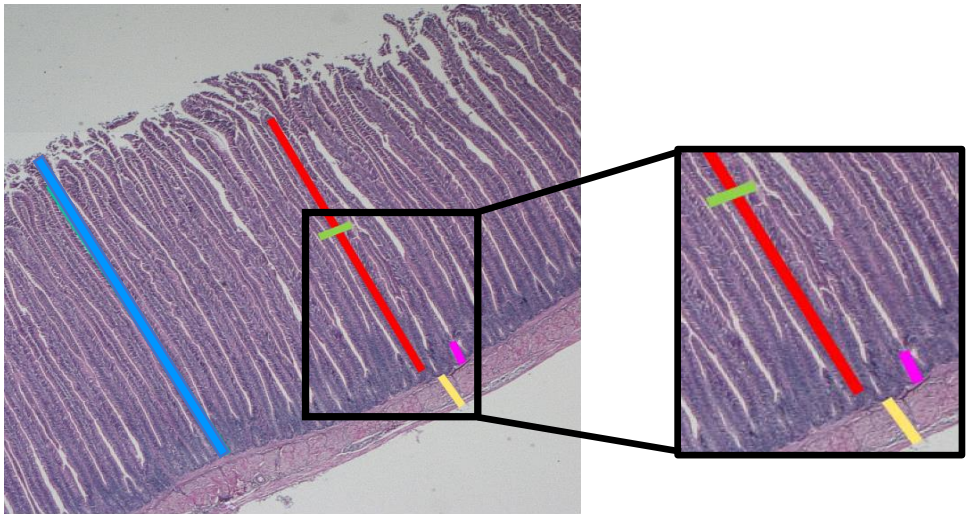

Figure 6. Morphometric indices evaluated in the gut (red line: villus height; pink line: crypt depth; green line: villus width; blue line: mucosa thickness; yellow line: muscular thickness).

7.3 Mucin staining intensity

7.3.1 Trials 1 and 2

The paraffin-embedded intestinal sections of the duodenum, jejunum, and ileum were also submitted to triple histochemical staining to evaluate the three different mucin subtypes.
Neutral mucins were stained magenta through the periodic acid-Schiff (PAS) staining. Sections were brought to water, immersed in 0.5% periodic acid for 20 minutes, washed in running tap water for 5 minutes, and immersed in Schiffer reactive for 30 minutes. Then, sections were rinsed in running tap water for 20 minutes, counterstained with Haematoxylin, dehydrated, and mounted on glass slides [129].
Acidic sialylated mucins were identified in blue by Alcian Blue (AB), pH 2.5 staining. Sections were brought to water, immersed in 8 G X Alcian blue in 3% acetic acid solution for 30 min, washed in running tap water for 5 min, dehydrated, and mounted on glass slides [163].
Acidic sulfated mucins were identified in purple-black by high iron diamine (HID) staining. Sections were brought to water, oxidized in 1% periodic acid solution for 10 minutes and washed in running tap water for 5 min. Sections were then immersed in the HID solution (120 mg metadiamine, 20 mg paradiamine and 1.4 ml 10% ferric chloride in 50 ml of distilled water) for 18 h, rinsed in tap water, dehydrated and mounted [163]. One slide per histochemical staining for each intestinal segment was examined by light microscopy. Five randomly selected high-power fields per slide were captured with a Nikon DS-Fi1 digital camera (Nikon Corporation, Tokyo, Japan) coupled to a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germania) using a 20× objective lens. NIS-Elements F software was used for image capturing. Mucin staining quantification was then performed by Image®-Pro Plus software (6.0 version, Media Cybernetics, Maryland, USA) by means of pixels classification. Mucin quantification was expressed as the percentage of the gut mucosal area (covering both the crypts and the villi) that was positive for the evaluated histochemical staining [164]. Total mucin content was obtained by adding the PAS, AB and HID percentage of each intestinal segment.

7.4 Intestinal gene expression

7.4.1 Trials 1 and 2

At slaughter, jejunum from 12 birds/treatment was aseptically collected, placed 24 hours in RNAlater (Sigma-Aldrich, MO, USA) at 4°C and then stored at -80°C. Total RNA was then extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with manufacturer's instructions. The RNA quality of every sample was quantified by Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wiington, DE, USA) and the ratio (OD260:OD280) ranged from 1.7 to 2.1. Afterwards, 2.0 µg of total RNA for each sample was reverse transcribed to cDNA by using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., CA, USA) according to manufacturer protocol and the cDNA was stored at -20°C. rt-qPCR was performed using a 7500 Real Time PCR system (Applied Biosystems, Waltham, MA) in a 20 µL reaction mixture containing 2 µL cDNA, 10 µL of SYBR Green Supermix kit (Bio-Rad Laboratories, Inc., CA, USA) and 0.1 µL of forward and reverse primers (40 mM) of the selected genes. Primers used for selected genes (IL-2, IL-4, IL-6, TNF-α, INF-γ, MUC-2) were designed based on the available sequences in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and synthetized by Macrogen Inc. (Amsterdam, the Netherlands) (Table 5 and 6). Thermal conditions for performing rt-qPCR were as follows: initial incubation at 95°C for 30 s; 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s following by a melting curve analysis (65-95°C with 0.5°C increments at 2-5 s/step). Relative standard curve method was performed using β -actin and GAPDH as internal control genes to normalize for RNA abundance. Each reaction was run in triplicate. Efficiency curves were performed for each primer set using log10 diluted cDNA in order to obtain efficiency-corrected relative quantification. Amplification efficiency between 90 and 110% was considered good with correlation coefficient (R^2) of 0.99 [165].

As for trial 2 sequences for the selected genes were not available in GenBank for Muscovy ducks (*Cairina moschata*) and primers were designed based on the predicted sequences of wild duck (*Anas platyrhyncos*), selected amplicons were purified using a commercial kit (NucleoSpin PCR & Gel Clean-up, Carlo Erba, Milano, Italy) and sequenced on both strands (Macrogen Europe, The Netherland). An average number of 6 individuals were sequenced and analysed in MegaX software (version 11, https://www.megasoftware.net). Sensus and consensus strands were aligned and the total length of the amplicon was compared with the available sequences of chicken (*Gallus gallus*) and wild duck (*Anas platyrhyncos*).

Table 5. Oligonucleotide primers used for chicken intestinal gene expression in trials 1.

Type	RNA Target	Primer Sequence	GenBank Accession no.
Reference gene	β-actin	F:5′-GAGAAATTGTGCGTGACATCA-3′ R:5′-CCTGAACCTCTCATTGCCA-3′	L08165.1
	GAPDH	F:5′-GGTGGTGCTAAGCGTGTTAT-3′ R:5′-ACCTCTGTCATCTCTCCACA-3′	K01458
Target gene	TNF-α	F:5′-CCCATCTGCACCACCTTCAT-3′ R:5′-CATCTGAACTGGGCGGTCAT-3′	AY765397.1
	IL-6	F:5′-CAAGGTGACGGAGGAGGAC-3′ R:5′-GGTAGGTCTGAAAGGCGAACA-3′	AJ309540
	INF-γ	F:5′-AGCTGACGGTGGACCTATTATT-3′ R:5′-GGCTTTGCGCTGGATTC-3′	Y07922.1
	IL-2	F:5′-TCTGGGACCACTGTATGCTCT-3′ R:5′-ACACCAGTGGGAAACAGTATCA-3′	AF000631
	IL-4	F:5′-CTTCCTCAACATGCGTCAGC-3′ R:5′-TGAAGTAGTGTTGCCTGCTGC-3′	AJ621735
	MUC-2	F: 5′-ACTCCTCCTTTGTATGCGTGA-3′ R: 5′-GTTAACGCTGCATTCAACCTT-3′	NM001318434.1

F: forward primer; GAPDH: glyceraldehyde-3-phosphate; IFN: interferon; IL: interleukin; MUC: mucin; R: reverse primer; TNF: tumour necrosis factor.

Table 6. Oligonucleotide primers used for duck intestinal gene expression in trial 2.

Type	RNA Target	Primer sequence	GenBank accession no.
Reference gene	β -actin	F:5′-CAGCCATGTATGTAGCCATCCA -3′ R:5′-CACCATCACCAGAGTCCATCAC-3′	EF667345.1
	GADPH	F:5′-CTCTGTTCGTGGACCTGACCT-3′ R:5′-CAGCAGCAGCCTTCACTACC-3′	AY436595.1
Target gene	TNF-α	F:5′-GGACAGCCTATGCCAACAA-3′ R:5′-CGATCATCTGGTTACAGGAAGG-5′	EU375296.1
	IL-6	F:5′-CAACGACGATAAGGCAGATGGT-3′ R:5′-GAGGATGAGGTGTGTGGTGATTT-3′	AB191038.1
	INF-γ	F:5′-TGACTACAAGAAGTTCAGAGACCT-3′ R:5′-GACTGGCTCCTTTTCCTTTTG-3′	AJ012254.1
	IL-2	F:5′-TTTACCCTGGGGCTACCTAACTTG-3′ R:5′-AGAACAGACACGTTATCACCCACA-3′	AY193713.1
	IL-4	F:5′-AAAGCCTCCACGGTTGTTT-3′ R:5′-TCACGATGTGCAGCAAGTT-3′	MF346730.1
	MUC-2	F:5′-GGGCGCTCAATTCAACATAAGTA-3′ R:5′-TAAACTGATGGCTTCTTATGCGG-3′	XM005024513.2

F: forward primer; GAPDH: glyceraldehyde-3-phosphate; IFN: interferon; IL: interleukin; MUC: mucin; R: reverse primer; TNF: tumour necrosis factor.

7.4.2 Trial 3

At slaughter, duodenum and jejunum from 16 birds/treatment was aseptically collected, placed 24 hours in RNA Later (Sigma-Aldrich, MO, USA) at 4°C and then stored at -80°C. Total RNA was then extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with manufacturer's instructions. The RNA quality of every sample was quantified by Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wiington, DE, USA) and the ratio (OD260:OD280) ranged from 1.7 to 2.0. Afterwards, RNA obtained from 4 chickens within each dietary group was pooled, in order to analyse 4 pooled samples per group. Afterwards, 1.5 μg of total RNA for each pool was reverse transcribed to cDNA and rt-qPCR was performed as previously described for trials 1 and 2. Primers used for selected genes (IL-2, IL-4, TNF-α, INF-γ, MUC-2; ZO-1 and CL-1) were designed based on the available sequences in GenBank and synthetized by Macrogen Inc. (Amsterdam, the Netherlands) (Table 7). Relative standard curve method was performed using β -actin and GAPDH as internal control genes to normalize for RNA abundance. Each reaction was run in triplicate. Efficiency curves were performed for each primer set using log10 diluted cDNA in order to obtain efficiency-corrected relative quantification. Amplification efficiency between 90 and 110% was considered good with correlation coefficient (R^2) of 0.99 [165].

Table 7. Oligonucleotide primers used for chicken intestinal gene expression in trials 3.

Type	RNA Target	Primer Sequence	GenBank Accession no.
Reference gene	β-actin	F:5′-GAGAAATTGTGCGTGACATCA-3′ R:5′-CCTGAACCTCTCATTGCCA-3′	L08165.1
	GAPDH	F:5′-GGTGGTGCTAAGCGTGTTAT-3′ R:5′-ACCTCTGTCATCTCTCCACA-3′	K01458
Target gene	TNF-α	F:5′-CCCATCTGCACCACCTTCAT-3′ R:5′-CATCTGAACTGGGCGGTCAT-3′	AY765397.1
	INF-γ	F:5′-AGCTGACGGTGGACCTATTATT-3′ R:5′-GGCTTTGCGCTGGATTC-3′	Y07922.1
	IL-2	F:5′-TCTGGGACCACTGTATGCTCT-3′ R:5′-ACACCAGTGGGAAACAGTATCA-3′	AF000631
	IL-4	F:5′-CTTCCTCAACATGCGTCAGC-3′ R:5′-TGAAGTAGTGTTGCCTGCTGC-3′	AJ621735
	MUC-2	F: 5′-ACTCCTCCTTTGTATGCGTGA-3′ R: 5′-GTTAACGCTGCATTCAACCTT-3′	NM001318434.1
	CL-1	F:5'-GTGTTCAGAGGCATCAGGTATC-3' R:5'-GTCAGGTCAAACAGAGGTACAA-3'	NM001013611.2
	ZO-1	F:5'-GCCAACTGATGCTGAACCAA-3' R:5'-GGGAGAGACAGGACAGGACT-3'	XM015278975

F: forward primer; GAPDH: glyceraldehyde-3-phosphate; IFN: interferon; IL: interleukin; MUC: mucin; R: reverse primer; TNF: tumour necrosis factor.

7.5 Microbiota evaluation

7.5.1 Trials 1 and 2

In trials 1 and 2, samples of caecal content were collected at slaughter from 18 birds/treatment (3 birds/replicate) and 12 birds/treatment (2 birds/replicate), respectively. Then, samples were stored at − 80°C prior DNA extraction and sequencing.

Total DNA was extracted from each sample using the RNeasy Power Microbiome KIT (Qiagen. Milan. Italy) following the manufacturer's instructions. One microliter of RNase (Illumina Inc. San Diego. CA) was added to digest RNA in the DNA samples with an incubation of 1 h at 37°C. DNA was quantified using Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wiington, DE, USA) and standardized at 5 ng/µl.

The extracted DNA was used to assess the microbiota by the amplification of the V3-V4 region of the 16S rRNA gene (F: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGA CAGCCTACGGGNGGCWGCAG-3'; R: 5'-GTCTCGTGGGCTCGGAGATGTGTATAA GAGACAGGACTACHVGGGTATCTAATCC-3') [166]. The PCR products were purified according to the Illumina metagenomic standard procedure (Illumina Inc. San Diego. CA). Sequencing was performed with a MiSeq Illumina instrument with V3 chemistry and generated 250 bp paired-end reads in accordance with the manufacturer's instructions.

7.6 Statistical analysis

7.6.1 Trials 1 and 2

Statistical analysis was conducted using R software version 4.0.4 (R Foundation for Statistical Computing, Vienna, Austria; https://www.r-project.org/). All statistical tests were bilateral, with an applied significance level of 0.05. The Shapiro-Wilk test was used to test the normality of the quantitative variables.

Quantitative variables were described as mean and standard deviation (SD) or median and interquartile range (IR) depending on data distribution.

Histological scores were analysed by One-way ANOVA test or the corresponding non-parametric Kruskall-Wallis test and Tukey post-hoc tests.

Data regarding histomorphometric indices and mucin staining intensity were analysed by a robust two-way ANOVA test (trimmed means method) using the "walrus" R package. The two-way ANOVA test allowed the evaluated variables to depend on three fixed factors (diet, intestinal segment, and the interaction between them). The interactions were evaluated by robust pairwise comparisons.

For rt-qPCR, Microsoft Excel was used to convert the quantification cycle (Cq) values to linear units called relative normalized expression in accordance with Taylor et al. [167]. Briefly, the average Cq of all samples in the control group for each target was determined and the relative difference (DCq) with the mean Cq per individual sample for each target gene was assessed. The relative quantities were calculated according to reaction efficiency (efficiency^DCq). For each sample, a normalization factor was determined from the geometric mean of the associated reference gene relative quantities. The relative normalized expression for each target gene was then calculated per sample by dividing the relative quantity by the normalization factor. Samples with relative normalized expression > 10 were identified as potential outliers and excluded from the analysis. A robust one-way ANOVA test was performed, followed by robust pairwise comparisons.

Regarding microbiota, paired-end reads were first merged using FLASH software with default parameters [168]. Joint reads were further quality filtered (at Phred < Q20) using QIIME 1.9.0 software through a multiple_split_libraries_fastq.py script [169] and the pipeline described in Biasato et al. [106]. OTU clustering was obtained at 97% of similarity by the pick_otus.py script and taxonomy assignment was assessed by Greengenes 16S rRNA gene database v. 2013 using the RDP Classifier, with a minimum confidence score of 0.80. OTU table was rarefied at the lowest number of sequence (12,337 reads) and display the higher taxonomy resolution. The vegan package of R was used to calculate the alpha diversity [170]. The diversity indices were further analysed using the Wilcoxon rank sum test to assess differences between the dietary treatments. Weighted UniFrac distance matrices and OTU table generated through QIIME were used to perform Adonis and Anosim

statistical tests in R environment. A Generalized Linear Model was used in order to test the importance of insect administration on the relative abundance of OTU.

7.6.2 Trial 3

R software version 4.0.4 (R Foundation for Statistical Computing, Vienna, Austria; https://www.r-project.org/) was used for statistical analysis. All statistical tests were bilateral, with an applied significance level of 0.05. The normality of data distribution was tested by Shapiro-Wilk test.
Quantitative variables were described as mean and standard deviation (SD) or median and interquartile range (IR) depending on data distribution.
Histological scores were analysed by One-way ANOVA test or the corresponding non-parametric Kruskall-Wallis test and Tukey post-hoc tests
Histomorphometric data were analysed by a robust two-way ANOVA test (trimmed means method) using the "walrus" R package. The interactions were evaluated by robust pairwise comparisons.
For rt-qPCR, Microsoft Excel was used to convert the quantification cycle (Cq) values to linear units called relative normalized expression in accordance with Taylor et al. [167]. Samples with relative normalized expression > 10 were identified as potential outliers and excluded from the analysis. A robust one-way ANOVA test was performed, followed by robust pairwise comparisons.

