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SUSTAVIANFEED

ALTERNATIVE ANIMAL FEEDS IN MEDITERRANEAN POULTRY BREEDS TO OBTAIN SUSTAINABLE PRODUCTS

Animal welfare and animal health evaluation

DELIVERABLE 3.3

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Partners

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Overall Summary

The document D3.3 Animal Welfare and Animal Health Evaluation is part of a European Union-funded project under the PRIMA program, which focuses on the use of alternative protein sources for sustainable poultry feed. This report examines the impact of Black Soldier Fly Larvae (BSFL) on poultry nutrition and welfare through studies conducted by three partner institutions: the University of Turin (UNITO), the University of Murcia (UMU), and EGE University (EGE).

The primary objective of the study was to assess the effects of BSFL supplementation in poultry diets on growth, behavior, welfare, and stress, with a particular focus on organic and low-impact farming systems. Each institution contributed with different approaches to this research. The University of Turin investigated how BSFL affected slow-growing chickens raised in organic systems, focusing on growth performance, behavior, welfare, and stress levels. Their findings suggested that live larvae provided greater enrichment benefits compared to dried larvae, promoting exploratory behavior and reducing aggression among birds.

At the University of Murcia, researchers studied laying hens raised in Mediterranean conditions and compared a conventional diet with two alternative diets: one plant-based and one supplemented with BSFL. The results indicated that alternative diets did not negatively impact the hens' welfare, demonstrating their viability as a sustainable feeding strategy. Meanwhile, EGE University focused on both local and commercial chicken breeds, particularly analyzing gut health and microbiota. Their study revealed that BSFL supplementation improved natural foraging behavior while also positively influencing the intestinal flora and immune response of the birds.

The findings highlighted the potential of BSFL to enhance poultry welfare and reduce stress. Behavioral observations showed that chickens receiving BSFL, particularly live larvae, exhibited lower levels of aggression and engaged in more exploratory behaviors. Stress indicators, such as cortisol metabolites from feces and feathers, confirmed that BSFL supplementation led to a reduction in chronic stress levels. In terms of sustainability, the use of BSFL as an alternative protein source aligns with circular agricultural principles. These larvae can be farmed on organic waste, reducing reliance on soybean meal and minimizing the environmental impact of poultry production.

Overall, the research suggests that BSFL can serve as an effective and sustainable alternative protein source, particularly for organic and free-range poultry systems. However, further studies are necessary to optimize its application and evaluate the long-term effects on poultry health and productivity.

Introduction

The study of the effects of Black Soldier Fly Larvae (BSFL) on poultry nutrition and welfare has gained significant attention due to the potential benefits of incorporating alternative, sustainable protein sources in animal feed. With the increasing need to reduce the environmental footprint of animal production, innovative approaches to nutrition and welfare are critical. This report presents the combined findings from four research institutions: the University of Turin (UNITO), the University of Murcia (UMU), and EGE University (EGE) and and InstitutSupérieurAgronomique de Chott Mariem (ISA-CM) each of which contributed to a broader understanding of how BSFL supplementation impacts the welfare, behavior, and physiological parameters of slow-growing and commercial chicken breeds.

The focus of the studies conducted by the three partners included growth performance, behavior, stress responses, and overall welfare in various rearing environments. The inclusion of BSFL, both live and dehydrated, as a protein source in poultry diets provides insight into the effectiveness of insects as feed, aligning with the goals of organic farming and sustainability. Each partner brought a unique approach to the research, adding valuable data to the overall findings, particularly regarding animal welfare, stress markers, and growth performance in alternative farming systems.

Objectives of the Study

The primary objective of the combined study was to explore the effects of BSFL supplementation on poultry nutrition and welfare across different rearing environments and breeds. Specifically, the research aimed to determine:

1. The impact of BSFL on growth performance, behavior, and welfare of slow-growing and commercial breeds.
2. How BSFL supplementation influences stress responses, physiological health, and overall well-being.
3. The sustainability and practicality of incorporating BSFL in poultry diets, particularly in organic and low-impact farming systems.

Contributions of Each Partner

University of Turin (UNITO)

UNITO focused on the effects of BSFL supplementation on slow-growing meat-type chicken breeds under organic farming conditions. The study at UNITO aimed to assess

growth performance, behavior, and welfare, emphasizing the enrichment value of live and dehydrated larvae. Chickens were supplemented with a 4% inclusion of either live or dehydrated BSFL, with behavioral observations and welfare assessments made at several stages of growth. The study highlighted that live larvae, due to their motility, provided more significant enrichment benefits compared to dehydrated larvae. The focus on organic farming practices is essential, as organic systems require natural and sustainable feeding approaches to ensure both productivity and welfare are maintained. UNITO's findings underscored the potential of BSFL to promote exploratory behavior, reduce aggression, and enhance the overall well-being of chickens in a more sustainable and eco-friendly manner.