8. RESULTS

8.1 Trial 1

The results of Trial 1 have already been published in:

- Colombino E., Biasato I., Ferrocino I., Bellezza Oddon S., Caimi C., Gariglio M., Dabbou S., Caramori M., Battisti E., Zanet S., Ferroglio E., Cocolin L., Gasco L., Schiavone A., Capucchio MT. Effect of Insect Live Larvae as Environmental Enrichment on Poultry Gut Health: Gut Mucin Composition, Microbiota and Local Immune Response Evaluation. Animals (Basel). 2021; 27;11(10):2819. doi: 10.3390/ani11102819. (IF= 3.232, Q1)
- Bellezza-Oddon S., Biasato I., Imarisio A., Pipan M., Dekleva D., Colombino E., Capucchio MT., Meneguz M., Bergagna S., Barbero R., Gariglio M., Dabbou S., Fiorilla E., Gasco L., Schiavone A., Black soldier fly and yellow mealworm live larvae for broiler chickens: Effects on bird performance and health status. J Anim Physiol Anim Nutr. 2021; 105 Suppl 1:10-18. doi: 10.1111/jpn.13567. (IF= 2.718, Q1)

8.1.1 Histomorphometric evaluation

As summarized in Table 8, diet and interaction between diet and intestinal segment did not influence the gut morphometric indices. However, all the evaluated morphometric indices depended on gut segment (P>0.001). In particular, the duodenum showed greater Vh, Cd and Vh/Cd than the other gut segments, with morphometric indices being also greater in the jejunum when compared to the ileum. Table 9 shows the histopathological alterations recorded in the main organs of the broiler chickens. Dietary inclusion of insect live larvae did not affect the severity of the histopathological scores in any of the organs (P>0.05). Regardless of diet, liver showed mild to moderate, multifocal to diffuse vacuolar degeneration of the hepatocytes (Figure 7A, B and C), as well as mild to moderate, multifocal lymphoplasmacytic infiltrates (Figure 7D). In the gut mild to moderate, multifocal to diffuse, mucosal lymphoplasmacytic infiltrates with or without GALT activation were observed (Figure 8). In addition, multifocal to diffuse follicular depletion was detected in the bursa of Fabricius (Figure 9A and B). No alterations were detected in the thymus. Spleen showed mild multifocal white pulp hyperplasia (Figure 9C and D). Even though there were no statistically significant differences, HI and TM spleen showed slightly higher white pulp hyperplasia compared to control (0.32 and 0.38 vs 0.05).

Table 8. Effects of insect live larvae on the gut morphology of broiler chicken (n=18/treatment).

	Diet (D)			Intestine (I)			P-value		
	C	**HI**	**TM**	**DU**	**JE**	**ILE**	**D**	**I**	**DxI**
Vh, *mean (SD)*	1.20 (0.39)	1.28 (0.42)	1.21 (0.42)	1.69[a] (0.22)	1.18[b] (0.24)	0.82[c] (0.21)	0.129	<0.001	0.866
Cd, *mean (SD)*	0.09 (0.02)	0.10 (0.02)	0.09 (0.02)	0. 10[a] (0.02)	0. 08[b] (0.02)	0.09[b] (0.02)	0.057	<0.001	0.576
Vh/Cd, *mean (SD)*	14.01 (4.73)	13.55 (4.39)	13.73 (4.59)	16.62[a] (3.62)	15.22[b] (4.01)	9.46[c] (2.26)	0.780	<0.001	0.949

C: control; Cd: crypt depth; DU: duodenum; HI: *Hermetia illucens*; ILE: ileum; JE: jejunum; TM: *Tenebrio molitor*; Vh: villus height; Means with superscript letters (a, b, c) denote significant differences between the dietary treatments and/or the gut segments.

Table 9. Effects of insect live larvae on the main organs of the broiler chickens (n=18/treatment).

	Diet			
	C	**HI**	**TM**	**P-value**
Liver				
Degeneration, *median (IR)*	0.00 (0.00-1.00)	0.00 (0.00-1.00)	0.00 (0.00-0.00)	0.365
Inflammation, median (IR)	1.00 (1.00-1.50)	1.00 (1.00-1.50)	1.00 (1.00-1.12)	0.321
Spleen, *mean (SD)*	0.05 (0.23)	0.32 (0.52)	0.38 (0.50)	0.063
Thymus	Absence of alterations			
Bursa of Fabricius, *median (IR)*	0.00 (0.00-0.75)	0.00 (0.00-0.00)	0.00 (0.00-1.00)	0.302
Gut, *median (IR)*	2.00 (1.00-2.62)	1.00 (0.75-2.00)	2.00 (1.00-2.25)	0.061

C: control; HI: *Hermetia illucens*; TM: *Tenebrio molitor*.

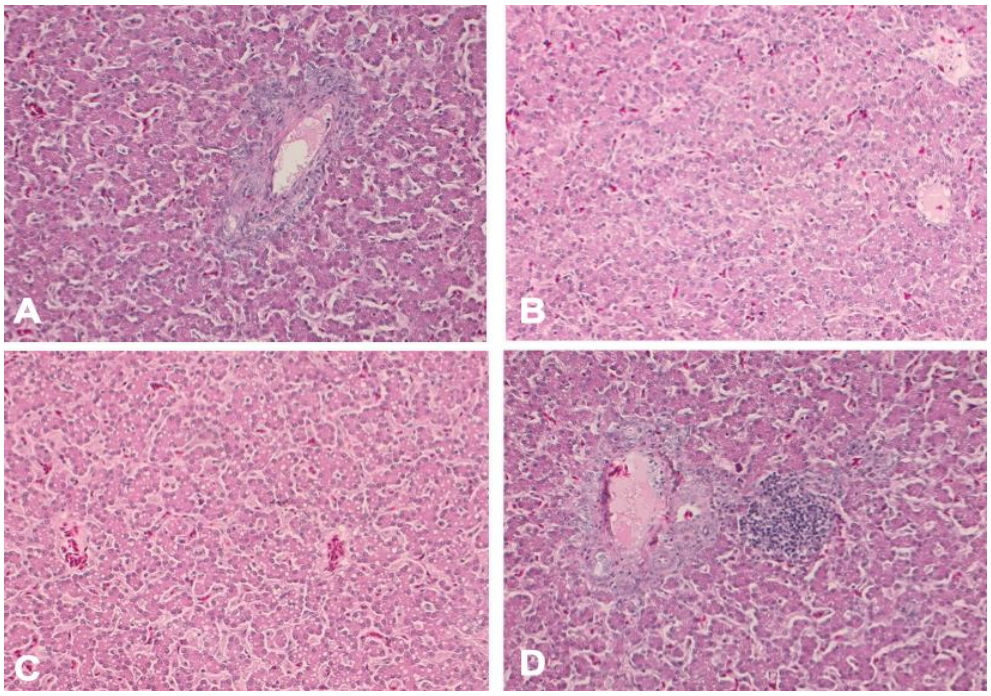

Figure 7. Main histopathological findings in chicken's liver. A) A normal liver (grade 0), 20x, Haematoxilin and Eosin (H-e). B) Mild and multifocal vacuolar degeneration (grade 1), 20x, H-e. C) Moderate and multifocal vacuolar degeneration (grade 2), 20x, H-e. D) Mild and multifocal lymphoplasmacytic inflammation (grade 1), 20x, H-e.

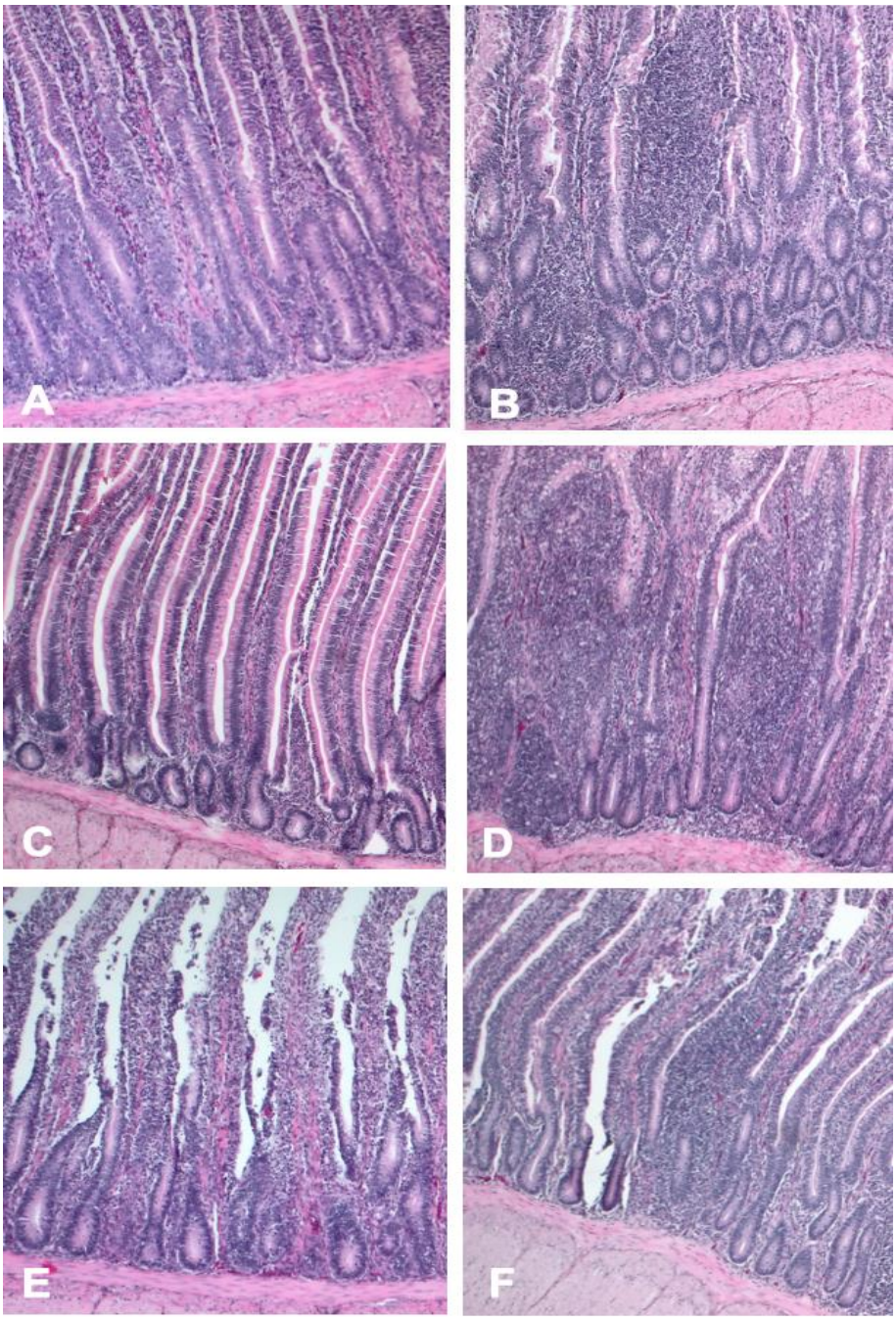

Figure 8. Main histopathological findings in chicken's gut. A) A normal duodenum (grade 0), 10x, Haematoxilin and eosin (H-e). B) Mild and multifocal lymphoplasmacytic duodenitis (grade 1), 10x, H-e. C) A normal jejunum (grade 0), 10x, H-e. D) Mild and multifocal lymphoplasmacytic jejunitis (grade 1), 10x, H-e. E) A normal ileum (grade 0), 10x, H-e. F) Mild and multifocal lymphoplasmacytic ileitis (grade 1), 10x, H-e.

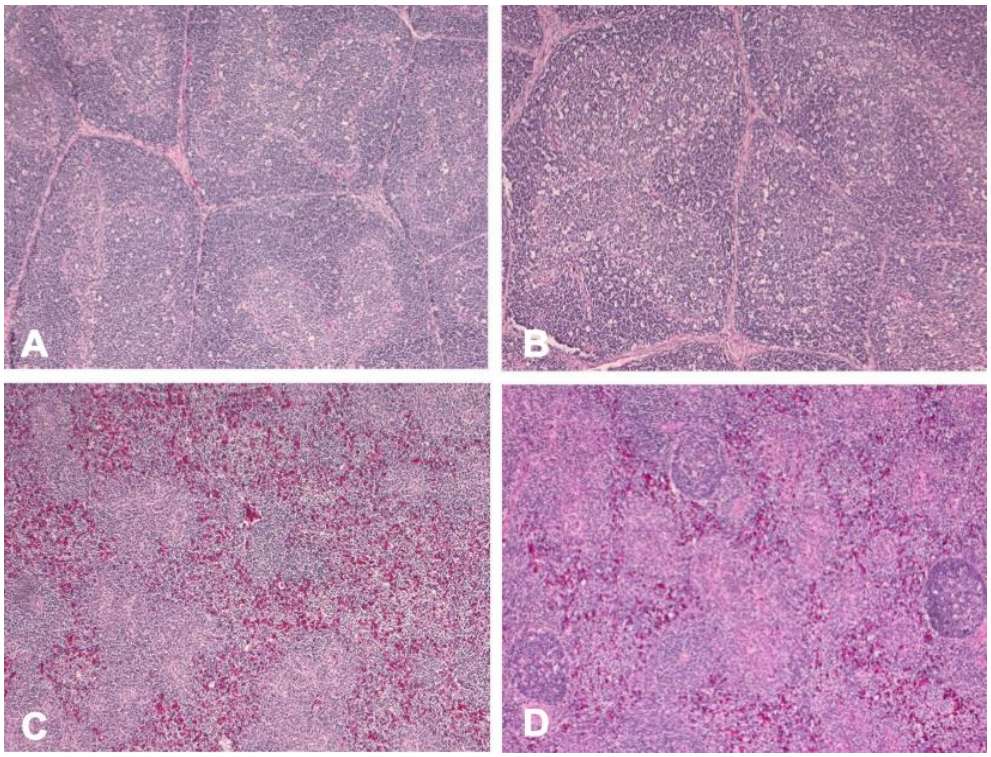

Figure 9. Main findings in chicken's bursa of Fabricius and spleen. A) A normal Bursa of Fabricius (grade 0), 10x, Haematoxylin and eosin (H-e). B) Bursa of Fabricius, mild and multifocal follicular depletion, 10x, H-e. C) A normal spleen (grade 0), 10x, H-e. D) Spleen, mild and multifocal white pulp hyperplasia (grade 1), 10x, H-e.

8.1.2 Mucin staining intensity

Data regarding mucin staining intensity in the gut of the evaluated chickens are reported in Table 10. Non-significant differences were recorded for neutral mucins, sialomucins, sulfomucins, and total mucins among the three dietary treatments (P>0.05) (Figure 10). On the contrary, significant differences were recorded for all the evaluated mucins among the intestinal segments, showing a proximo-distal increasing gradient from duodenum to ileum (P<0.001).

Table 10. Mucin histochemical quantification in the small intestine of the broiler chickens (n=12/treatment).

	Diet (D)			**Intestine (I)**			**P-value**		
	C	**HI**	**TM**	**DU**	**JE**	**ILE**	**D**	**I**	**DxI**
Neutral mucins, *mean (SD)*	3.15 (1.18)	3.15 (1.07)	3.56 (1.39)	2.16[a] (0.63)	3.63[b] (0.88)	4.08[b] (1.16)	0.194	<0.001	0.439
Sialomucins, *mean (SD)*	2.75 (0.85)	2.97 (1.43)	3.34 (2.28)	1.96[a] (0.60)	3.39[b] (1.20)	3.72[b] (2.13)	0.659	<0.001	0.922
Sulfomucins, *mean (SD)*	3.48 (1.58)	3.30 (1.30)	3.90 (2.04)	2.23[a] (0.91)	4.08[b] (1.79)	4.37[b] (1.32)	0.544	<0.001	0.293
Total mucins, *mean (SD)*	9.39 (3.19)	9.43 (3.12)	10.81 (4.42)	6.35[a] (1.49)	11.10[b] (2.65)	12.17 [b] (3.44)	0.217	<0.001	0.697

C: control; DU: duodenum; HI: *Hermetia illucens*; ILE: ileum; JE: jejunum; TM: *Tenebrio molitor*;. Means with superscript letters (a, b, c) denote significant differences between the gut segments.

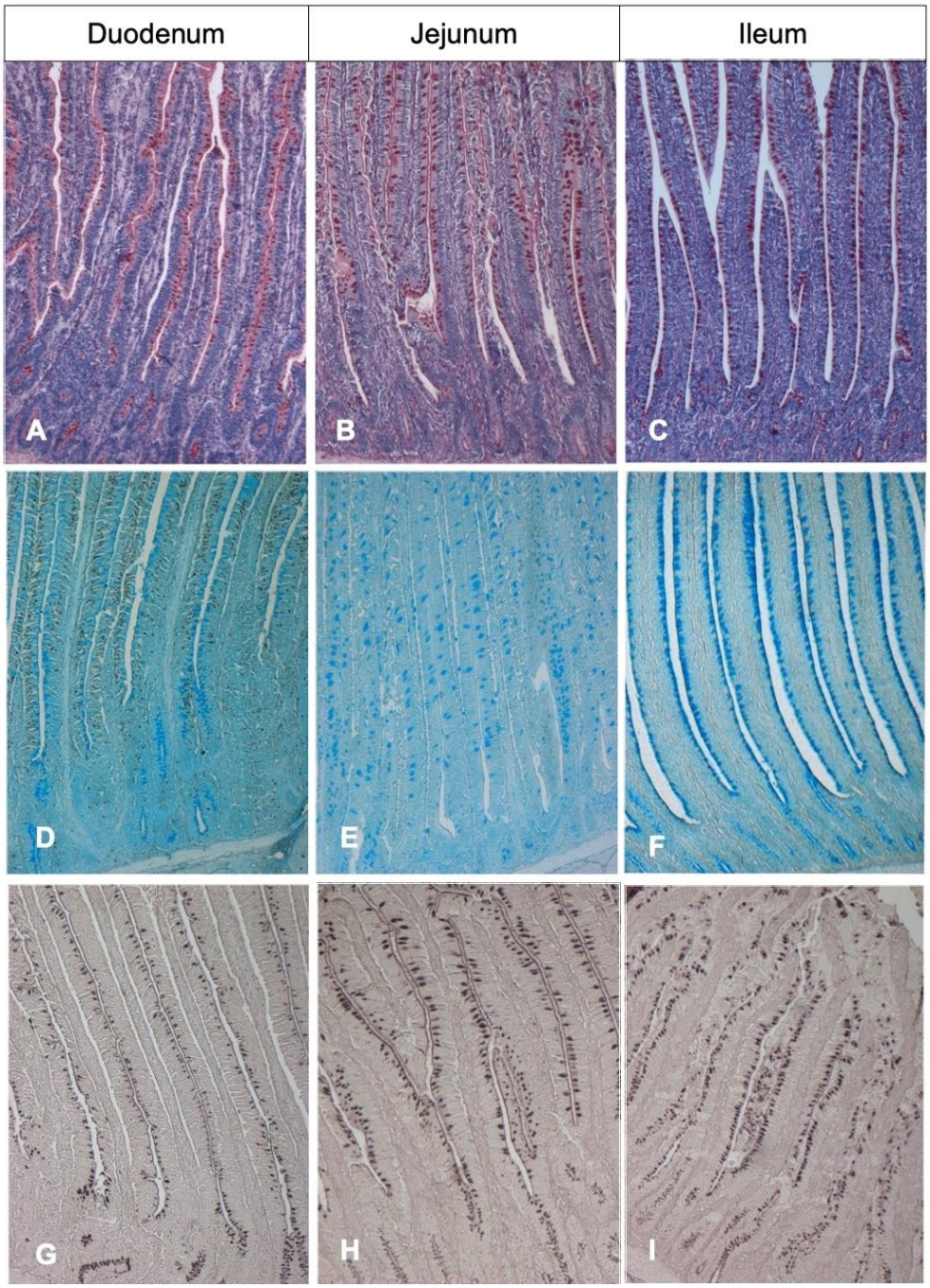

Figure 10. Mucin staining intensity in chicken's gut. A, B, C) Periodic Acid Schiff staining, 20x. D, E, F) Alcian Blue ph 2.5 staining, 20x. G, H, I) High Iron Diamine staining, 20x.

8.1.3 Intestinal gene expression

Cytokines and MUC-2 transcription levels recorded in the jejunum of the broiler chickens are summarised in Table 11. IL-2 expression was influenced by diet, being lower in TM group when compared to the other groups (P=0.044). The other evaluated cytokines and MUC-2 expression were not influenced by diet (P>0.05).

Table 11. Relative mRNA expression of gut cytokines and MUC-2 in jejunal tissue of broilers chickens (n=12/treatment).

	Diet			
	C	**HI**	**TM**	**P-value**
IL-2, *mean (SD)*	1.88[a] (0.02)	2.42[a] (0.16)	0.57[b] (0.17)	0.044
IL-4, *mean (SD)*	2.21 (0.28)	1.20 (0.43)	1.80 (0.00)	0.961
INF-γ, mean (SD)	1.26 (0.40)	0.95 (0.45)	1.48 (0.21)	0.860
TNF-α, *mean (SD)*	1.06 (0.06)	0.64 (0.51)	1.04 (0.66)	0.125
IL-6, *mean (SD)*	1.86 (0.44)	0.42 (0.11)	1.21 (0.45)	0.146
MUC-2, *mean (SD)*	1.22 (0.33)	1.52 (0.52)	1.58 (0.37)	0.444

HI: *Hermetia illucens*; IL: interleukin; INF: interferon; MUC: mucin; TM: *Tenebrio molitor*; TNF: tumour necrosis factor. Reference genes (β-actin and GAPDH) were used for normalization of the real-time PCR. Means with superscript letters (a, b, c) denote significant differences between the dietary treatments.

8.1.4 Microbiota evaluation

At the end of the trial, 54 caecal samples were sequenced. After sequencing, 18,491,109 reads were obtained and after the quality filtering, 995,342 reads were used for the downstream analysis with an average value of 18,432 ± 6,093 reads/sample. No differences in alpha or beta diversity were observed among the three dietary treatments (P>0.05). The microbiota in the three diets was characterized by the presence of Rikenellaceae and Ruminococcaceae families. At genus level, *Bacteroides*, *Faecalibacterium*, *Barnesiella*, *Helicobacter* and *Phascolarctobacterium* were the most abundant ones (Figure 11). However, it was possible to observe that the minor OTU fraction (relative abundance <5%) was influenced by HI and TM live larvae inclusion (P<0.05). In details, HI inclusion was characterized by the presence of *Clostridium*, *Saccharibacteria* (TM7) and Victivallaceae, while TM showed a higher abundance of *Collinsella*. On the other hand, *Eubacterium* was enriched in both HI and TM groups compared to C (Figure 12).

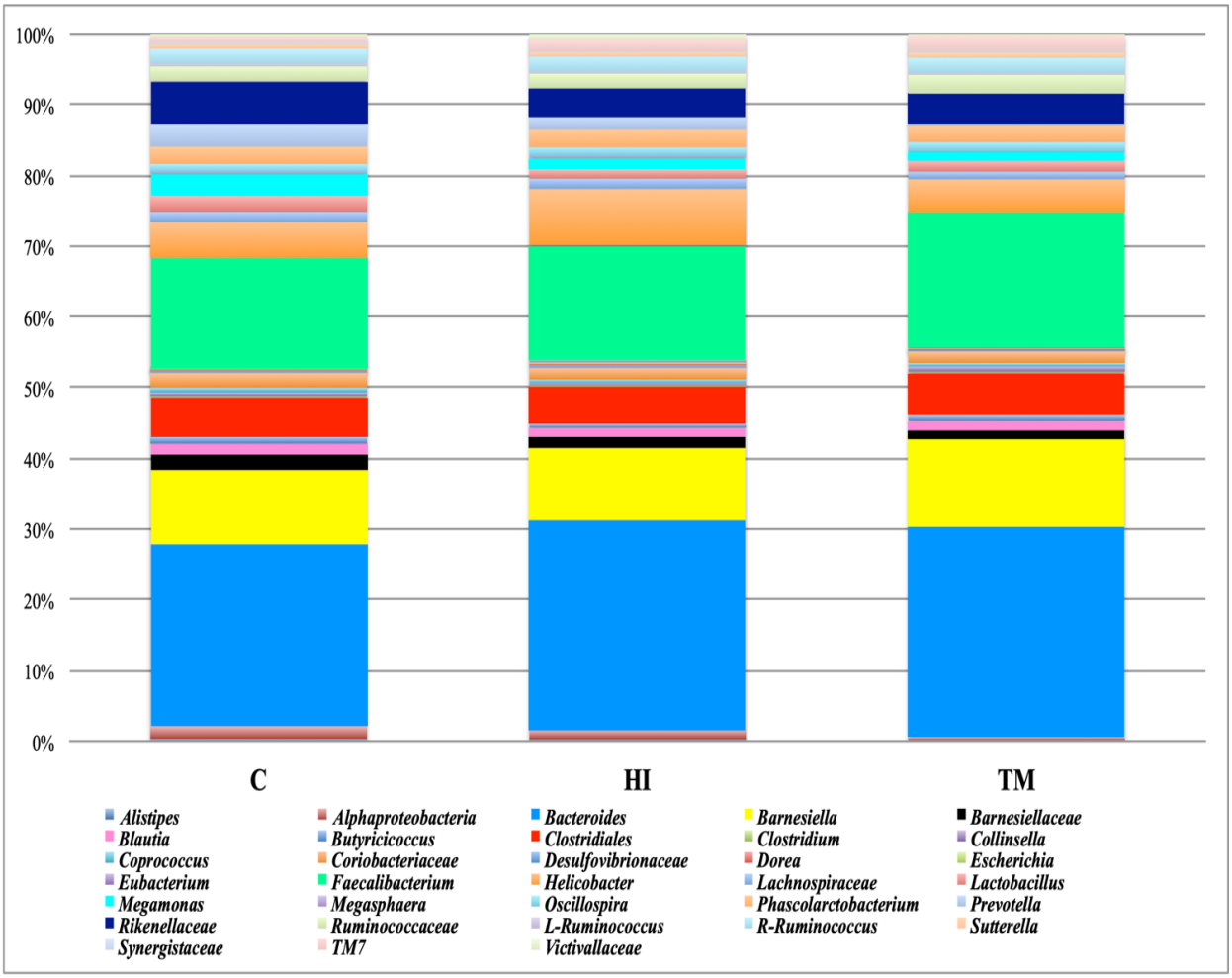

Figure 11. Composition of the caecal microbiota in the three dietary treatments (C: control; HI: *Hermetia illucens*; TM: *Tenebrio molitor*).

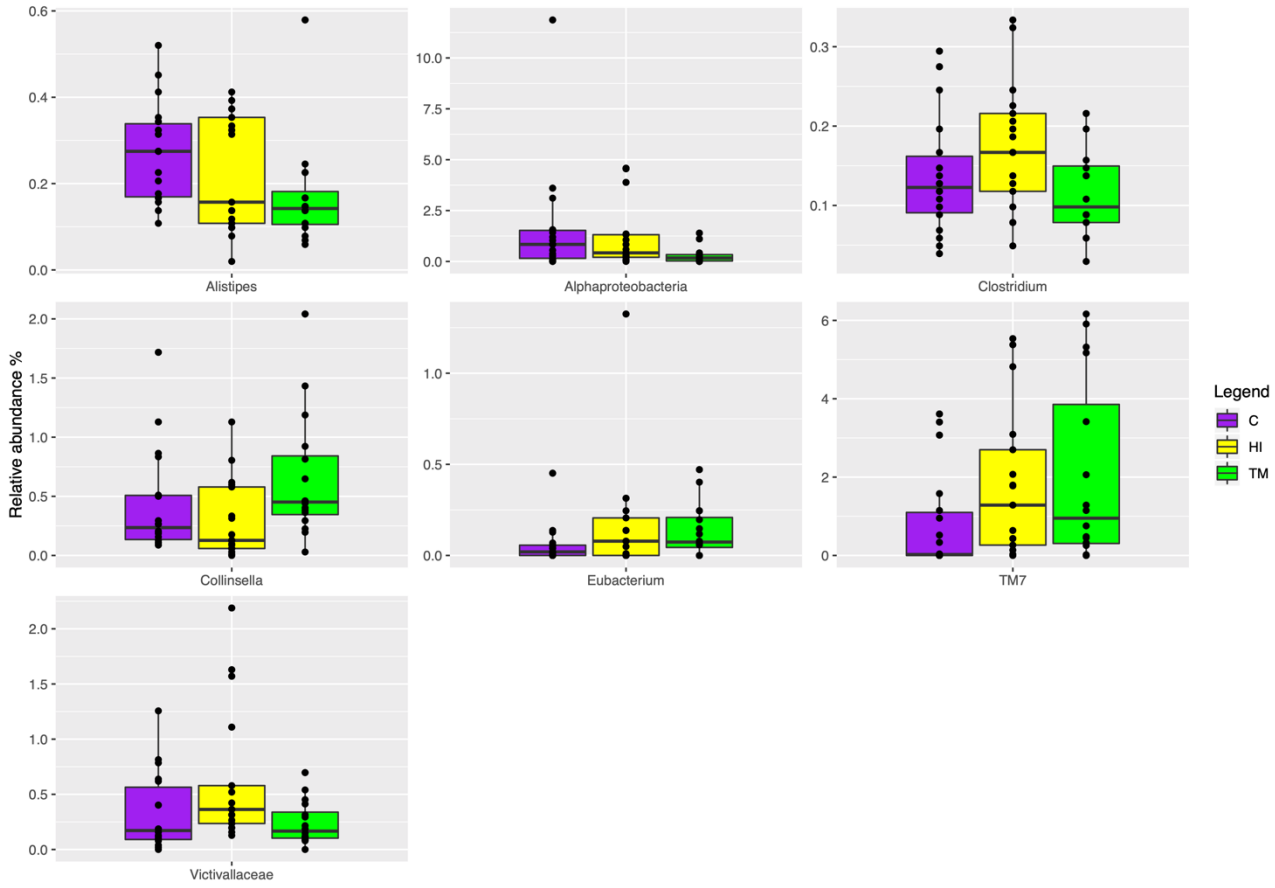

Figure 12. Differentially abundant OTU as a function of the dietary treatments (C: control; HI: *Hermetia illucens*; TM: *Tenebrio molitor*).

8.2 Trial 2

8.2.1 Histomorphometric evaluation

Data regarding morphometric evaluation are reported in Table 12. Non-significant differences were recorded among the dietary treatments for Vh, Cd and Vh/Cd in duodenum, jejunum and ileum. Regardless of diet, Vh and Cd depended on the intestinal segment, showing a proximo-distal decreasing gradient from duodenum to ileum (P<0.001).

Table 13 summarizes the histopathological findings in the main organs of the duck of the present trial. Diet did not influence the severity of the observed histopathological lesions in liver, thymus, bursa of Fabricius and gut (P>0.05). Regardless of diet, liver showed from mild to severe multifocal to diffuse vacuolar degeneration (Figure 13A, B, C and D) along with mild multifocal lymphoplasmacytic inflammation (Figure 13E). An absent to mild multifocal lymphoplasmacytic enteritis was also recorded in the small intestine (Figure 14). Bursa of Fabricius and thymus presented from absent to mild follicular depletion and cortical depletion, respectively (Figure 15A, B, C and D). On the contrary, spleen showed a statistically significant difference among dietary treatments, being the white pulp hyperplasia greater in TM and HI groups compared to control (P=0.025) (Figure 15E and F).

Table 12. Effects of insect live larvae on gut morphology of the Muscovy ducks (n=12/treatment)

	Diet (D)			**Intestine (I)**			**P-value**		
	C	**HI**	**TM**	**DU**	**JE**	**ILE**	**D**	**I**	**DxI**
Vh, *mean (SD)*	0.79 (0.24)	0.78 (0.23)	0.84 (0.24)	1.03^{a} (0.18)	0.76^{b} (0.23)	0.62^{c} (0.23)	0.147	<0.001	0.833
Cd, *mean (SD)*	0.06 (0.02)	0.07 (0.02)	0.07 (0.02)	0.08^{a} (0.02)	0.06^{b} (0.02)	0.05^{c} (0.02)	0.256	<0.001	0.880
Vh/Cd, *mean (SD)*	12.17 (3.29)	11.12 (3.29)	12.87 (3.29)	12.35 (3.29)	12.13 (3.29)	11.63 (3.29)	0.063	0.594	0.910

C: control; Cd: crypt; DU: duodenum; HI: *Hermetia illucens*; ILE: ileum; JE: jejunum; TM: *Tenebrio molitor*; Vh: villus height; depth. Means with superscript letters (a, b, c) denote significant differences between the gut segments.

Table 13. Effects of insect live larvae inclusion on the main organs of the Muscovy ducks (n=12/treatment)

	Diet			
	C	**HI**	**TM**	**P-value**
Liver				
Degeneration, *median (IR)*	0.00 (0.0-1.0)	0.50 (0.0-1.5)	0.50 (0.0-1.1)	0.454
Inflammation, median (IR)	0.00 (0.0-0.0)	0.00 (0.0-0.5)	0.00 (0.0-0.1)	0.110
Spleen, *mean (SD)*	0.03^{a} (0.13)	0.35^{b} (0.45)	0.28^{b} (0.25)	0.025
Thymus, *median (IR)*	0.00 (0.0-0.5)	0.00 (0.0-0.1)	0.00 (0.0-0.5)	0.438
Bursa of Fabricius, *median (IR)*	0.50 (0.0-0.6)	0.25 (0.0-0.5)	0.50 (0.0-1.0)	0.306
Gut, *median (IR)*	0.75 (0.0-1.0)	1.00 (0.0-1.6)	0.0 (0.0-2.0)	0.716

C: control; HI: *Hermetia illucens*; TM: *Tenebrio molitor.* Means with superscript letters (a, b) denote significant differences between the dietary treatments.

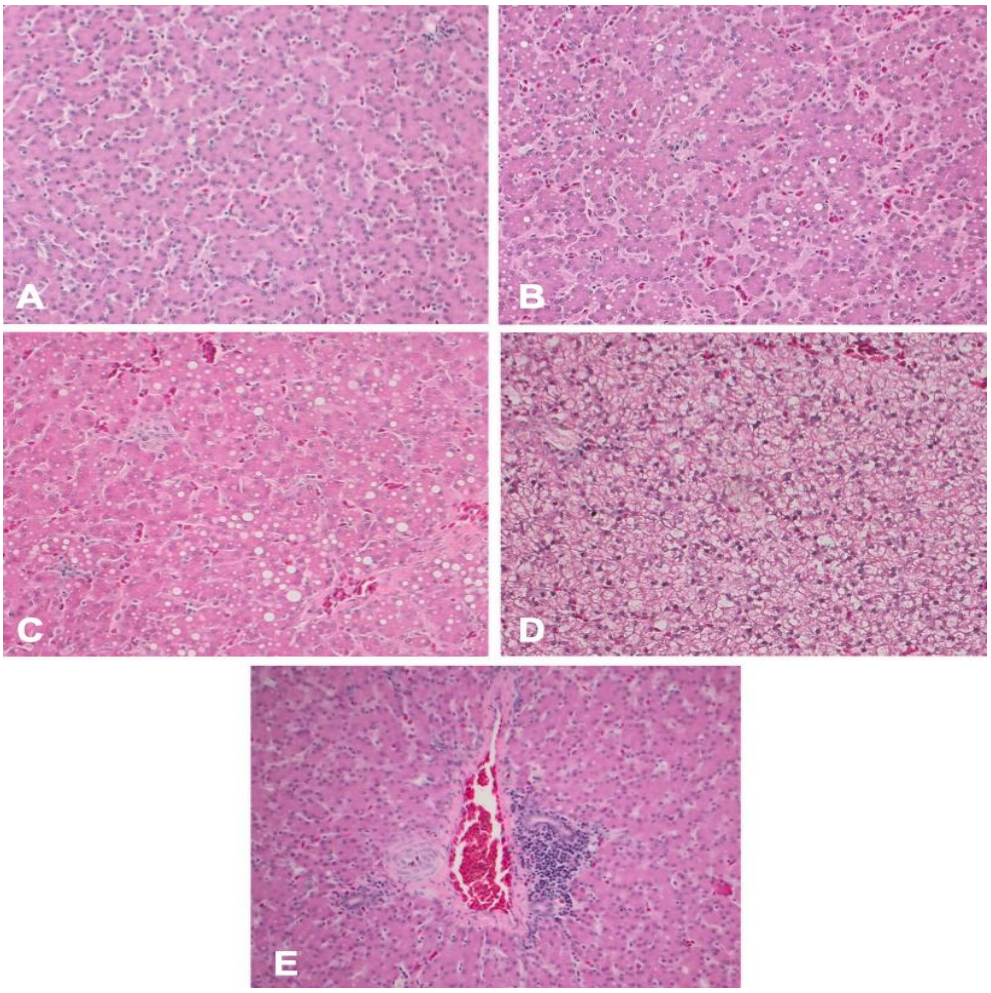

Figure 13. Main histopathological findings in duck's liver. A) A normal liver (grade 0), 20x, Haematoxilin and Eosin (H-e). B) Mild and multifocal vacuolar degeneration (grade 1), 20x, H-e. C) Moderate and multifocal vacuolar degeneration (grade 2), 20x, H-e. D) Severe and diffuse vacuolar degeneration (grade 3), 10x, H-e. E) Mild and multifocal lymphoplasmacytic inflammation (grade 1), 20x, H-e.