University of Murcia (UMU)

A parallel study was conducted by UMU in Spain, focusing on the welfare, physiological health, stress markers and digestive status of hens, comparing three diets: one with inclusion of usual ingredients, and two alternative diets in which soybean meal was partially substituted: one with alternative ingredients of plant origin, and one supplemented with BSFL. The study at UMU used a Mediterranean-adapted breed of laying hens and assessed the impact of these alternative diets on their welfare parameters, including the absence of injuries, diseases and the appropriateness of behaviors such as pecking. In addition, the UMU study incorporated welfare assessments such as keel bone deformation, footpad dermatitis and tonic immobility tests, providing a comprehensive analysis of the physical and emotional well-being of the birds. In addition, other indicators were studied, such as: behavior by video recording, feather corticosterone, biochemical and hematological variables. Digestibility, tissue histomorphometry and organ histopathology studies were also carried out. The results of UMU demonstrated that the alternative diets were suitable for maintaining optimal hen welfare, without affecting any of the parameters studied.

EGE University (EGE)

EGE University's contribution to the study focused on both local and commercial meat-type chicken breeds, examining a range of behaviors, welfare parameters, and physiological health indicators. EGE's research extended to microbial analysis, providing insights into the gut health of chickens supplemented with BSFL. This component of the research is particularly relevant in understanding the long-term effects of BSFL on the immune system and gut microbiota. EGE's findings revealed that BSFL supplementation had a positive impact on behavior, with birds exhibiting more

natural foraging activities, reduced aggression, and better health outcomes. The inclusion of microbiota and histomorphological analysis provided a unique dimension to the study, indicating that BSFL may also enhance gut health and disease resistance in poultry.

ISA-CM

ISA-CM (Institut Supérieur Agronomique de Chott Mariem) played a key role in evaluating the impact of alternative and insect-based diets on the welfare and behavior of Mediterranean poultry breeds, particularly laying hens. Their study focused on comparing three dietary treatments: a conventional control diet, a plant-based alternative diet, and a diet enriched with 5% dried Black Soldier Fly Larvae (BSFL). The main goal was to assess whether these innovative feed strategies could maintain or enhance poultry welfare without compromising health or productivity.

The ISA-CM team conducted extensive welfare assessments using adapted Welfare Quality® protocols. These included physical checks for injuries (keel bone deformation, footpad dermatitis, and plumage condition), evaluation of disease symptoms, behavioral analysis through video recordings, corticosterone levels in feathers as a stress biomarker, and physiological evaluations (hematological and biochemical profiles). Importantly, behavioral observations were recorded over 15 weeks to assess feeding, comfort, social interactions, movement, and nesting activity across different times of day.

Findings showed that neither the alternative plant-based nor the BSFL-supplemented diets negatively affected hen welfare. On the contrary, in some cases, insect-fed hens showed improved indicators—for example, fewer comb abnormalities and efficient insect consumption over time. Corticosterone levels, both in primary and interscapular feathers, did not differ significantly between treatments, indicating that stress levels remained low across all groups. Additionally, the hens adapted quickly to BSFL inclusion, with decreasing larvae consumption times over the weeks, suggesting increasing acceptance and palatability.

ISA-CM's results contribute important evidence that BSFL can be integrated into laying hen diets without adverse effects on welfare or stress, supporting the feasibility of using insects as a sustainable protein source in Mediterranean poultry systems. This aligns with the broader goals of the PRIMA project: promoting environmental sustainability, animal well-being, and innovative feed solutions in traditional farming contexts.

Relevance of BSFL as a Sustainable Protein Source

In light of the increasing demand for sustainable agricultural practices, BSFL offer a highly efficient solution to the challenge of providing alternative protein sources. BSFL can be farmed on organic waste, converting low-value substrates into high-quality protein. This makes them an attractive option not only for their nutritional value but also for their role in reducing waste and enhancing the sustainability of farming systems. The integration of BSFL in poultry diets aligns with the principles of circular economy and eco-friendly agriculture, as they require minimal land, water, and resources compared to traditional protein sources like soy and fishmeal.

Additionally, BSFL supplementation provides environmental enrichment, particularly when live larvae are used, simulating natural foraging behaviors in chickens. This enrichment is particularly beneficial for slow-growing breeds and organic farming systems, where animal welfare is a critical concern. The motility and sensory engagement provided by live BSFL can reduce stress and improve the overall well-being of poultry, making them a valuable tool in improving both productivity and welfare outcomes in organic and free-range systems.

UNITO

Introduction

This report presents the findings of a study conducted at the University of Turin to investigate the effects of dietary supplementation with live and dehydrated Black Soldier Fly Larvae (BSFL) on slow-growing chicken breeds. BSFL are known for their high nutritional value, and the objective of the study was to determine whether they could serve as a viable alternative protein source, particularly in organic farming systems where growth performance, animal welfare, and environmental enrichment are key considerations.

The focus of the research was to assess various parameters including the birds' growth performance, behavioral patterns, stress indicators, and overall welfare conditions. The study was carried out under controlled organic farming conditions, and comparisons were made between birds fed a basal diet, birds supplemented with live BSFL, and birds supplemented with dehydrated BSFL. This research is particularly important because it addresses the growing need for sustainable protein sources in poultry production and investigates how these dietary supplements might enhance the welfare of slow-growing chicken breeds, which are often more reactive and responsive to environmental stimuli than fast-growing strains.