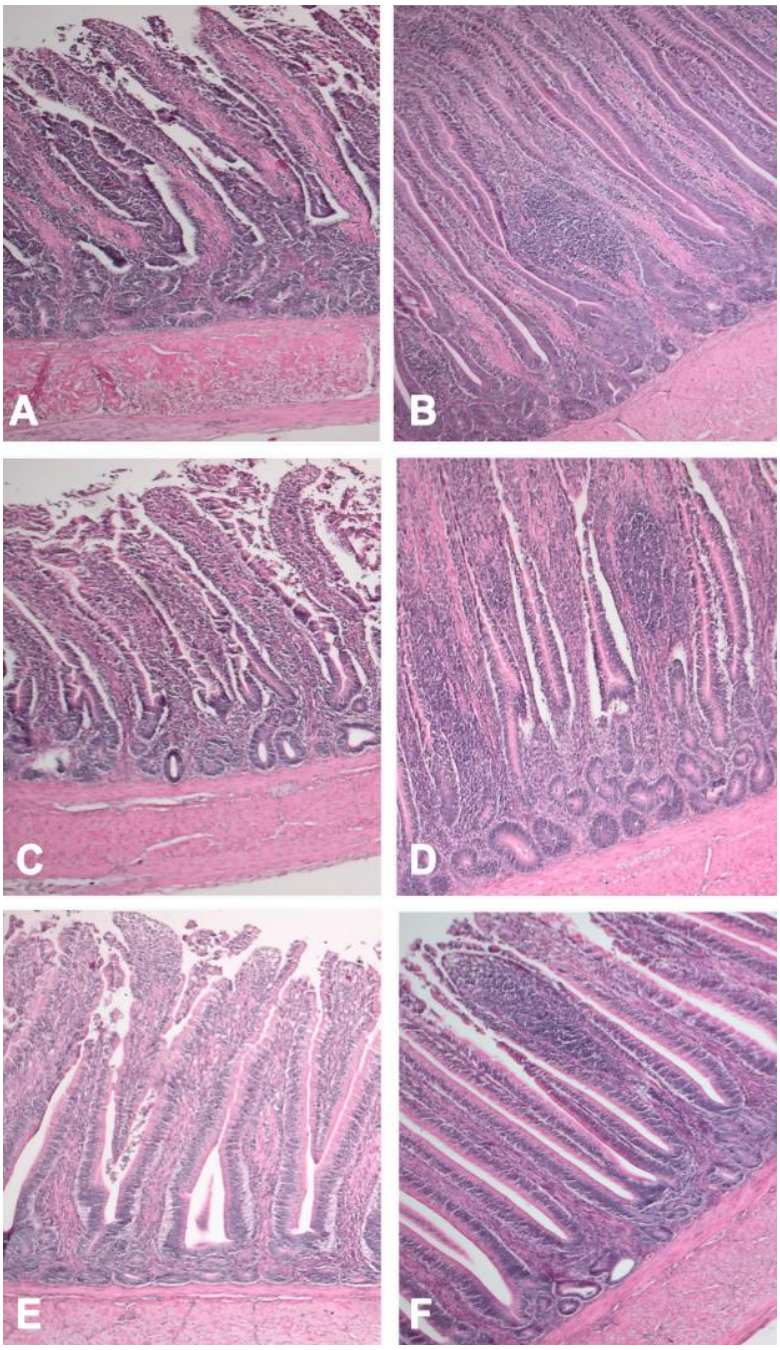

Figure 14. Main histopathological findings of duck's gut. A) A normal duodenum (grade 0), 10x, Haematoxilin and eosin (H-e). B) Mild and multifocal lymphoplasmacytic duodenitis (grade 1), 10x, H-e. C) A normal jejunum (grade 0), 10, H-e. D) Mild and multifocal lymphoplasmacytic jejunitis (grade 1), 10x, H-e. E) A normal ileum (grade 0), 10x, H-e. F) Mild and multifocal lymphoplasmacytic ileitis (grade 1), 10x, H-e.

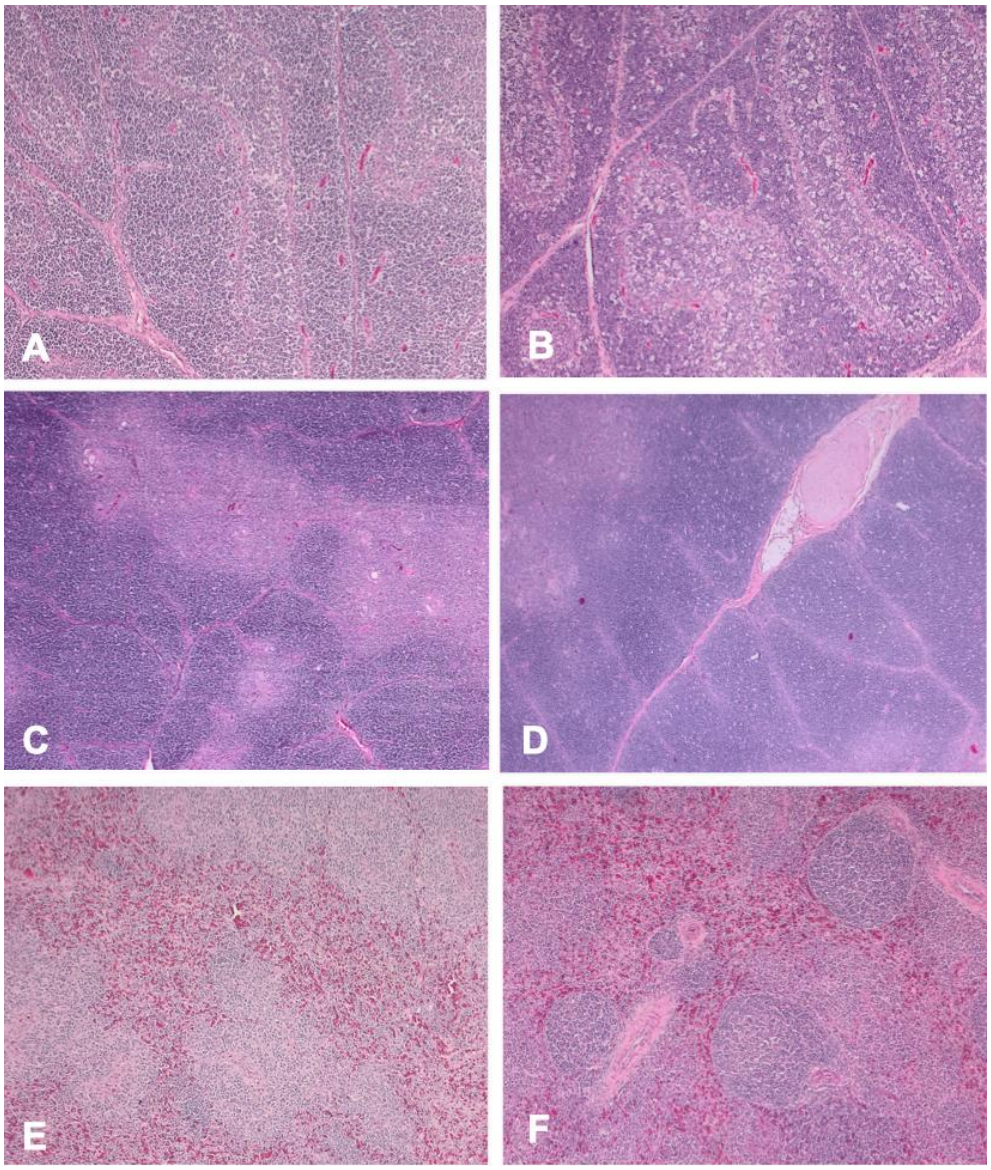

Figure 15. Main findings in chicken's Bursa of Fabricius, thymus and spleen. A) A normal Bursa of Fabricius (grade 0), 10x, Haematoxylin and eosin (H-e). B) Bursa of Fabricius, mild and multifocal follicular depletion, 10x, H-e. C) A normal thymus (grade 0), 5x, H-e. D) Thymus, mild and multifocal cortical depletion, 5x, H-e. E) A normal spleen (grade 0), 10x, H-e. F) Spleen, mild and multifocal white pulp hyperplasia (grade 1), 10x, H-e.

8.2.2 Mucin staining intensity

Table 14 reports the results for histochemical quantification of mucin in the duck's gut. Non-significant differences were observed for all the evaluated mucins among dietary treatments (P>0.05). However, sialomucins, sulfomucins and total mucins depended on gut segment, showing a proximo distal increasing gradient from duodenum to ileum (P=0.001) (Figure 16).

Table 14. Mucin histochemical quantification in the small intestine of the Muscovy ducks (n=12/treatment)

	Diet (D)			**Intestine (I)**			**P-value**		
	C	**HI**	**TM**	**DU**	**JE**	**IL**	**D**	**I**	**DxI**
Neutral mucins, *mean (SD)*	3.81 (1.48)	3.72 (1.24)	3.63 (1.12)	3.44 (1.19)	3.91 (1.48)	3.81 (1.13)	0.822	0.696	0.126
Sialomucins, *mean (SD)*	3.73 (1.79)	3.61 (1.68)	3.88 (2.37)	2.78^{a} (1.64)	3.98^{b} (1.81)	4.50^{c} (2.03)	0.991	0.001	0.129
Sulfomucins, *mean (SD)*	3.40 (1.77)	3.43 (1.55)	3.41 (1.43)	2.46^{a} (0.92)	3.46^{b} (1.27)	4.35^{c} (1.82)	0.957	0.001	0.154
Total mucins, mean (SD)	10.45 (3.55)	10.86 (3.28)	10.81 (3.80)	8.69^{a} (2.50)	10.95^{b} (3.37)	12.51^{c} (3.60)	0.933	0.001	0.171

C: control; DU: duodenum; HI: *Hermetia illucens*; IL: ileum; JE: jejunum; TM: *Tenebrio molitor*. Means with superscript letters (a, b, c) denote significant differences between the gut segments.

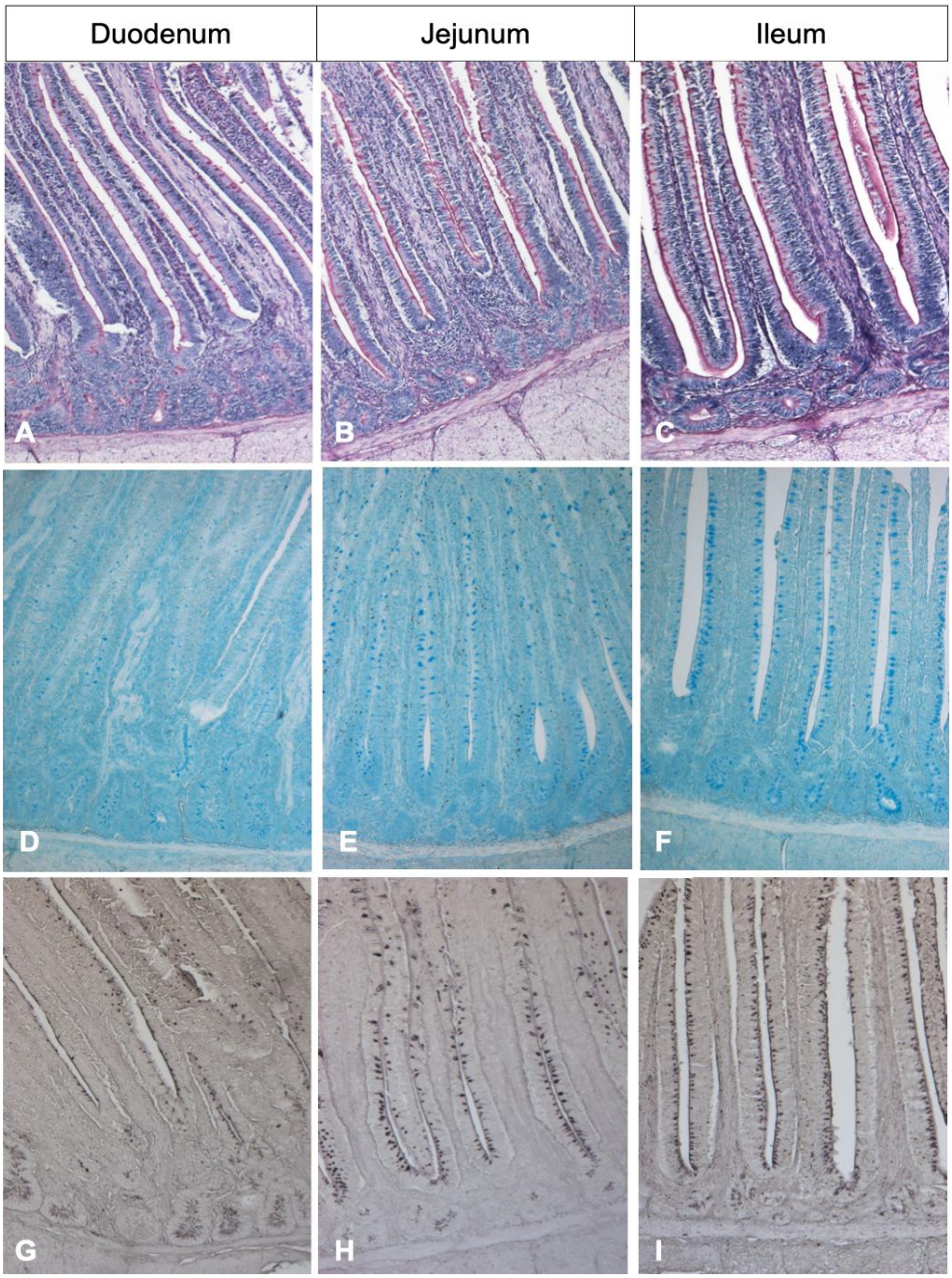

Figure 16. Mucin staining intensity in chicken's gut. A, B, C) Periodic Acid Schiff staining, 20x. D, E, F) Alcian Blue ph 2.5 staining, 20x. G, H, I) High Iron Diamine staining, 20x.

8.2.3 Intestinal gene expression

Cytokines and MUC-2 transcription levels in the jejunum of Muscovy ducks are summarised in Table 15. IL-6 transcription levels were influenced by diet, being higher in TM group when compared to the other groups (P=0.009). The other evaluated cytokines and MUC-2 were not influenced by diet (P>0.05).

Table 15. Relative mRNA expression of gut cytokines and MUC-2 in jejunal tissue of Muscovy ducks (n=12/treatment)

	Diet			
	C	**HI**	**TM**	**P-value**
IL-2, *mean (SD)*	0.87 (0.39)	0.68 (0.36)	0.82 (0.32)	0.716
IL-4, *mean (SD)*	1.31 (1.34)	0.93 (0.87)	1.89 (1.37)	0.348
INF-γ, mean (SD)	0.89 (0.42)	0.71 (0.52)	1.10 (0.62)	0.296
TNF-α, *mean (SD)*	1.13 (0.52)	0.89 (0.63)	1.48 (0.66)	0.126
IL-6, *mean (SD)*	1.09^{ab} (0.46)	0.85^{a} (0.54)	1.30^{b} (0.46)	0.009
MUC-2, *mean (SD)*	1.71 (1.46)	0.77 (0.68)	1.79 (1.13)	0.086

HI: *Hermetia illucens*; IL: interleukin; INF: interferon; MUC: mucin; TM: *Tenebrio molitor*; TNF: tumor necrosis factor. Reference genes (B-actin and GAPDH) were used for normalization of the real-time PCR. Means with superscript letters (a, b) denote significant differences between the dietary treatments and/or the gut segments

8.2.4 Microbiota evaluation

At the end of the trial 2, 36 caecal samples were obtained and sequenced. After sequencing and quality filtering 1,420,947 reads were used for downstream analysis with an average value of 33,832 reads/sample. No significant differences in alpha diversity measures (Shannon and Chao1 indexes) were observed among the three experimental treatments (P>0.05). In all the three dietary treatments the microbiota was characterized by the presence of Ruminococcacae and Desulfovibrio family. At genus level *Bacterioides*, *Faecalibacterium* and *Bilophila* were the most abundant ones (Figure 17). However, the minor OTU fraction (relative abundance <5%) varied among the dietary treatments (P<0.05, Figure 18). In particular, HI and TM groups showed a higher presence of *Helicobacter*, *Elusimicrobium*, and *Succinatimonas* and a lower abundance of Coriobacteriaceae and *Phascolarctobacterium* compared to control.

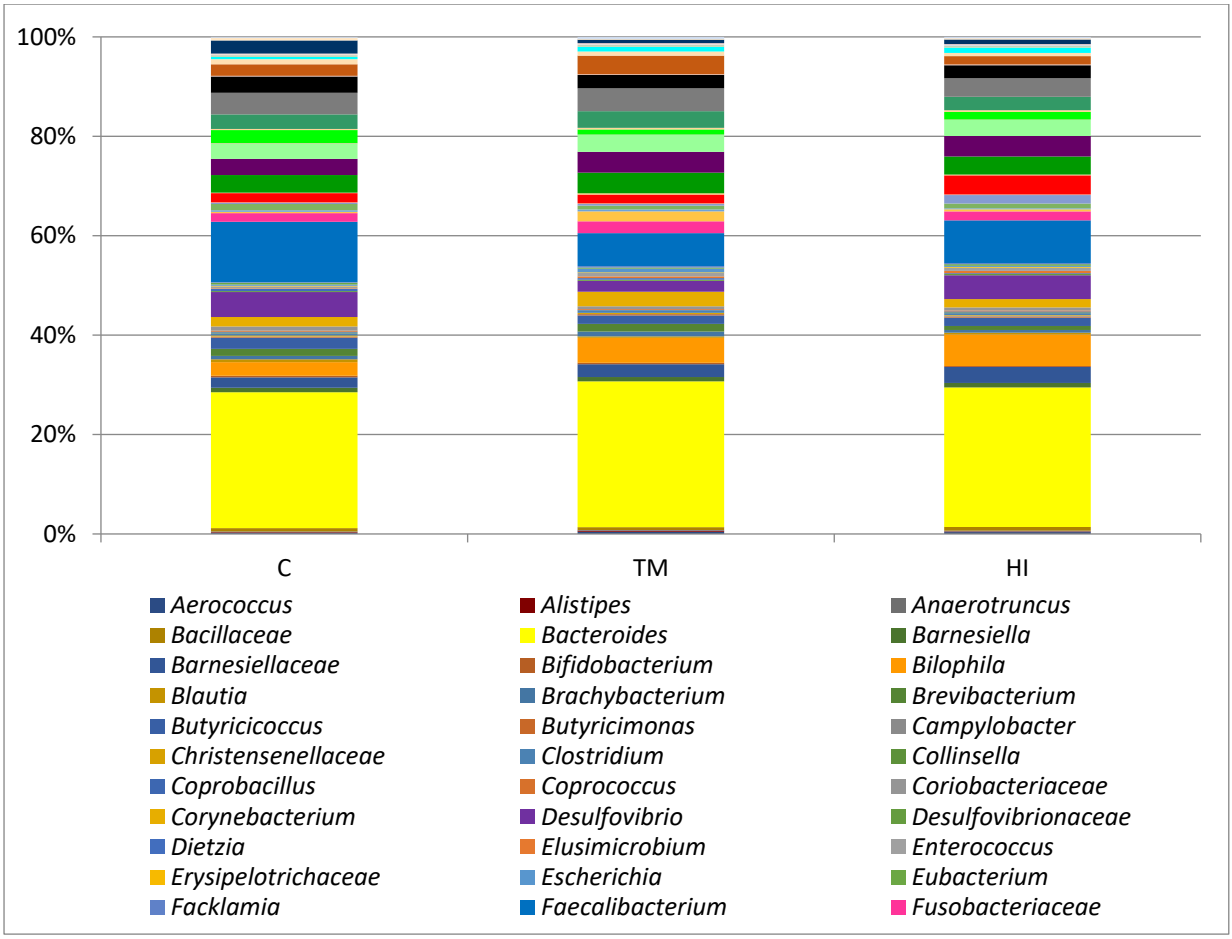

Figure 17. Composition of the caeca microbiota in the three dietary treatments (C: control; HI: *Hermetia illucens*; TM: *Tenebrio molitor*).

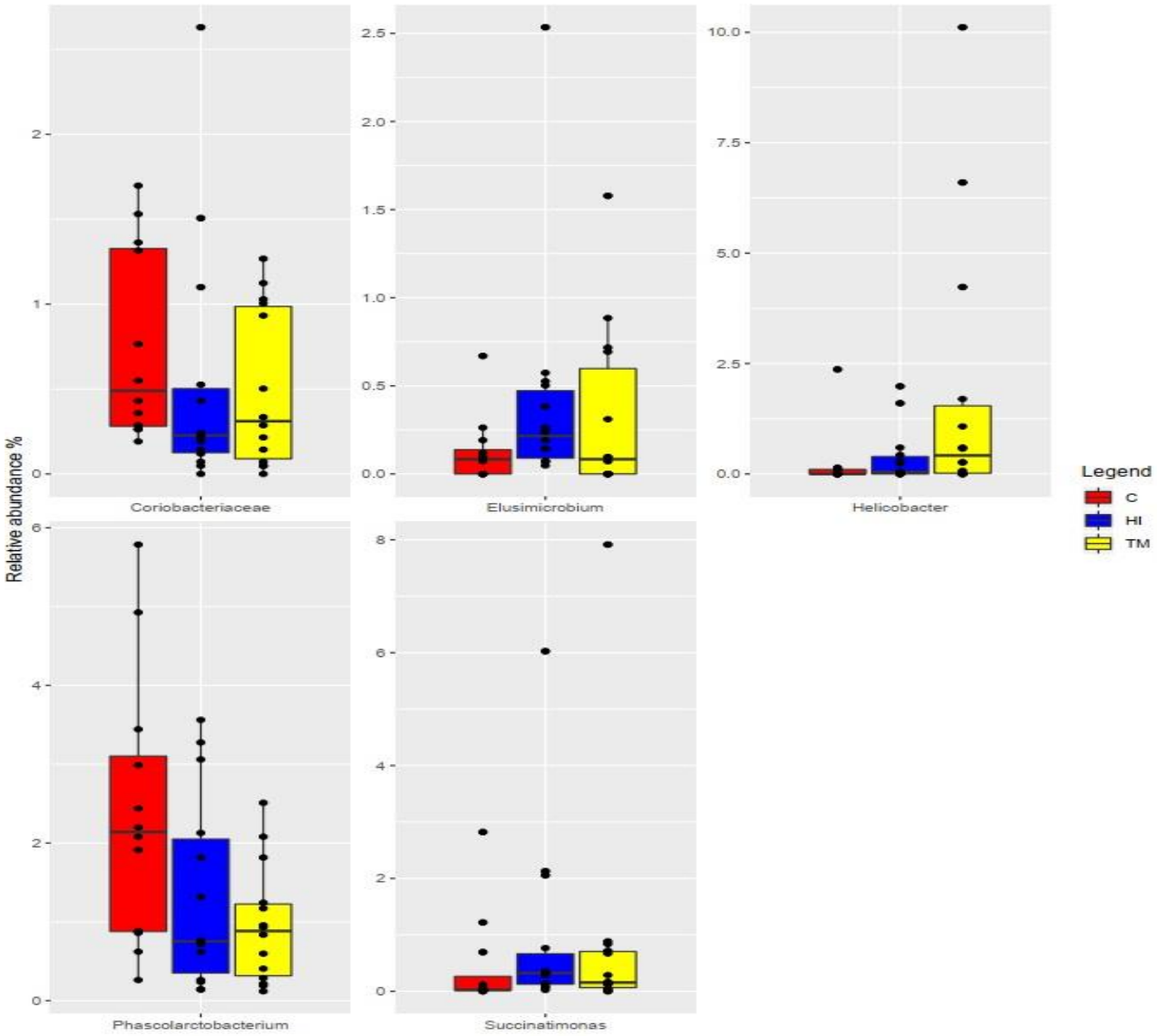

Figure 18. Differentially abundant OTU as a function of the dietary treatments (C: control; HI: *Hermetia illucens*; TM: *Tenebrio molitor*).

8.3 Trial 3

The results of Trial 3 have already been published in:

- Colombino E., Karimi M., Ton Nu MA., Tilatti AA., Bellezza-Oddon S., Calini F., Bergamino C., Fiorilla E., Gariglio M., Gai F., Capucchio MT., Schiavone A., Gasco L., Biasato I. Effect of feeding a thermomechanical, enzyme-facilitated, co-processed yeast and soybean meal on growth performance, organ weights, leg health and gut development of broiler chickens. Poult Sci. 2023. doi:10.1016/j.psj.2023.102578 (IF=4.014, Q1)

8.3.1 Histomorphometric evaluation

The gut morphology of the broiler chickens on day 3 and 10 is summarized in Table 15 and 16, respectively. Dietary ASG inclusion and the diet per intestinal segment interaction did not influence the morphometric indices ($P>0.05$). On the contrary, they depend on gut segment ($P<0.001$). In fact, a proximo-distal decreasing gradient was recorded for all the evaluated morphometric indices from duodenum to jejunum. Moreover, the duodenal Vh, Vh/Cd, Vw, VSA, muscular and mucosal thickness as well as the jejunal Vh, Vh/Cd, Vw, VSA and muscular thickness were greater in 10-day-old chicks when compared to 3-day-old birds.

Table 17 and 18 summarized the histopathological findings in the main organs of the chickens at day 3 and 10, respectively. Diet did not influence the severity of the observed histopathological lesions both at day 3 and 10 ($P>0.05$). Regardless of diet, different grades of vacuolar degeneration along with mild multifocal lymphoplasmacytic inflammation both at day 3 and 10 were observed in the liver (Figure 19 and 20). Gut showed from absent to mild multifocal lymphoplasmacytic inflammation (Figure 21-22). Bursa of Fabricius presented from absent to mild follicular depletion at day 10 (Figure 23).

Table 15. Effects of the dietary functional feed inclusion on the gut morphology of the broiler chickens on day 3 (n=16/dietary treatment).

	Diet (D)			**Intestine (I)**		**P-value**		
	C	**T1**	**T2**	**DU**	**JE**	**D**	**I**	**DxI**
Vh, *mean (SD)*	0.57 (0.18)	0.61 (0.18)	0.57 (0.18)	0.73^{a} (0.13)	0.46^{b} (0.09)	0.400	0.001	0.996
Cd, *mean (SD)*	0.07 (0.02)	0.06 (0.01)	0.07 (0.01)	0.07^{a} (0.02)	0.06^{b} (0.01)	0.801	0.003	0.920
Vh/Cd, *mean (SD)*	10.19 (8.64)	9.40 (2.73)	8.59 (3.21)	11.43^{a} (6.78)	7.33^{b} (2.31)	0.099	0.001	0.658
Vw, *mean (SD)*	0.07 (0.01)	0.07 (0.01)	0.07 (0.01)	0.07^{a} (0.01)	0.07^{b} (0.01)	0.323	0.042	0.383
VSA, *mean (SD)*	0.13 (0.05)	0.13 (0.05)	0.12 (0.05)	0.17^{a} (0.05)	0.09^{b} (0.02)	0.303	0.001	0.731
MT, mean (SD)	0.70 (0.20)	0.73 (0.21)	0.70 (0.20)	0.87^{a} (0.15)	0.56^{b} (0.10)	0.478	0.001	0.925
MuT, *mean (SD)*	0.11 (0.03)	0.12 (0.07)	0.10 (0.02)	0.12^{a} (0.06)	0.01^{b} (0.02)	0.553	0.001	0.411

C: Control; Cd: crypt depth; DU: duodenum; JE: jejunum; MT: mucosa thickness; MuT: muscular thickness; T1 = 20% inclusion of functional pro protein in the pre-starter phase and 10% in the starter phase; T2 = 5% inclusion of functional protein in the pre-starter and starter phases; Vh: villus height; VSA: villus surface area; Vw: villus width. Means with superscript letters (a, b) denote significant differences between the gut segments.

Table 16. Effects of the dietary functional feed inclusion on the gut morphology of the broiler chickens on day 10 (n=16/dietary treatment).

	Diet (D)			**Intestine (I)**		**P-value**		
	C	**T1**	**T2**	**DU**	**JE**	**D**	**I**	**DxI**
Vh, *mean (SD)*	0.82 (0.38)	0.80 (0.36)	0.76 (0.33)	1.07^{a} (0.18)	0.49^{b} (0.14)	0.055	0.001	0.606
Cd, *mean (SD)*	0.06 (0.02)	0.09 (0.12)	0.09 (0.16)	0.10^{a} (0.16)	0.06^{b} (0.01)	0.574	0.001	0.783
Vh/Cd, *mean (SD)*	12.90 (5.13)	12.48 (6.06)	12.05 (4.74)	16.39^{a} (4.54)	8.63^{b} (2.32)	0.351	0.001	0.955
Vw, *mean (SD)*	0.10 (0.02)	0.09 (0.01)	0.10 (0.01)	0.10^{a} (0.02)	0.09^{b} (0.01)	0.635	0.001	0.705
VSA, *mean (SD)*	0.27 (0.17)	0.24 (0.13)	0.24 (0.12)	0.37^{a} (0.10)	0.14^{b} (0.05)	0.145	0.001	0.455
MT, mean (SD)	0.93 (0.41)	0.89 (0.38)	0.82 (0.34)	1.21^{a} (0.20)	0.55^{b} (0.13)	0.225	0.001	0.725
MuT, *mean (SD)*	0.15 (0.04)	0.15 (0.05)	0.13 (0.04)	0.17^{a} (0.03)	0.12^{b} (0.03)	0.320	0.001	0.800

C: Control; Cd: crypt depth; DU: duodenum; JE: jejunum; MT: mucosa thickness; MuT: muscular thickness; T1 = 20% inclusion of functional pro protein in the pre-starter phase and 10% in the starter phase; T2 = 5% inclusion of functional protein in the pre-starter and starter phases; Vh: villus height; VSA: villus surface area; Vw: villus width. Means with superscript letters (a, b) denote significant differences between the gut segments.

Table 17. Effects of the dietary functional feed inclusion on the main organs in 3-days-old chickens (n=16/treatment)

	Diet			
	C	**T1**	**T2**	**P-value**
Liver				
Degeneration, *median (IR)*	1.00 (1.00-1.50)	1.00 (1.00-1.50)	1.50 (1.50-2.10)	0.267
Inflammation, *median (IR)*	1.00 (0.00-1.00)	1.00 (0.00-1.00)	1.00 (0.00-1.00)	0.843
Bursa of Fabricius, *median (IR)*	1.00 (0.00-1.00)	1.00 (0.00-1.00)	1.00 (0.20-1.00)	0.925
Gut, *median (IR)*	0.00 (0.00-0.50)	0.00 (0.00-0.20)	0.00 (0.00-0.00)	0.560

C: Control; T1 = 20% inclusion of functional pro protein in the pre-starter phase and 10% in the starter phase; T2 = 5% inclusion of functional protein in the pre-starter and starter phases.

Table 18. Effects of the dietary functional feed inclusion on the main organs in 10-days-old chickens (n=16/treatment)

	Diet			
	C	**T1**	**T2**	**P-value**
Liver				
Degeneration, *median (IR)*	0.00 (0.00-0.00)	0.25 (0.00-1.00)	0.00 (0.00-0.50)	0.180
Inflammation, *median (IR)*	1.00 (0.00-1.00)	1.00 (0.90-1.00)	1.00 (0.90-1.00)	0.532
Bursa of Fabricius, *median (IR)*	1.00 (0.50-1.50)	1.00 (1.00-1.50)	1.50 (1.00-1.50)	0.130
Gut, *median (IR)*	0.50 (0.00-1.00)	1.00 (0.50-1.12)	0.75 (0.50-1.50)	0.452

C: Control; T1 = 20% inclusion of functional pro protein in the pre-starter phase and 10% in the starter phase; T2 = 5% inclusion of functional protein in the pre-starter and starter phases.

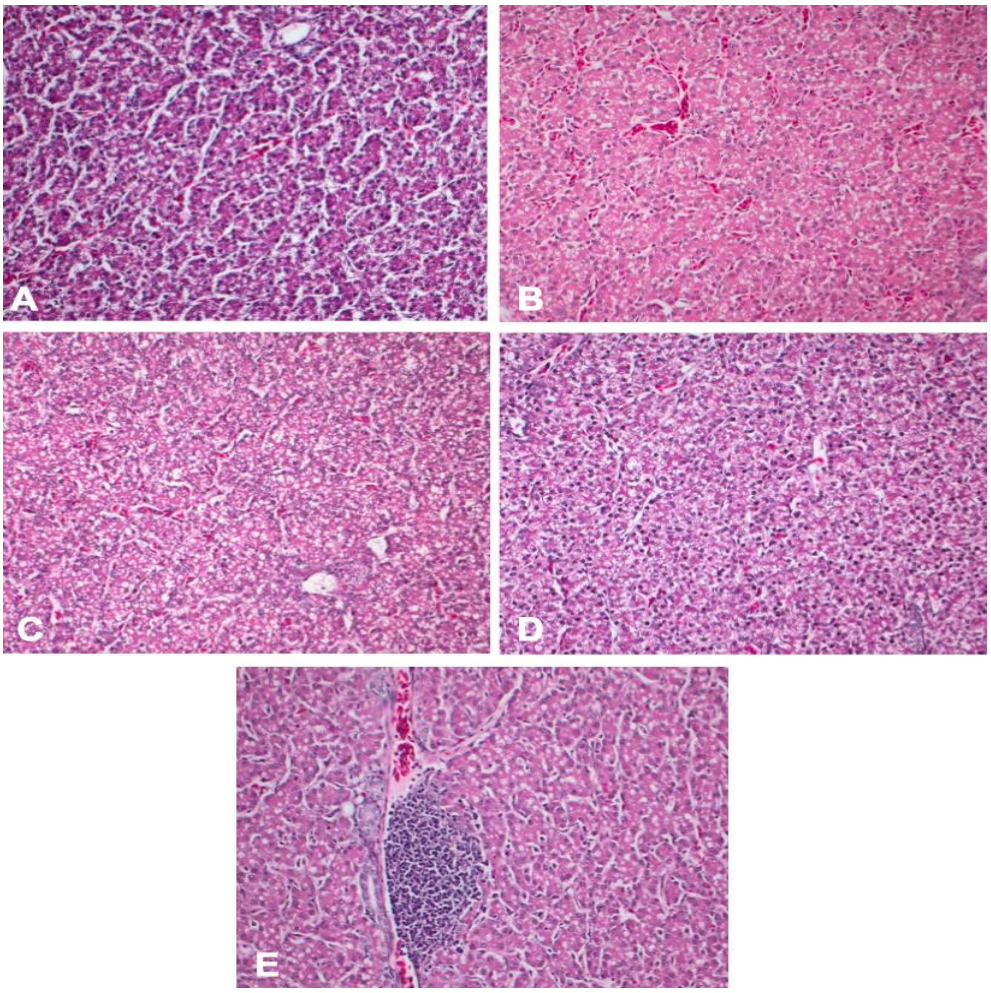

Figure 19. Main histopathological findings in chicks's liver at day 3. A) A normal liver (grade 0), 20x, Haematoxilin and Eosin (H-e). B) Mild and multifocal vacuolar degeneration (grade 1), 20x, H-e. C) Moderate and multifocal vacuolar degeneration (grade 2), 20x, H-e. D) Severe and diffuse vacuolar degeneration (grade 3), 10x, H-e. E) Mild and multifocal lymphoplasmacytic inflammation (grade 1), 20x, H-e.

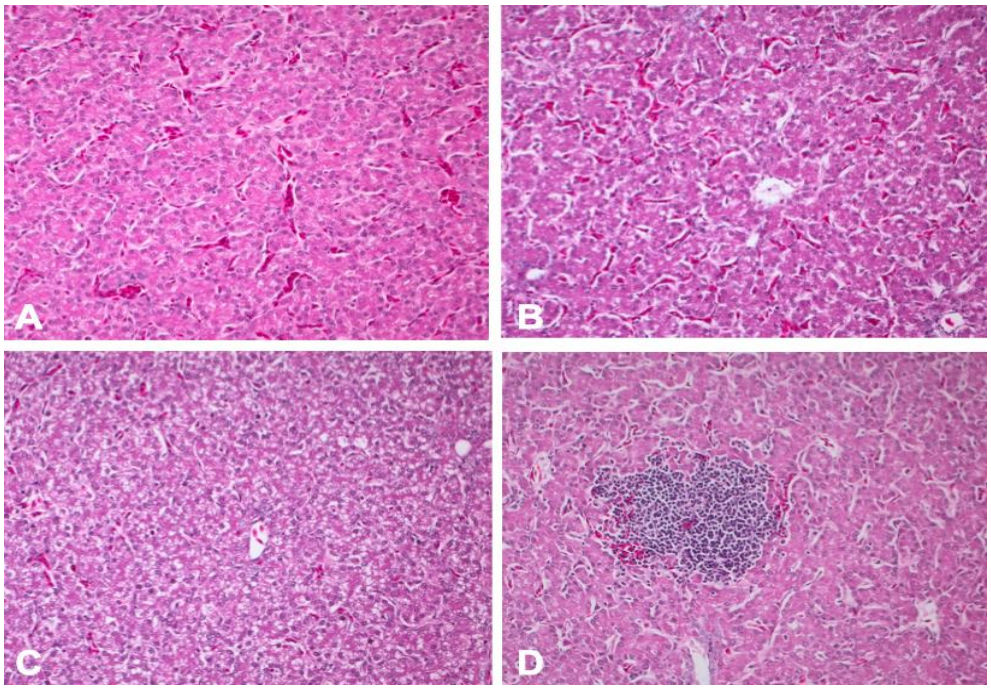

Figure 20. Main histopathological findings in chicks's liver at day 10. A) A normal liver (grade 0), 20x, Haematoxilin and Eosin (H-e). B) Mild and multifocal vacuolar degeneration (grade 1), 20x, H-e. C) Moderate and multifocal vacuolar degeneration (grade 2), 20x, H-e. D) Mild and multifocal lymphoplasmacytic inflammation (grade 1), 20x, H-e.