Materials and Methods

Animals and Experimental Design

The experiment was conducted at the research facility of the University of Turin, following approval by its Bioethical Committee (Protocol n°814715). A total of 144 male chickens, from fertilized eggs obtained at the university's avian conservation facility, were used in the study. These eggs were incubated, and after sexing, only males were selected. Organic farming practices were adhered to throughout the trial.

Chickens were raised in environmentally controlled brooders until they reached 38 days of age. At this point, the birds were selected based on their average live weight, with 144 birds distributed into 18 pens (8 birds per pen). The pens were equipped with rice husk litter, and the birds were allowed access to outdoor spaces from 63 days of

age. Housing conditions included natural ventilation and lighting, with regular monitoring of environmental temperatures.
The study employed three dietary treatments: a control group (C) fed a basal diet, a group (D-BSFL) supplemented with 4% dehydrated BSFL, and a group (L-BSFL) supplemented with 4% live BSFL. The trial lasted for 108 days, from May to October 2022, covering the summer to early autumn season. Growth performance, behavioral observations, and welfare parameters were monitored throughout the trial.

2.1 Behavioral Observations

Behavioral observations were made to assess the impact of BSFL supplementation on the birds' activities. The behaviors were categorized into two macro-categories: exploratory behaviors and agonistic behaviors. Video recordings were analyzed using the Behavioral Observation Research Interactive Software (BORIS), focusing on specific behaviors such as ground pecking, scratching, aggressive pecking, and fighting. The observations were conducted at three different time points: T1 (52 days), T2 (94 days), and T3 (136 days).

Table 1. Ethogram for evaluating the effects on behavior of feeding a slow growing autochthonous chicken breed dehydrated and live black soldier fly larva

Macro-category	Category		
Agonistic behaviors	Wing flap	Bird flaps wings < 0.5 m in front of other birds, regardless the body orientation of the other bird and the intensity of the flapping activity.	
	Raised hackle	Body horizontal, head towards opponent, hackles of one (count as 1) or both (count as 2) raised.	Katajamaa et al., 2018
	Chasing	Bird follows another bird, both running.	Katajamaa et al., 2018
	Aggressive peck	Bird moves swiftly towards opponent miming to giving or gives aggressive peck. Head over opponent.	Katajamaa et al., 2018
	Sparring/fighting	Bird involved in aggressive encounter. Peck/peck attempt, both	Katajamaa et al., 2018

		birds active, running, jumping, or flying.	
Indoor exploration	Plate/larvae exploration	Foraging behavior larvae/plate related (when larvae are not provided) (within 1 bird length from the plate, regardless bird orientation). Walking or standing, while approaching/head close to ground/plate, eyes focusing on ground/plate items, pecking at the ground/plate, moving litter backwards by means of the claws.	
	Free pen exploration (Ground pecking/+scratching/+ object pecking)	Foraging activity NOT larvae/plate related (>1 bird length from the plate). Walking or standing, body axes with head towards/on the ground, moving litter backwards by means of the claws	Katajamaa et al., 2018)
Outdoor exploration	-	Explore the outdoor area (bird completely outside, thus not visible from the inside anymore)	

Welfare and Stress Parameters

Welfare was assessed using animal-based parameters including plumage condition, skin integrity, and leg health. Stress levels were evaluated by analyzing excreta corticosterone metabolites (ECM) and heterophil-to-lymphocyte (H/L) ratios. Fear responses were also evaluated using the Tonic Immobility (TI) test and the Avoidance Distance (AD) test, which are standard methods for measuring fear and stress in poultry.

Gut Morphometry

This study was conducted on the Bianca di Saluzzo chicken breed to assess the effects of Black Soldier Fly Larvae (BSFL) supplementation on gut morphology. A total of 144 male chickens were used and assigned to one of three treatment groups: a control group (C) fed a standard basal diet, a group receiving dehydrated larvae (DL), and another fed live larvae (LL). BSFL were included at 5% of expected daily dry matter intake, consistent throughout the rearing period (39–174 days of age).

Birds were slaughtered at two time points (147 and 174 days of age), and gut samples were collected from 12 birds per group per time point (n = 72 in total). The mid-jejunum (section before Meckel's diverticulum) was selected for morphometric evaluation due to its key role in nutrient absorption.

Histological Processing and Morphometric Analysis

Gut samples (~5 cm) were rinsed with 0.9% physiological saline, fixed in 10% buffered formalin, dehydrated, and embedded in paraffin wax. Tissue sections (5 µm thick) were stained with hematoxylin and eosin (H&E) for histological examination.

Microscopy was performed using a Zeiss Axiophot microscope at 2.5x magnification, and images were captured with a Nikon DS-Fi1 camera. Morphometric measurements were carried out using ImageJ software and the Fiji image processing package.

For each bird, 10 intact and well-oriented villi and 10 corresponding crypts were analyzed. The following metrics were assessed:

- Villus height (VH): distance from the villus tip to the crypt junction.
- Crypt depth (CD): depth from base of villus to submucosa.
- Villus height/crypt depth ratio (VH/CD).
- Villus width (VW) and villus surface area (VA) using the formula:

$VA = (2\pi \times VW/2) \times VH$.

- Mucosae and muscularis layer thicknesses were also measured.

Statistical Analysis

A general linear model (GLM) was used for statistical analysis. The effects of diet (C, DL, LL), age (147, 174 days), and their interaction were tested. Differences were considered significant at $p < 0.05$. All data are presented as least square means ± SEM.

Microbiota

Sample Collection and DNA Extraction

At 174 days of age, caecal contents were collected from 48 Bianca di Saluzzo chickens across three dietary groups: control (C), dehydrated larvae (DL), and live larvae (LL). All samples were collected using sterile instruments and immediately stored at −80 °C to preserve microbial integrity.

Sequencing and Bioinformatics

Microbial DNA was extracted and the 16S rRNA gene was amplified targeting the V3-V4 regions. Amplicons were sequenced using the Illumina MiSeq platform (V2 chemistry, 250-bp paired-end reads). Sequences were processed using QIIME 2. Primer removal was conducted with Cutadapt, denoising with DADA2, and taxonomy assignment was performed using the SILVA reference database. Amplicon Sequence Variants (ASVs) with low abundance (fewer than five reads in two or more samples) were excluded to ensure data quality.

Statistical Analysis

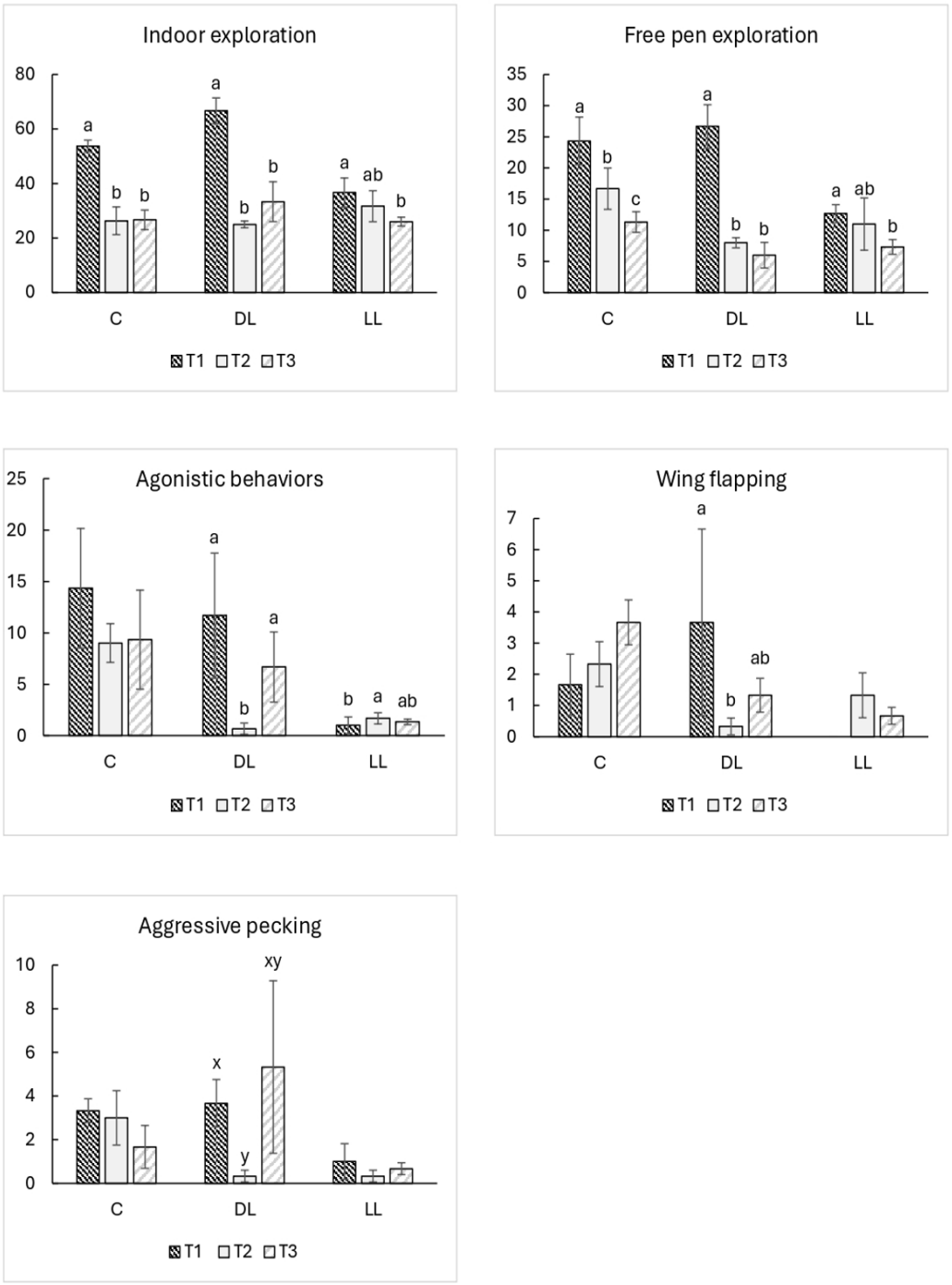
Microbiota alpha-diversity indices (Shannon, Simpson, Chao1) were calculated using the vegan package in R. Spearman correlation was performed to assess associations between microbial ASVs and volatile fatty acids (VFAs), and plotted using corrplot. Statistical significance was considered at $p < 0.05$ with FDR correction.