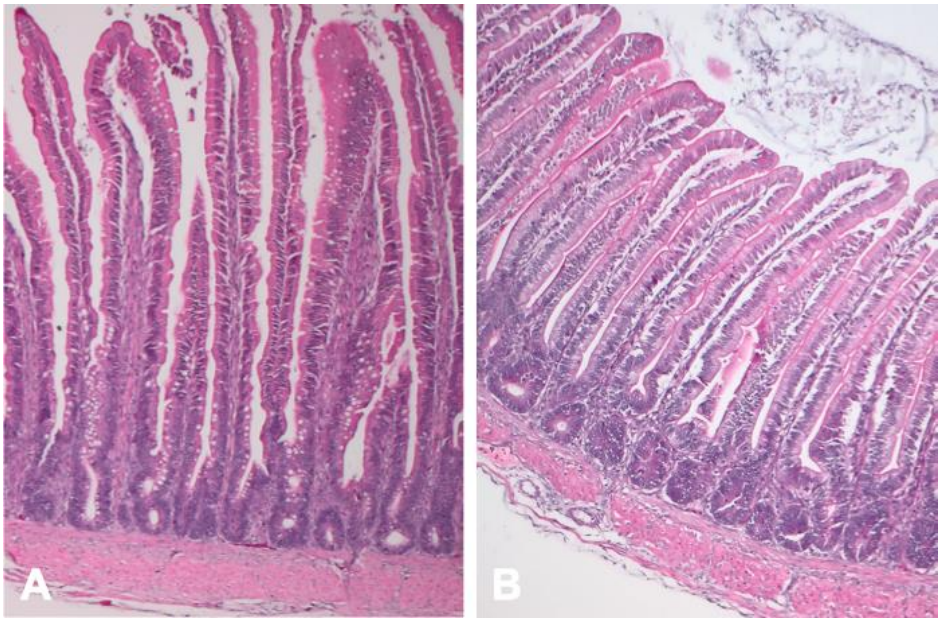

Figure 21. Main findings in chick's gut at day 3. A) A normal duodenum (grade 0), 10x, Haematoxilin and eosin (H-e). B) A normal jejunum (grade 0), 10x, H-e.

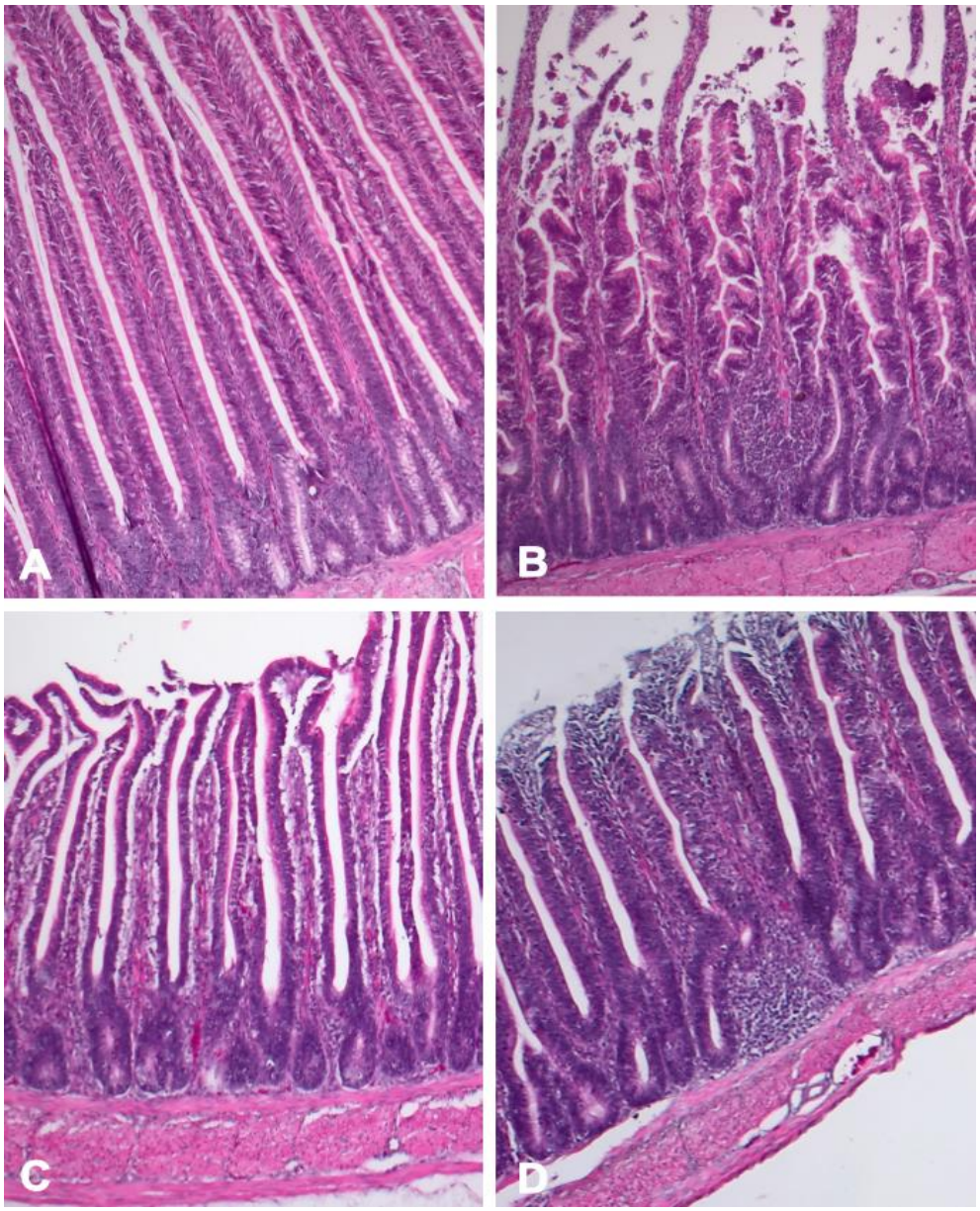

Figure 22. Main histological findings of chick's gut at day 10. A) A normal duodenum (grade 0), 10x, Haematoxilin and eosin (H-e). B) Mild and multifocal lymphoplasmacytic duodenitis (grade 1), 10x, H-e. C) A normal jejunum (grade 0), 10, H-e. D) Mild and multifocal lymphoplasmacytic jejunitis (grade 1), 10x, H-e.

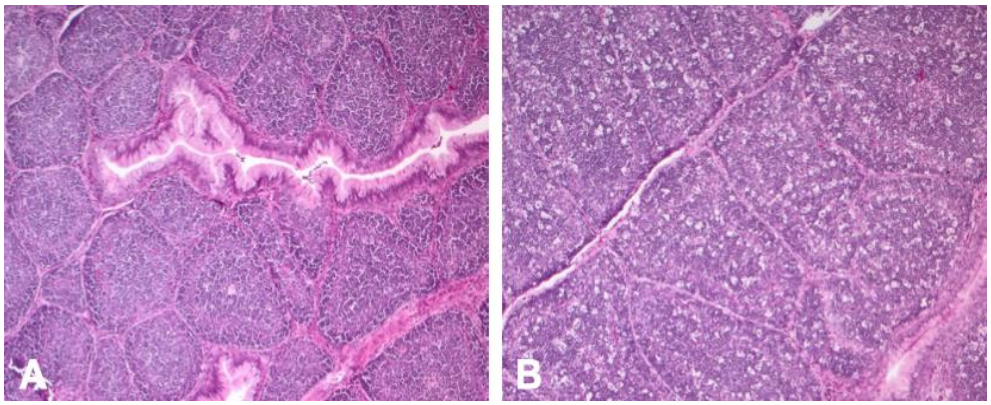

Figure 23. Main findings of chicks' Bursa of Fabricius at day 10. A) A normal Bursa of Fabricius (grade 0), 10x, Haematoxylin and eosin (H-e). B) Mild and multifocal follicular depletion, 10x, H-e.

8.3.2 Intestinal gene expression

The cytokine transcription levels in 3-day-old broiler chickens are summarized in Table 19 and Figure 23. The expression of IL-2 was influenced by dietary treatments (P = 0.054) and intestinal segment (P = 0.038), being 63.8% lower in the T1 and T2 birds when compared to the C group, and being 1.9 times higher in the jejunum than in the duodenum (Table 19). Furthermore, INF-γ and TNF-α transcription levels were influenced by the interaction between diet and intestinal segment (P = 0.058 and P<0.001, respectively) while IL-2 showed a statistical tendency (P = 0.083) (Figure 24). In particular, both T1 and T2 groups had lower pro-inflammatory cytokines when compared to C group in the duodenum (P ≤ 0.05), but not in the jejunum (P>0.05) (Table 19). On the contrary, IL-4 was not influenced by diet (P>0.05), but it only depends on the gut segment (P = 0.010), being higher in the jejunum than in the duodenum (Table 19). Furthermore, MUC-2 was influenced by the interaction between diet and intestinal segment (P = 0.016), being 30.9% and 18.18% lower in the duodenum of T1 birds compared to C and T2 groups, respectively. Moreover, ZO-1 showed a statistical tendency among dietary treatments at day 3, being lower in T1 compared to C and T2 groups (P = 0.085). Differently, no significant differences among the dietary treatments and between the intestinal segments were identified for all the evaluated cytokines, mucin, and tight junction transcription levels at day 10 (P> 0.05, Table 20).

Table 19. Effects of the dietary functional feed inclusion on the relative mRNA expression of cytokines, mucin and tight junction related genes in duodenum and jejunum of 3-day-old broiler chickens (n=4/dietary treatment).

	Diet (D)			**Intestine (I)**		**P-value**		
	C	**T1**	**T2**	**DU**	**JE**	**D**	**I**	**DxI**
IL-2, mean (SD)	1.27[a] (0.70)	0.46[b] (0.24)	0.46[b] (0.27)	0.52[a] (0.17)	0.98[b] (0.26)	0.054	0.038	0.083
IL-4, mean (SD)	1.01 (0.56)	0.51 (0.23)	0.57 (0.24)	0.27[a] (0.12)	1.11[b] (0.21)	0.423	0.010	0.240
INF-γ, mean (SD)	1.10[a] (0.44)	0.45[b] (0.28)	0.52[b] (0.23)	0.40[a] (0.30)	0.98[b] (0.30)	0.020	0.001	0.058
TNF-α, mean (SD)	1.02[a] (0.22)	0.63[b] (0.25)	0.61[b] (0.49)	0.45[a] (0.28)	1.06[b] (0.13)	0.005	0.001	0.001
MUC-2, mean (SD)	1.10 (0.25)	0.76 (0.40)	0.90 (0.64)	0.89 (0.62)	0.95 (0.24)	0.069	0.774	0.016
ZO-1, mean (SD)	1.00 (0.13)	0.79 (0.24)	0.91 (0.68)	0.94 (0.56)	0.86 (0.21)	0.085	0.682	0.105
CL-1, mean (SD)	1.20 (0.49)	1.10 (0.61)	1.62 (0.58)	1.11 (0.43)	1.51 (0.34)	0.706	0.387	0.119

C: control; CL: claudin; DU: duodenum; JE: jejunum; IFN: interferon; IL: interleukin; MUC: mucin; T1 = 20% inclusion of functional pro protein in the pre-starter phase and 10% in the starter phase; T2 = 5% inclusion of functional protein in the pre-starter and starter phases; TNF: tumor necrosis factor; ZO: zona occludens. Reference genes (B-actin and GAPDH) were used for normalization of the real-time PCR. Means with superscript letters (a, b) denote significant differences between the dietary treatments and the gut segments.

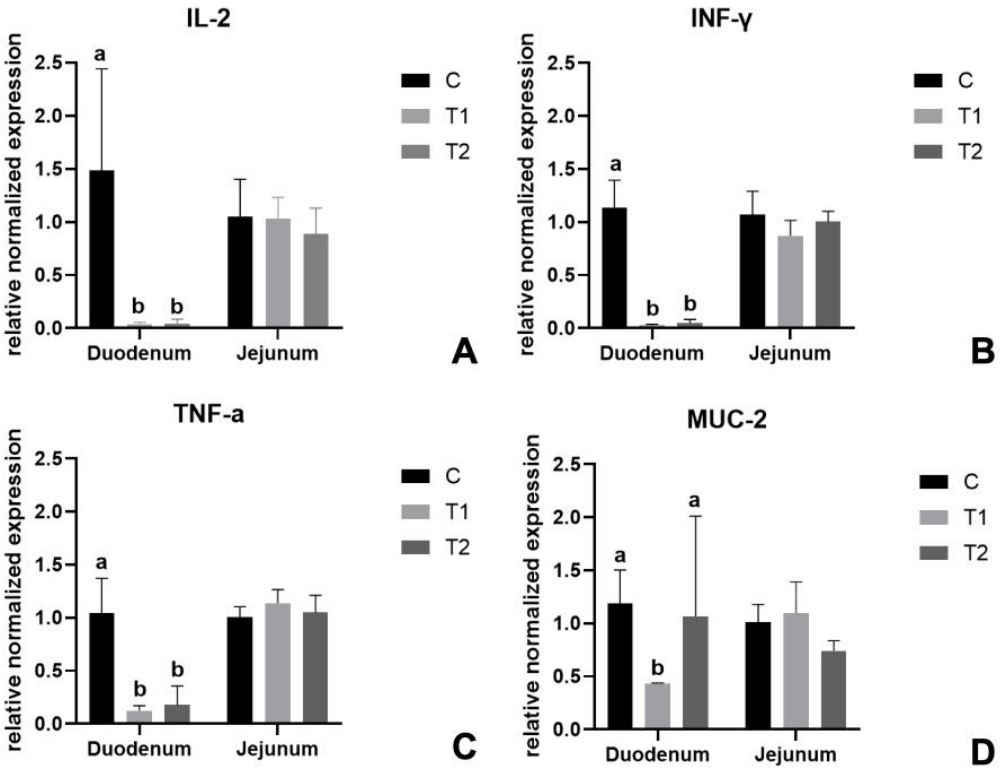

Figure 24. Effects of the dietary functional feed inclusion on the relative mRNA expression of cytokines and mucin related genes in duodenum and jejunum of 3-day-old broiler chickens depending on the interaction between the diet and the gut segment (n=4/dietary treatment). C = control diet; T1 = 20% inclusion of functional protein in the pre-starter phase and 10% in the starter phase; T2 = 5% inclusion of functional protein in the pre-starter and starter phases. Graph bars with different superscript letter (a, b, c) denote significant differences among the experimental treatment.

Table 20. Effect of the dietary functional feed inclusion on the relative mRNA expression of cytokines related genes in duodenum and jejunum at day 10.

	Diet (D)			Intestine (I)		P-value		
	C	**T1**	**T2**	**DU**	**JE**	**D**	**I**	**DxI**
IL-2, mean (SD)	1.46 (0.73)	2.37 (1.29)	1.86 (1.08)	2.09 (1.16)	1.71 (1.01)	0.322	0.418	0.793
IL-4, mean (SD)	1.34 (0.19)	1.57 (0.47)	1.67 (0.83)	1.64 (0.64)	1.41 (0.45)	0.382	0.362	0.593
INF-γ, mean (SD)	1.40 (0.47)	1.50 (0.63)	1.68 (0.91)	1.49 (0.57)	1.56 (0.79)	0.763	0.803	0.470
TNF-α, mean (SD)	1.34 (0.11)	1.50 (0.31)	1.38 (0.25)	1.50^{a} (0.25)	1.32^{b} (0.20)	0.249	0.056	0.120
MUC-2, mean (SD)	1.39 (0.31)	1.41 (0.24)	1.38 (0.44)	1.49 (0.33)	1.29 (0.30)	0.970	0.151	0.169
ZO-1, mean (SD)	1.38 (0.24)	1.22 (0.12)	1.12 (0.39)	1.30 (0.17)	1.18 (0.24)	0.097	0.152	0.448
CL-1, mean (SD)	1.49 (0.70)	1.64 (0.58)	1.79 (0.52)	1.65 (0.66)	1.63 (0.54)	0.676	0.961	0.857

C: control; CL: claudin; DU: duodenum; JE: jejunum; IFN: interferon; IL: interleukin; MUC: mucin; T1 = 20% inclusion of functional pro protein in the pre-starter phase and 10% in the starter phase; T2 = 5% inclusion of functional protein in the pre-starter and starter phases; TNF: tumor necrosis factor; ZO: zona occludens. Reference genes (β-actin and GAPDH) were used for normalization of the real-time PCR.

9. DISCUSSION

Due to the similarity of the experimental design and results, Trial 1 and 2 will be discussed together. On the contrary, trial 3 will be discussed separately due to the different feed ingredient administered to the broiler chickens.

9.1 Trial 1 and 2

9.1.1 Histomorphometric evaluation

Both in trials 1 and 2 non-significant differences among dietary treatments were recorded for all the evaluated morphometric indices ($P>0.05$). It has been demonstrated that in poultry the small intestine should be physiologically characterized by long villi and shallow crypts: longer villi guarantee an optimal nutrient utilization [101], while shallower crypts reflect the maintenance of the villi without the need of renewal [171]. On the contrary, lower villi and deeper crypt determine an impairment in nutrient digestion and absorption as well as poor growth performances [104]. Although no histomorphologic investigations have been performed so far in poultry reared with insect live larvae as environmental enrichment, previous studies using insect meals reported a dose-depending effect on gut morphometry in chickens. Particularly, Biasato et al. [106] did not observe any significant differences in free-range chickens fed 7.5% of TM meal. On the contrary, chickens fed with 15% of TM or HI meal showed an impairment of gut morphometry, showing lower villi and higher crypt depth [108, 109]. Regarding ducks, the results of trial 2 were in accordance with Gariglio et al. [112] who observed that 3, 6 and 9% of HI meal inclusion did not impair Muscovy ducks' gut development. The results of Trial 1 and 2 seems to be in accordance with those observed when low doses of insect meal (<15%) were fed to poultry, suggesting that the dietary inclusion of insect live larvae in broiler chickens and Muscovy duck did not negatively influence the gut development, the intestinal morphology and, consequently, the animals' health.