Results

Behavioral Observations

Exploratory behaviors, such as ground pecking and scratching, were significantly influenced by time and diet. Birds in the DL group exhibited a higher frequency of indoor exploratory behaviors compared to the LL and control groups, especially during the early phases of the trial. However, overall exploration decreased as the birds aged. Agonistic behaviors, including aggressive pecking and chasing, were more frequent in the control group than in the BSFL-supplemented groups. LL birds consistently showed the lowest levels of aggression (Fig 1).

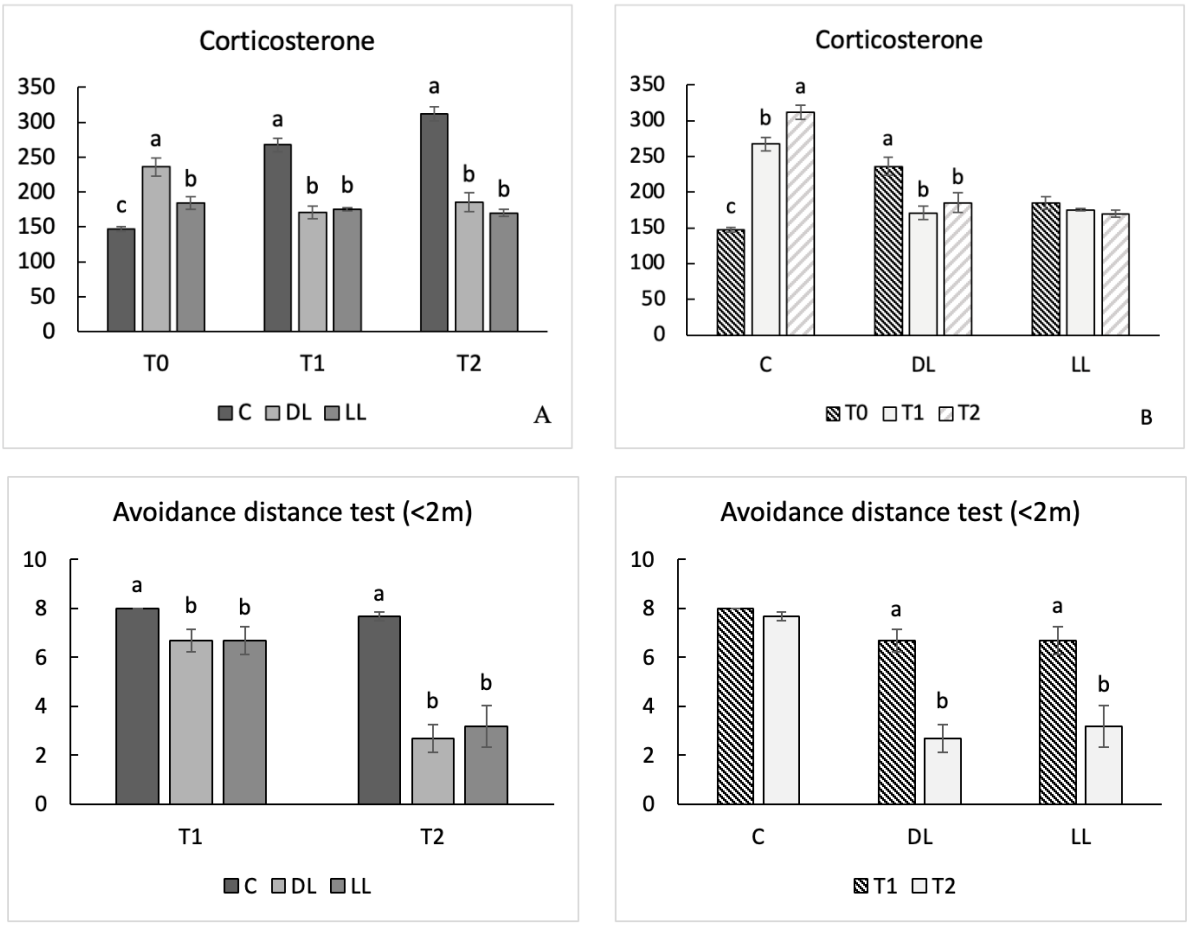
Figure 1. Effects on behavior of feeding a slow growing autochthonous chicken breed dehydrated and live black soldier fly larva, including enrichment treatment (E), time (T), and their interactions (E×T) (n=3).