Regardless of dietary treatment, morphometric indices also showed a proximo-distal decreasing gradient from duodenum to ileum. This finding is in accordance with previous findings [107, 173] and with the physiological development of the absorption processes. Indeed, the duodenum is the intestinal segment in which most of the nutrient digestion and absorption processes take place receiving the physical, chemical and hormonal stimuli caused by the presence of the diet in the lumen [174]. Thus, it needs longer and slender villi with shallow crypts to guarantee a higher total luminal absorptive area and a satisfactory action of digestive enzymes [104]. It is important to highlight that intestinal structure and functionality are strictly linked with growth performances as an adequate digestion and absorption of nutrients derived from feed is fundamental for assuring an optimal animal's growth [91]. Non-significant differences were observed for live weight, daily feed intake and average daily gain both in trial 1 and 2, suggesting that insect live larvae did not affect the productive performances [175].

In both trials, HI and TM live larvae inclusion did not influence the severity of the histopathological alterations observed in gut, liver, thymus and Bursa of Fabricius, suggesting that they did not have any adverse effects on animal general health. These results are in accordance with previous studies

using different HI and TM inclusion levels in broiler chickens [107–109, 176] and Muscovy ducks [177]. However, greater white pulp hyperplasia was recorded in the spleen of HI and TM groups of both trials. This finding can explain the higher spleen weight recorded at slaughter both in Trial 1 and 2 [177] and it is in accordance with Bovera et al. [70]. The highest white pulp hyperplasia of the spleen can be attributed to the insect chitin content which provoked an increase in the activity of the immune system, indicating a better disease resistance and immune response of the birds [178]. In conclusion, the obtained results demonstrated that HI and TM live larvae inclusion in broiler chickens and Muscovy ducks did not have any adverse effects on gut morphometry and animal health.

9.1.2 Mucin staining intensity

Regarding mucin composition, in trial 1 and 2 non-significant differences were recorded among the three dietary treatments for all the evaluated mucins (neutral mucins, sialomucins, sulfomucins, and total mucins) ($P>0.05$). Furthermore, MUC-2 gene expression was comparable in C, HI, and TM groups ($P>0.05$) in both trials.

Mucins are the major constituents of the mucus layer and they protect the gut from acidic chyme, digestive enzymes, and pathogens [179]. Particularly, MUC-2 is the major type of secretory mucin in the intestine [180]. Previous studies have demonstrated that dietary factors can alter mucin secretion and, as a consequence, digesta viscosity, integrity of the mucus layer, and nutrient absorption [181]. The lack of effects observed after the administration of insect live larvae at the dosage used in the present study is in accordance with Biasato et al. [106]. However, in two subsequent studies, Biasato et al. [131] and Biasato et al. [155] observed a higher mucin staining intensity in chickens fed 5% of HI or TM meal [108, 131, 132]. These heterogeneous results can be due to the different amounts of chitin and the different form in which insects were provided. Thanks to heating-drying process defatted or full-fat insect meals are more concentrated in nutrients, while insect live larvae are richer in water [182]. For this reason, fresh whole insect had a lower content of nutrients and chitin and their dose need to be doubled in chickens to obtain similar results (e.g. 10.48% TM live larvae inclusion correspond to 4% TM meal) [182]**.** In fact, insect meal normally contained 6% of chitin on dry matter and higher levels of nutrients, which can influence mucin expression. In this study insects were administered based on 5% of the expected daily feed intake, which is far less compared to the 5% of TM or HI meal used in the above-mentioned studies [155, 164].

This hypothesis is supported by the more pronounced effects observed on mucin composition in chickens fed higher doses of HI and TM meals [108, 132]. However, the mechanisms through which chitin can modulate MUC-2 transcription levels and mucin secretion are still unclear [183]. It has

been hypothesized that chitin and chitosan could reach the gut and affect intestinal glycosylation, further affecting the MUC2 secretion [184].

Regardless of diet, all the evaluated mucins in trial 1 and sialomucins, sulfomucins and total mucin in trial 2 showed a proximo-distal increasing gradient, being lower in the duodenum when compared to ileum and jejunum ($P<0.001$). This is in accordance with the physiological development of digestion process and microbiota colonization of the chicken gastrointestinal tract, being already reported in previous studies using HI and TM meals in poultry nutrition [11, 17, 22, 37]. In particular, Forder et al. [129] observed an increased GC density along the GIT, suggesting that the distal ileum may be predisposed for bacterial colonization, needing a higher amount of mucin in order to avoid pathogens attachment and invasion.

To conclude, the obtained results demonstrated that insect live larvae did not impair gut mucin composition and MUC-2 transcription levels.

9.1.3 Intestinal gene expression

Considering local immune response, in trial 1 and 2 the expression of intestinal cytokines was not influenced towards "pro" or "anti" inflammatory patterns by feeding HI and TM live larvae. Solely, pro-inflammatory IL-2 was down-regulated in the jejunum of the TM group ($P=0.044$) in trial 1 and anti-inflammatory IL-6 was up-regulated in the jejunum of the TM group in trial 2 ($P=0.009$).

To date, IL-2 is mainly produced by activated $CD4^+$ T cells in secondary lymphoid organs and it influence the differentiation of T helper cell as well as the homeostasis of regulatory T cells [185]. Moreover, IL-6 has both proinflammatory and anti-inflammatory properties [143]. On one hand, it is a potent inducer of the acute-phase protein response [186]. On the other, it down-regulates the synthesis of the proinflammatory cytokines without affecting the synthesis of anti-inflammatory ones [143]. No previous studies are available on the effects of HI and TM meal or live larvae dietary inclusion on poultry gut mucosal immune system However, many studies have shown that the innate immune response can be modulated by dietary supplementation [187–189].

Particularly, the only available study evaluating the effect of HI meal inclusion (3.0, 6.0 and 9.0%) has been performed in Brown layer chickens, showing no changes in IL-2; IL-6 and TNF-α in the ileum [110]. The different results herein obtained can be attributed to the fact that the two studies have used two different chicken breed and evaluated two different intestinal segments. It is well known that chitin digestibility can be influenced by feeding behaviours and genetics as well as their action changes along the gut segments [76]. On the contrary, the results herein obtained are partially in accordance with the study of Yu et al. [190] , who investigated the effects of 4.0% and 8.0% HI meal dietary inclusion on pig mucosal immune response, showing a down-regulation of pro-inflammatory cytokines and an up-regulation of anti-inflammatory cytokines [190]. Therefore,

the reduction of IL-2 in the duodenum of TM chickens in trial 1 and the up-regulation of IL-6 in TM group of trial 2 should be considered beneficial for the mucosal homeostasis, helping to avoid aberrant immune response. The greatest effects observed in the TM group compared to HI group could be due to different chitin content of TM and HI live larvae. Particularly, in the present study TM showed a lower chitin content compared to HI. This result is in accordance with recent literature that reports that TM larvae are less rich in chitin than HI [64, 74, 76, 191, 192]**.** Thus, as chitin can be sensed by the innate immune system through specific membrane-bound receptors and induce cytokine's production [86], the lower concentration of this active bio-compound in TM larvae could explain the lower transcription levels of IL-2 and the higher levels of IL-6 [86]. This result seems also to support the immunoregulatory property attributed to chitin and chitosan, even if further studies are required to clarify the underlying mechanisms [14].

9.1.4 Microbiota evaluation

In Trial 1 and 2, no significant differences were observed in alpha diversity measures, suggesting that the insect live larvae at the administered doses have no negative effect on gut microbiota diversity. Regardless of diet, in both trials the composition of the microbiota of caecal samples was characterized by a high presence of *Bacteroides* and *Faecalibacterium*. These results are in accordance with Qi et al. [193], Clavijo et al. [144] and Zhu et al. [194] that reported *Bacteroides* and *Faecalibacterium* as some of the most representative genus and family bacteria in chickens' and/or Pekin and Muscovy ducks' microbiota. To date, *Faecalibacterium* genus represents an important butyrate producer, with positive effects on enterocytes nourishment and on intestinal mucin production [195, 196]. Moreover, Bacteroides are more abundant in the ceca than in any other intestinal segment as they have one of the highest hydrolytic activities among all known genera, being able to degrade non-digestible carbohydrates and to produce SCFAs [197]. As it is known, SCFAs are the main energy substrate for enterocytes and they have been shown to have anti-inflammatory and anti-oxidant properties, especially butyric acid [198]. In addition, in Trial 1, caecum was also colonized by *Helicobacter*, *Barnesiella* and *Phascolarctobacterium*. Among the abovementioned bacterial genera, *Helicobacter* can play a positive role in the ceca because some members can produce the hydrogenase enzyme, which favour the production of SCFAs [144]. However, *Helicobacter* genus also included potential pathogenic and zoonotic species that can colonize chicken gut, highlighting the need of further studies to clarify its role in chicken's gastrointestinal diseases [199]. The presence of *Barnesiella* genus is considered a positive finding as it is part of the Porphyromonadaceae family, whose presence is correlated with resistance to infection by *Salmonella enterica* serovar *typhimurium* and to the ability of fermenting sugars producing mainly acetic and propionic acids, which are important nutrients for the enterocytes [200].

Regarding *Phascolarctobacterium* spp., they were previously reported in poultry ceca [193, 201]. They are saccharolytic, succinate-utilizing bacteria able to produce propionate, which inhibits fat synthesis in the liver and could reduce the animal feed intake, representing a potential negative shift in the microbiota composition [202]. However, the animal feed intake and growth performances of the three experimental groups of the trial 1 were not negatively affected, showing that this increase of *Phascolarctobacterium* genus had no negative effects on birds' performances [175].

In trial 2 Desulfovibrio family and *Bilophila* genus were also recorded in all the dietary treatments. On one hand, the presence of Desulfovibrio bacteria could be beneficial for the animals as they consume hydrogen for sulphate reduction, helping in the removal of the free hydrogen produced during anaerobic fermentations [203, 204]. On the other, it has also been reported that this family is able to degrade intestinal mucin, weakening or damaging the intestinal barrier [193]. Scarce data are available regarding *Bilophila* genus. It seems to be a consistent member of the anaerobic colonic flora of poultry and it seems to be involved in bile acid metabolism but further studies are needed to clarify its role [205].

Furthermore, HI and TM live larvae influenced the minor OTU fraction both in trial 1 and 2. In particular, in Trial 1 Victivillaceae family, Saccharibacteria (TM7) and *Clostridium* genus were higher in HI group than in the others. No previous studies are available on the presence of the Victivillaceae family in the chicken's ceca. However, it has been previously isolated in human ceca, and these anaerobic bacterial species seem to be involved in the polysaccharide fermentation process thanks to their cellobiose-degrading capacity [206]. Moreover, Saccharibacteria has previously been detected in ducks, but its role in poultry microbiota still remains unclear [207]. *Clostridium* is one of the most abundant bacterial genera inhabiting the chicken ceca, and some species are capable of producing butyric acid, which has been reported to positively influence intestinal morphometry and resistance to pathogens, as well as to show remarkable anti-inflammatory properties [208]. Moreover, Collinsella was more abundant in the TM group in comparison to HI and C diets. Collinsella seems to influence the host lipid metabolism by altering intestinal cholesterol absorption, decreasing glycogenesis in the liver and increasing triglyceride synthesis [209]. In both the HI and the TM groups an increase in Eubacterium was also detected, being beneficial for the gut mucosa as these bacteria are involved in the hydrolysis of starch and other macromolecules, with the subsequent formation of SCFAs [210].

In Trial 2, HI and TM groups showed a highest presence of *Helicobacter*, *Elusimicrobium*, and *Succinatimonas* and a lower abundance of Coriobacteriaceae and *Phascolarctobacterium* compared to control. As already reported for trial 1, *Helicobacter* role in chicken cecum is still controversial [144]. Some species of *Helicobacter* genus can stimulate the production of SCFAs but some others -especially *Helicobacter pylori*- could depress mucin synthesis, determining a worsening in terms of

intestinal development [128, 204]. However, the gut structure and development of HI and TM live larvae fed groups were not negatively affected, showing that this increase in *Helicobacter* genus had no negative effects on birds' performance and welfare.

Succinatimonas spp., belonging to the family *Succinivibrionaceae*, is able to digest glucose and other carbohydrates to produce SCFAs, especially acetate and succinate that can benefit enterocytes development [211]. Moreover, Coriobacteriaceae and *Elusimicrobium* are normal components of the birds' gastrointestinal microbiota and their variation has no biological significance. However, some members of the Coriobacteriaceae family have been implied in intestinal infection and further studies are needed to understand how and when they start to become detrimental to the host [212].

To date, a greater change in the microbiota composition was detected in previous studies after the administration of insect meal in poultry diet. On one hand, low dosages of insect meal generally decrease pathogenic bacteria and increase SCFAs-producing bacteria [106, 213] On the other, a high dosage of insect meal increase mucolytic bacteria and decrease the bacterial syntheses of SCFAs, with an impairment of gut health [131, 132, 155]. This discrepancy between the results herein obtained and the previous studies could be due to the administration form of insects, being insect live larvae less rich in nutrients and chitin that insect meals [182]. Moreover, it cannot be excluded that larvae microbiota can play a role in modulating the host microbiota. A few studies are available on the HI and TM microbiota composition, but the results are still extremely heterogenous. In fact, similar to mammals, the insect's microbiota depends on the composition of rearing substrates, which are far from being standardized [214–216].

In conclusion, the increase of Vicitivillaceae family, *Collinsella*, *Eubacterium* and *Succinatomonas* genera represent a positive outcome, as they participate in the hydrolysis of starch and polysaccharides producing SCFAs, which are known for their anti-inflammatory properties, their ability to modulate oxidative stress and for being a main energy substrate for enterocytes [24, 28]. However, the increase of *Helicobacter* and *Clostridium* genera recorded in insect-fed groups must be taken into consideration as they encompass both enteric pathogens and SCFAs-producing bacteria in poultry (although no negative effects had not been registered in Trial 1 and 2).

9.2 Trial 3

9.2.1 Histomorphometric evaluation

Independently of the age, feeding ASG did not significantly influence the gut morphology of the broiler chickens, thus suggesting no ASG-related negative effects on intestinal development, health, and functionality, and reasonably explaining the unaffected small intestine weights. These results are in contrast with the available literature, as previous studies reported that β-glucans contained in yeast can improve gut morphology [113]. In particular, Morales-López et al. [217]; Ding et al. [218] and Teng et al. [113] found greater Vh and Cd in duodenum and jejunum/ileum after the administration of yeast β-glucans. Moreover, Cox et al. [42]reported ameliorated Vh and Cd after β-glucans administration in *Eimeria*- or coccidian-challenged broiler chickens. The Vh, Cd, and the Vh/Cd are important indicators of intestinal digestion and absorption capacity, as an increase in Vh and Vh/Cd, and a shallowing of crypts indicate an improvement in gut nutrient digestion and absorption [219]. However, in most of these above-mentioned studies, the chickens were reared under stress conditions. It has been reported that prebiotics, including β-glucans and MOS, are helpful in improving gut health under disease and stress conditions, including extremes temperature, high stocking density, poor management or infectious diseases, which are invariably present in commercial broiler production [41]. The present study was conducted under good hygienic conditions (strict biosecurity measures, clean litter, good ventilation, and low stocking density), thus implying a minimum bacterial challenge. Under such conditions, the birds may not have required any functional feed to ameliorate their gut health. Furthermore, in the above-mentioned research, the birds received the functional feed until slaughtering age (35 days), while in the present study chickens only received the functional feed for 10 days, thus representing another potential factor that prevented the observation of any beneficial effects on gut morphometry. As a final aspect to consider, morphometric indices showed a proximo-distal decreasing gradient from the duodenum to the ileum in both the C- and the ASG-fed birds, which is in accordance with the physiological processes of nutrient absorption in poultry as already mentioned above for trial 1 and 2 [173]. Moreover, a greater development of the duodenal and jejunal segments was also identified in 10 days broiler chickens of age when compared to 3 days broiler chickens in both the C and the ASG-fed birds. This is due to the physiological growth of the chicks as the gut morphometric indices (especially, Vh, Cd and total absorptive area) have previously been reported to increase with age[220].

Furthermore, feeding ASG in pre-starter and starter diets did not significantly influence the development or the severity of the histopathological changes detected in liver, gut or bursa of

Fabricius of the chickens, thus suggesting that ASG did not negatively affect gut health and animal health.

9.2.2 Intestinal gene expression

Feeding ASG to broilers seemed also to drive the intestinal immune response towards an "anti-inflammatory pattern". Indeed, it down-regulated pro-inflammatory cytokines (IFN-γ, TNF- α, and IL-2) in the duodenum of broiler chicks in the pre-starter phase (0-3 days). On the contrary, it did not influence the expression of anti-inflammatory IL-4. Inflammation plays a key role in protecting tissues after infection, but the uncontrolled inflammatory reaction characterized by a high release of pro-inflammatory cytokines would lead to tissue damage and high nutrient consumption [221]. For this reason, the results obtained in the present study can be considered a positive outcome, helping in maintaining a proper balance of the intestinal cytokine levels and regulating the innate immune response. Very few studies are available on the effects of functional ingredients on gut inflammatory cytokines in poultry, especially in the pre-starter and starter phases. However, it is well established that diet could be used to drive the intestinal immune response in poultry [221] and that β-glucans or MOS improved disease resistance against pathogens, enabling a low immune status and maximizing nutrients utilization for growth, rather than for the activation of the immune system in basal conditions [222] On the other side, in the case of bacterial infections, prebiotics improve the immune response, helping in controlling the disease [223]. Indeed, Johnson et al. [224] reported a decrease in pro-inflammatory cytokines after the administration of yeast β-glucans in chickens with necrotic enteritis, suggesting a controlled response situation. Furthermore, Janardhana et al. [225] reported no significant differences in both the pro- and the anti-inflammatory cytokine transcription levels in chickens fed a functional feed containing MOS in basal conditions. Differently, Yitbarek et al. [226] demonstrated that chickens receiving the same functional feed and infected with *Clostridium perfringens* presented high levels of pro-inflammatory cytokines (IL-12 and IFN- γ), supporting a pro-inflammatory effect via T-helper type-1 cell-associated pathways to control the early stages of the infection. These results are extremely heterogeneous, and they demonstrated that the interpretation of immune response is difficult because there is an active, homeostatic balance between pro-inflammatory and anti-inflammatory responses continuously occurring in the gut [224]. However, the results of the present study seem to suggest that β-glucans and MOS produce a low immune status in basal conditions in treated groups, even though further studies should be conducted in order to clarify the efficacy of such functional ingredient during a bird challenge.

On the contrary, MUC-2 transcription levels were similar in the duodenum of the C and T2 groups, but resulted to be lower in the T1 chickens ($P = 0.016$). Particularly, the MUC-2 gene encodes for

secretory MUC-2, which is the primary gel-forming mucin in the gut [180]. Despite the statistical significance of the interaction between diet and gut segment, all the dietary treatments displayed normal levels of MUC-2 expression according to previous works, and the differences recorded for the T1 group are still unclear [227, 228]. A possible explanation can be found in the different dosages of FB in the diets. In fact, Duangnumsawang et al. [126] reported that thermal processing of poultry feed may reduce the mucus shedding in the lumen, reducing the stimulus to secrete mucins by goblet cells and, as a consequence, lowering the expression of the MUC-2 gene. The T1 group received a higher percentage of ASG in their first three days of life, which could have had a protective role for the mucus layer, reducing the need of mucins to replace it. Moreover, mucin transcription levels showed an increasing gradient from the duodenum to the jejunum. This is in accordance with the physiological development of mucin along the gut, and Forder et al. [129] previously suggested that this can be due to an increase in bacterial colonization from the duodenum to the ileum that stimulates mucin production. Furthermore, the major effects observed in the duodenum of the treated groups may be due to the richness of the ASG in highly digestible nutrients and fast digestible protein fraction. As a results, proteins reach their highest concentration in the duodenum, where they are rapidly absorbed. This can reasonably explain the immunomodulatory effect mainly seen in the duodenum, and, as a consequence, the lower effects in the jejunum, where the functional feed did not reach a sufficient concentration.

Regarding tight junctions, ZO-1 showed a statistical tendency at day 3, being lower in T1 compared to C and T2 groups ($P=0.085$). Non-significant differences were observed for CL-1 in the birds at both day 3 and 10. Tight junctions, which seal the paracellular space between adjacent epithelial cells, are required for the maintenance of the mucosal barrier [116] Zona occludens-1 (ZO-1) is located at the cytoplasmic surface of the cell membrane, close to the tight junction's strands, and it is thought to be a functionally critical tight junction component. Moreover, Claudin-1 (CL-1) is a pore-sealing claudin whose increased expression leads to a very tight epithelium, increasing transepithelial electrical resistance and decreasing solute permeability of the epithelial monolayer [118]. Previous studies have demonstrated that dietary protein content and amino acids composition, along with probiotics and prebiotics administration, can improve tight junctions' transcription levels in chickens challenged with *Eimeria/Salmonella* spp. or environmental stressors (e.g., heat stress) [121, 229–231]. As already mentioned above, the statistical tendency observed for the lower expression of ZO-1 in T1 group could be due to the higher dosage of ASG received by the birds during their first three days of life, which reduce the damage to the intestinal mucosa thanks to its lower content in ANFs [126]. However, the lack of more pronounced effects recorded in the present study on tight junctions could be attributed to the optimal conditions in which chickens were reared.

10. CONCLUSION

The aim of this PhD project is to assess whether insect live larvae (*Hermetia illucens* and *Tenebrio molitor*) or functional feed (Alphasoy Gold) can have a beneficial effect on gut health of poultry using a multidisciplinary approach including histopathological, histochemical and biomolecular techniques. For this purpose, three experimental trials were carried out. The first two focused on the administration of insect live larvae (HI and TM) in chickens and ducks, respectively. The third trial consisted in the administration of a functional feed, obtained by the enzyme-treatment and thermomechanical co-process of soybean meal and hydrolyzed yeast. Different gut-health related parameters have been evaluated to assess the effects of these innovative feed ingredients in chickens and ducks: gut morphometry, histology, mucin, local immune response, tight junctions and microbiota.

Regarding gut morphology, the unaffected morphometric indices and the preservation of the physiological proximo-distal decreasing gradient of Vh and Cd from duodenum to jejunum/ileum provide positive information about the feasibility of including insect live larvae or the tested functional feed in poultry diet, suggesting that both can preserve a proper intestinal structure and function. Indeed, an adequate nutrient digestion and absorption can be assured, which is crucial for reaching optimal growth performances. These results are supported by the unaffected tight junction transcription levels in Trial 3, confirming the maintenance of an appropriate gut barrier function, which is fundamental both for the digestion process and for the protection against pathogens.

The lack of modification both of mucin's staining intensity and MUC-2 gene expression (Trial 1 and 2) can be also considered a positive outcome, suggesting that insect live larvae did not impair the integrity of the mucous layer, ensuring an adequate protection of the gut mucosa from the potential pathogens that can inhabit the lumen. Moreover, the lower MUC-2 transcription level recorded in T1 group in Trial 3 suggests that the thermal processing of soybean meal in chicks along with the combination with β-glucan can improve diet digestibility, reducing intestinal inflammation and the need of mucous synthesis from goblet cells, making it a suitable option to increase growth performances in young birds.

Similarly, the reduction of pro-inflammatory cytokine IL-2 (Trial 1) and the increase of cytokine IL-6 (Trial 2) in TM group suggest that TM live larvae can positively modulate the local immune response towards an "anti-inflammatory pattern". Similarly, the reduction of pro-inflammatory cytokines (IL-2, TNF-α and INF-γ) in the duodenum of three-days-old chicks in Trial 3 indicates that β-glucans can exert anti-inflammatory properties in the gut of young birds. Overall, both TM live larvae and the tested functional feed seem to improve disease resistance against pathogens, enabling a low immune status and maximizing nutrients utilization for growth, rather than for the activation of the immune system in basal conditions.

These results are further supported by the lack of significant histopathological alterations in the gut, the liver and the main lymphoid organs related to the dietary treatments in all the three trials, confirming that both insect live larvae and the tested functional feed can guarantee an adequate animal 's health. Particularly, the greater white pulp hyperplasia recorded in Trial 1 and 2 can confirm the immunomodulatory properties of chitin.

The lack of major effects on the gut microbiota composition both in Trial 1 and 2 can be attributed to the low amount of nutrients provided by the HI and TM live larvae. However, the increase of SCFA-producing bacteria (Vicitivillaceae family, *Collinsella*, *Eubacterium* and *Succinatomonas* genera) represent a positive outcome while the increase of *Helicobacter* and *Clostridium* genera must be carefully considered.

In conclusion, the present PhD project provides new insights about the use of insect live larvae and functional feed in poultry nutrition. The results herein obtained suggest that both insects and β-glucans can act as probiotic/prebiotic in poultry farming, showing positive effects on gut health immune response and gut microbiota, without impairing gut morphometry, mucin and tight junctions.

Although the use of insect meal seems to be more promising in intensive poultry farming due to the higher amount of nutrients and chitin, insect live larvae should be taken into consideration for extensive poultry farming as environmental enrichment to increase the welfare of poultry as well as maintain and reinforce their gut health. Also, β-glucans derived from yeast should be considered a valuable prebiotic in poultry nutrition, being able to enhance gut local immune response in the first days post-hatching. However, further studies are needed to deeply investigate the most efficient dosage of chitin and to determine if other insect's components rather than chitin can exert a prebiotic action both in chickens and ducks. Finally, further studies should be performed to evaluate the effects of functional feed combining thermomechanical co-process soybean meal and hydrolyzed yeast on the gut microbiota.

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12. RESEARCH PRODUCTS

12.1 Articles

12.1.1 Articles related to the PhD project

- Bellezza-Oddon S., Biasato I., Imarisio A., Pipan M., Dekleva D., **Colombino E**., Capucchio MT., Meneguz M., Bergagna S., Barbero R., Gariglio M., Dabbou S., Fiorilla E., Gasco L., Schiavone A., Black soldier fly and yellow mealworm live larvae for broiler chickens: Effects on bird performance and health status. J Anim Physiol Anim Nutr. 2021; 105 Suppl 1(Suppl 1):10-18. doi: 10.1111/jpn.13567 (IF=2.718, Q1).
- **Colombino E**., Biasato I., Ferrocino I., Bellezza Oddon S., Caimi C., Gariglio M., Dabbou S., Caramori M., Battisti E., Zanet S., Ferroglio E., Cocolin L., Gasco L., Schiavone A., Capucchio MT. Effect of Insect Live Larvae as Environmental Enrichment on Poultry Gut Health: Gut Mucin Composition, Microbiota and Local Immune Response Evaluation. Animals (Basel). 2021; 27;11(10):2819. doi: 10.3390/ani11102819 (IF=3.232, Q1).
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12.1.2 Articles not related to the PhD project

- **Colombino E**., Ferrocino I., Biasato I., Cocolin LS., Prieto-Botella D., Zduńczyk Z., Jankowski J., Milala J., Kosmala M., Fotschki B., Capucchio MT., Juśkiewicz J. Dried fruit pomace inclusion in poultry diet: growth performance, intestinal morphology and physiology. J Anim Sci Biotechnol 2020; 19;11:63. doi: 10.1186/s40104-020-00464-z.
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- **Colombino E**., Prieto-Botella D., Capucchio MT. Gut Health in Veterinary Medicine: A Bibliometric Analysis of the Literature. Animals (Basel). 2021; 3;11(7):1997. doi: 10.3390/ani11071997.
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- Tran HQ., Prokešová M., Zare M., Gebauer T., Elia AC., **Colombino E**., Ferrocino I., Caimi C., Gai F., Gasco L., Stejskal V. How Does Pikeperch (Sander lucioperca) Respond to Dietary Insect Meal Hermetia illucens? Investigation on Gut Microbiota, Histomorphology, and Antioxidant Biomarkers. Front Mar Sci 2021; 27(8):680942. doi: 10.3389/fmars.2021.680942.
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composition, and histomorphological features. Anim Feed Sci Technol. 2022; 289:115314. doi: 10.1016/j.anifeedsci.2022.115314.

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- Salogni C., Capucchio MT., **Colombino E**., Pozzi P., Pasquali P., Alborali GL. Bacterial polyarthritis in post-weaning pigs in a high-density swine breeding area in Italy. J Vet Diagn Invest 2022; 20:10406387221090903. doi: 10.1177/10406387221090903.
- Masucci M., Capucchio MT., Buttitta R., **Colombino E**., Mignacca AS. Congenital hydrocephalus in three sheep: Clinical, electroencephalographic and pathological features. Vet Med-Czech 2022; 67(01):51– 58. doi: 10.17221/8/2021-VETMED.
- **Colombino E**., Raspa F., Perotti M., Bergero D., Vervuert I., Valle E., Capucchio MT., Gut health of horses: effects of high fibre vs high starch diet on histological and morphometrical parameters. BMC Vet Res 2022; 8;18(1):338. doi: 10.1186/s12917-022-03433-y.

12.2 Congress abstracts and posters

12.2.1 Congress Abstracts and Posters related to the PhD project

- **Colombino E**., Bellezza-Oddon S., Biasato I., Gariglio M., Zanet S., Battisti E., Ferroglio E., Gasco L., Schiavone A., Capucchio MT. Effects of insect live larvae on mucin dynamics in poultry: histochemical and biomolecular investigations. 74° Convegno SISVet-Virtual Edition 23-26th June 2021.
- **Colombino E**., Biasato I., Karimi M., Ton Nu MA., Bellezza-Oddon S., Gariglio M., Bergamino C., Battisti E., Zanet S., Fiorilla E., Gai F., Gasco L., Schiavone A., Capucchio MT. Effects of a functional protein on gut local immune response and morphometrical indices in poultry. 4° Cutting Edge European Society of Veterinary Pathology (ESVP) Virtual Congress, 15-17 September 2021.

12.2.2 Congress Abstracts and Posters not related to the PhD project

- **Colombino E**., Biasato I., Ferrocino I., Dabbou S., Nery J., Soglia D., Gasco L., Cocolin L.S., Capucchio M.T., Schiavone A., Tenebrio molitor meal inclusion in broiler chicken's diet: A multidisciplinary approach to gut health. Symposium on gut health in production of food animals, St. Louis (Missouri, USA), 4-6 November 2019.
- Biasato I., **Colombino E**., Ferrocino I., Dabbou S., Vincenti V., Imarisio A., Schiavone A., Cocolin L.S., Gasco L., Capucchio M.T., Hermetia illucens meal inclusion in piglets: Effects on gut health. Symposium on gut health in production of food animals, St. Louis (Missouri, USA), 4-6 November 2019.
- Capucchio MT., **Colombino E**., Nespro A., Gaviglio C., Mioletti S., Vercelli A., Puccinelli MP., Giai Via M. Necrotizing encephalopathy with elevated urinary organic acids in a British shorthair cat: clinical and pathological features. 74° Convegno SISVet-Virtual Edition 23-26 June 2021.
- Raspa F., **Colombino E**., Capucchio MT., Cavallini D., Vervuert I., Bottero MT., Pattono D., Dalmasso A., Bergero D., Valvassori E., Valle E. Effects of feeding managements on microbial contamination of mesenteric lymph nodes and liver and on intestinal histo-morphology in horses. European Society of Veterinary and Comparative Nutrition (ESVCN) Congress 2021-Virtual edition, 9-11 September 2021.
- **Colombino E**., Zoppi S., Alborali GS., Bonvegna M., Caruso C., Cucco I., Mioletti S., Sona B., Tarantola M., Tomassone L., Tursi M., Vercelli C., Dondo A., Capucchio MT. Bacterial and viral pathogens in swine of north-west Italy. 4° Cutting Edge European Society of Veterinary Pathology (ESVP) Virtual Congress, 15-17 September 2021.
- **Colombino E**., Perotti M., Bellezza-Oddon S., Biasato I., Bongiorno V., Gariglio M., Schiavone A., Renna M., Gasco L., Capucchio MT., Zaniboni L. Effects of Hermetia illucens live larvae as environmental enrichment on poultry gut health. Insect to feed the World, Quebec City (Canada) 12-16 June 2022.
- **Colombino E**., Prandi I., Quaranta G., Mauthe von Degerfeld M., Capucchio MT. Main causes of death in insectivorous bats in Turin province. 75° Convegno SISVet, Lodi (Bergamo, Italy), 15-18 June 2022.
- Cucco I., **Colombino E**., Perotti M., Zoppi S., Alborali GL, Bonvegna M., Caruso C., Mannelli A., Mioletti S., Sona B., Tarantola M., Tomassone L., Tursi M., Vercelli C., Dondo A., Capucchio MT. Main causes of death in pig farms in piedmont: anatomo-histopathological and microbiological investigations. XLVII Meeting Annuale Società Italiana di Patologia ed Allevamento dei Suini, Lazise (Verona, Italy) 9-10 June 2022.

- **Colombino E**., Perotti M., Bellezza-Oddon S., Biasato I., Bongiorno V., Gariglio M., Schiavone A., Renna M., Gasco L., Capucchio MT., Zaniboni L. Effects of Hermetia illucens live larvae as environmental enrichment on poultry gut health. Insects to feed the world, Québec city (Canada), 12-16 June 2022.
- Mignacca SA., Capucchio MT., Bianco C., Garcia-Ara A., **Colombino E**., Lingual fossa ulcers in bovine tongue: gross and histological features. Annual European Society of Veterinary Pathology/European College of Veterinary Pathology 2022, Athens, 7-10 September 2022.
- Mignacca SA., **Colombino E**., Capucchio MT. Intrapancreatic accessory spleen in slaughtered pigs: investigations on occurrence and histomorphology. Annual European Society of Veterinary Pathology/European College of Veterinary Pathology 2022, Athens, 7-10 September 2022.
- **Colombino E**., Biasato I., Bongiorno V., Gai F., Zambotto V., Gariglio M., Prai F., Cardello M., Gasco L., Schiavone A., Capucchio, MT. Can black soldier fly live larvae supplementation modify gut histomorphology of organic chickens? Insecta 2022, Gießen (Germany), 14-16 September 2022
- **Colombino E**., Lelli D., Quaranta G., Leopardi S., Guidetti C., Robetto S., De Benedictis P., Orusa R., Mauthe Von Degerfeld M., Capucchio MT. Bat's surveillance: bat health vs human health. V Congresso Nazionale di Ecopatologia della Fauna, Udine, 14-17 September 2022.
- Prandi I., **Colombino E**., Perotti M., Cardello M., Vacchetta M., Bertolotti L., Tomassone L., Mauthe Von Degerfeld M., Quaranta G., Capucchio MT. Causes of admission, mortality and gross pathology in european hedgehogs in piedmont region: preliminary data. V Congresso Nazionale di Ecopatologia della Fauna, Udine, 14-17 September 2022.

12.3 Congresses and Seminars

- Symposium on gut health in production of food animals, St. Louis (Missouri, USA), 4-6 November 2019.
- Gross Pathology Review Course, Davis-Thompson Foundation, 17-21 May 2021
- Virtual Descriptive Veterinary Pathology Course, Davis-Thompson Foundation, 21-25 Junio 2021
- 74° Convegno SISVet-Virtual Edition 23-26 June 2021.
- Resident's Day "Skills in scientific communication", 4° Cutting Edge European Society of Veterinary Pathology (ESVP) Virtual Congress, 15-17 September 2021.
- 4° Cutting Edge European Society of Veterinary Pathology (ESVP) Virtual Congress, 15-17 September 2021.

13. ACTIVITIES

13.1 Stay Abroad

4th of October, 2021- 14 th of November, 2021, Training in necropsy and related-microbiological and histological investigations under the supervision of Prof. Luis Gómez Gordo- Department of Animal Pathology, Veterinary Faculty of Cáceres, University of Extremadura (Spain).

15th of November, 2021- 22nd of December, 2021, Training in necropsy and biopsy under the supervision of Prof. Lluís Luján - Department of Animal Pathology, Veterinary Faculty of Zaragoza, University of Zaragoza (Spain).

10th of January, 2022- 25th of February 2022, Training in confocal microscopy and immunofluorescence under the supervision of Prof. Eduardo de Puelles Martínez de la Torre - Instituto de Neurociencias-CSIC, Miguel Hernandez University, Alicante (Spain).