Welfare and Stress Parameters

The analysis of welfare parameters revealed no significant differences in physical condition between the groups. All birds displayed good feather quality, skin integrity, and leg health, suggesting that BSFL supplementation did not adversely affect their welfare. However, ECM analysis revealed that stress levels were significantly higher in the control group compared to the BSFL-supplemented birds, with the LL group showing the lowest levels of corticosterone. The AD test indicated that birds in the LL and DL groups were less fearful of humans than those in the control group, suggesting a positive effect of BSFL on reducing fear responses (Fig 2.).

Figure 2. Corticosterone and avoidance distance test results



Gut Morphometry

The morphometric analysis (Table 3 in the source) revealed:

- No significant differences in villus height, crypt depth, or villus area among the dietary groups (C, DL, LL).
- Age effect was observed in villus height and height-to-crypt depth ratio, with older birds (174 days) displaying taller villi and a higher ratio than at 147 days ($p < 0.05$).
- Mucosal and muscularis thickness remained statistically unchanged across all groups.
- Histopathological scoring of the intestines showed no dietary influence on inflammation or degenerative changes, with all groups averaging mild scores (around 1.1–1.2 out of 3).

Jejunal Morphometry

The morphometric parameters across diets and age groups are summarized in Table 3. Overall:

- Villus height showed a significant increase with age ($p = 0.004$), averaging 0.887 mm at 174 days versus 0.747 mm at 147 days.
- The VH/CD ratio also increased significantly with age ($p = 0.008$), indicating enhanced absorptive potential.
- No dietary effect was observed for villus height, crypt depth, or their ratio across C, DL, and LL groups.
- Villus area, mucosal thickness, and muscularis layer width showed no significant variation among treatments.
- Histopathological scores for gut tissue ranged between 1.1 and 1.2, indicating mild changes, with no significant differences attributable to diet or age ($p > 0.05$).

Microbiota

Microbial Diversity and Composition

Analysis revealed no significant differences in overall microbiota alpha-diversity among the three dietary treatments, suggesting similar richness and evenness of microbial communities.

Differential Taxa

However, specific taxonomic changes were observed:

- Negativibacillus abundance was significantly increased in both DL and LL groups compared to the control.
- Faecalibacterium, a known SCFA-producing genus, was significantly more abundant in the LL group than in C or DL.

Correlation with Volatile Fatty Acids

A strong positive correlation was found between Negativibacillus and propionic acid concentration. Additionally, butyric acid positively correlated with the thrombogenicity index (TI), while lactic acid showed a negative correlation with TI, suggesting potential impacts on host health and meat quality.

Discussion

The findings of this study indicate that Black Soldier Fly Larvae (BSFL), both live and dehydrated, have a noteworthy impact on the behavior, stress levels, and overall welfare of slow-growing chickens. This research aligns with the growing interest in sustainable and natural enrichment strategies in poultry farming, particularly within organic systems where welfare is a primary concern. The use of BSFL offers a dual benefit, providing both nutritional value and environmental enrichment.

Behavioral Impacts of BSFL Supplementation

The behavioral observations revealed that BSFL supplementation, particularly in its live form, encouraged higher levels of exploratory behavior among the chickens. Exploratory behaviors, such as ground pecking and scratching, were more frequent in the DL group compared to the control group. This trend was even more pronounced in the LL group, where the live larvae acted as a strong stimulant for the birds to engage in natural foraging behaviors. These findings suggest that the movement of live larvae may have played a crucial role in triggering innate exploration and feeding behaviors, which are critical to maintaining animal welfare and reducing stress in poultry.

This result is particularly significant in slow-growing breeds, which are typically more responsive to environmental enrichment compared to fast-growing commercial strains. The opportunity to engage with live larvae appears to satisfy their natural instinct to forage, a behavior often restricted in commercial poultry settings. Furthermore, the study demonstrates that live larvae offer more

engagement than dehydrated larvae, likely due to the additional sensory stimulation provided by the movement of live insects. This aligns with the literature suggesting that dynamic, interactive enrichment sources yield better welfare outcomes in livestock species.

The agonistic behaviors, such as aggressive pecking and chasing, were significantly lower in the BSFL-supplemented groups, with the LL group exhibiting the lowest levels of aggression. This reduction in aggressive behaviors may be attributed to the enrichment provided by BSFL, which diverts the birds' attention from negative social interactions toward the larvae. It is well-known that chickens, especially in confinement, can exhibit aggressive tendencies, leading to welfare concerns like feather pecking. The use of BSFL, therefore, shows potential as a mitigation strategy against such behaviors by promoting positive social dynamics within flocks.

Stress Reduction and Welfare Implications

In addition to behavioral improvements, BSFL supplementation also had a significant effect on stress reduction. The corticosterone metabolite analysis (ECM), which serves as a reliable indicator of chronic stress in chickens, revealed that birds supplemented with BSFL had lower stress levels compared to the control group. This was particularly evident in the LL group, where stress levels were the lowest, suggesting that live larvae not only provide physical enrichment but may also help mitigate the effects of stressors in the rearing environment.

The results from the Avoidance Distance (AD) test support these findings. Birds in both the LL and DL groups showed less fear toward humans, an important indicator of improved welfare. The reduced fear responses can be linked to the positive reinforcement associated with BSFL supplementation, where the provision of larvae could have led to a more favorable perception of human interaction. This aspect of BSFL supplementation is especially valuable in organic farming systems, where animal-human interactions are more frequent due to manual management practices. Enhanced human-animal relationships foster improved welfare and reduce the negative impacts of handling on birds.

Interestingly, the Tonic Immobility (TI) test, which measures the bird's response to a simulated predator attack, did not show significant differences between the groups. This suggests that while BSFL supplementation enhances overall welfare and reduces stress linked to environmental conditions, it may not alter the bird's fundamental fear responses to life-threatening stimuli. However, the lower ECM levels and improved AD test results point to the conclusion that BSFL primarily mitigates chronic stress, rather than acute stress reactions.

Comparative Value of Live vs. Dehydrated BSFL

While both live and dehydrated BSFL offered similar benefits in terms of growth performance and stress reduction, live larvae had a slight advantage in promoting natural behaviors and reducing aggression. The greater satisfaction likely associated with live larvae stems from their motility, which simulates a more natural foraging experience compared to dehydrated larvae. The increased engagement and reduced aggression seen in LL groups are evidence that live BSFL serve as a more effective environmental enrichment tool.

However, the use of live larvae also presents practical challenges, including management logistics and the need for live feed handling. Dehydrated larvae, on the other hand, are easier to manage and store, making them a viable alternative for farmers looking for simpler enrichment solutions. Future research should further investigate the long-term implications of using dehydrated BSFL, particularly in terms of the balance between convenience and enrichment value.

Environmental and Sustainability Considerations

From an environmental perspective, the use of BSFL in poultry diets aligns with the principles of sustainability in agriculture. Black Soldier Fly larvae are highly efficient at converting organic waste into high-quality protein, making them an eco-friendly alternative to traditional protein sources like soybean meal. By incorporating BSFL into poultry diets, farmers can reduce the environmental footprint of their operations, particularly in terms of land use, water consumption, and greenhouse gas emissions. Furthermore, the larvae themselves can be sourced from waste streams, contributing to circular agriculture and waste reduction initiatives.

Future Research Directions

While this study provides valuable insights into the benefits of BSFL supplementation, there are several areas that warrant further investigation. First, it would be beneficial to expand the study to include different breeds of chickens and larger sample sizes to confirm the generalizability of the findings. The potential of BSFL to improve welfare in fast-growing broiler chickens, which are more prone to welfare issues like leg disorders and stress, should also be explored.

Additionally, research should examine the long-term effects of BSFL supplementation on gut health and immune function. The chitin content in the

exoskeleton of BSFL has been suggested to have prebiotic properties, which could enhance gut microbiota and improve overall health in poultry. Investigating the impact of BSFL on gut health and disease resistance could provide further justification for their inclusion in poultry diets.

Gut Morphometry

The intestinal morphology of poultry plays a pivotal role in nutrient digestion and absorption, directly influencing feed efficiency, growth performance, and overall health. In this study, the morphometric evaluation of the jejunum in Bianca di Saluzzo chickens revealed that dietary supplementation with Black Soldier Fly Larvae (BSFL), whether in dehydrated (DL) or live (LL) form, did not significantly alter key histomorphometric parameters when compared to a conventional diet. These findings are particularly relevant for slow-growing indigenous breeds, which are increasingly valued in agroecological and free-range systems due to their adaptability and meat quality traits.

The stability observed in villus height, crypt depth, and the villus height-to-crypt depth ratio across treatments indicates that BSFL inclusion does not compromise gut integrity. The jejunal villi, central to nutrient absorption, maintained their structural dimensions regardless of insect supplementation. This suggests that the gut mucosa of chickens can tolerate novel protein sources like BSFL without experiencing morphological atrophy or stress-related degeneration.

Notably, while age influenced gut development—evident in taller villi and an improved VH/CD ratio at 174 days—the absence of diet × age interactions further supports the benign nature of BSFL supplementation. This physiological maturation is consistent with the known ontogenic development of the gut in poultry, where villus height increases with age, enhancing the absorptive surface area to meet higher metabolic demands.

The findings align with previous studies exploring the impact of insect-based feeds on gut histology. For instance, Biasato et al. (2017) and Bovera et al. (2016) reported minimal changes in intestinal morphology in broilers and quails fed insect meals. Similarly, Anas et al. (2024) observed beneficial effects on intestinal villi height and tight junction integrity in laying hens supplemented with BSFL oil, although this effect may be influenced by the specific form of inclusion (oil vs. whole larvae), bird genotype, and duration of feeding.

In contrast, extreme inclusion rates of poorly processed insect meals have been associated with crypt hyperplasia or villus erosion, particularly in younger birds or in combination with high-chitin content. The present study, however, utilized

moderate inclusion levels (5%) and evaluated birds at later developmental stages, factors likely contributing to the homeostasis observed in gut architecture.

The histological scoring of gut tissues, which showed mild and non-significant inflammation across all groups, further supports the intestinal compatibility of BSFL diets. Inflammation or degenerative changes in gut mucosa are often early markers of dietary stress, infection, or immune reactivity. The absence of such lesions implies that BSFL does not elicit adverse immune responses at the gut level, a finding reinforced by the unchanged systemic immune markers (IgM, IgA, IgG, IL-6) measured in the same birds.

This contrasts with some traditional high-protein supplements (e.g., fish meal), which have been linked to gut irritation and immune dysregulation when used excessively or without proper processing. The relatively high digestibility of BSFL proteins, combined with their balanced amino acid profile and functional lipids (e.g., lauric acid), may contribute to maintaining gut immune tolerance and epithelial integrity.

Although the morphometric parameters remained stable across diets, the numerically higher villus area in the LL group suggests a potential trend toward improved absorptive surface area, which could partly explain the enhanced final body weight observed in these birds. Even small increases in villus surface can translate to improved nutrient assimilation efficiency, especially under free-range conditions where feed intake variability is higher.

Importantly, the crypt depth, often used as a proxy for epithelial turnover and regeneration, remained unaffected by diet, suggesting that cell renewal rates were not elevated—a common sign of tissue stress or damage. This reinforces the conclusion that gut homeostasis was preserved under BSFL inclusion.

One potential concern in using whole larvae is the presence of chitin, a structural polysaccharide in insect exoskeletons, which may be poorly digested by monogastric animals. However, chitin can also function as a prebiotic, modulating microbial populations and promoting gut health. In this study, despite the presence of chitin in both DL and LL diets, no signs of mucosal irritation or crypt hyperplasia were detected, indicating either adequate tolerance or adaptive microbial fermentation.

Indeed, the microbiota analysis from this same trial (as reported in the full study) showed a selective increase in Faecalibacterium spp., a known short-chain fatty acid (SCFA) producer, particularly in the LL group. These SCFAs, including butyrate and propionate, are well documented to enhance epithelial cell energy supply, reinforce tight junctions, and reduce inflammation—all of which may contribute to the preservation of gut morphology in BSFL-fed birds.

Implications for One Health and Agroecological Systems

From a broader perspective, the ability to integrate BSFL into poultry diets without disrupting gut health holds immense value in sustainable agriculture. Indigenous breeds like Bianca di Saluzzo are ideally suited for low-input, diversified systems, where locally available, alternative feed resources are essential for resilience. Maintaining gut health is a cornerstone of disease resistance, nutrient efficiency, and welfare, all of which are critical within the One Health framework, which emphasizes the interconnectedness of animal, human, and environmental health. The fact that BSFL can be reared on organic waste streams adds an additional layer of ecological circularity, closing nutrient loops and reducing feed-food competition.
While the current findings confirm the short- to medium-term safety of BSFL inclusion from a gut morphometry perspective, longer-term studies are warranted to assess potential cumulative effects, especially under field conditions. Furthermore, the dose-response relationship between BSFL levels and intestinal adaptation remains underexplored, and studies integrating functional gene expression, gut barrier protein assays, and microbiota metabolites (e.g., SCFA quantification) would provide mechanistic insights.
Finally, the potential synergistic effects between BSFL and phytogenic feed additives, probiotics, or enzymes (e.g., chitinase) could be explored to further optimize gut function and performance outcomes in both indigenous and commercial poultry lines.

Microbiota

The gut microbiota plays an essential role in the overall health and productivity of poultry, influencing not only digestion and nutrient absorption but also immune regulation, metabolic balance, and even meat quality. In the context of sustainable poultry production, especially for indigenous breeds like the Bianca di Saluzzo, understanding how alternative protein sources—such as black soldier fly larvae (BSFL)—modulate gut microbial ecosystems is both timely and vital.
One of the most interesting findings of this study is that while overall microbial diversity metrics—such as Shannon, Simpson, and Chao1 indices—did not significantly differ among dietary treatments, the composition of the microbiota was clearly influenced by the form of BSFL inclusion, particularly in the live larvae (LL) group. This suggests that the functional dynamics of the microbiota may shift

even when overall richness and evenness remain stable, highlighting a qualitative rather than quantitative impact.

A key taxon that emerged in this context was Faecalibacterium, which was significantly more abundant in the LL group. This genus is widely recognized as a beneficial member of the gut ecosystem, primarily due to its ability to produce butyrate, a short-chain fatty acid (SCFA) known for its anti-inflammatory properties, role in maintaining epithelial integrity, and function as a major energy source for colonocytes. The increased presence of Faecalibacterium in chickens supplemented with live larvae may indicate a positive shift toward a more protective, anti-inflammatory gut environment.

This shift gains further relevance when considered alongside the VFA profile. Although the study did not find significant differences in butyric acid levels across groups, propionic acid concentrations were notably higher in both BSFL-fed groups (DL and LL), with a particularly strong correlation observed with the genus Negativibacillus. Propionic acid is another SCFA with significant metabolic importance. It is absorbed and metabolized by the liver, where it can influence gluconeogenesis, lipid metabolism, and satiety signaling. In the context of poultry, increased propionic acid levels may be linked to improved feed efficiency and weight gain, as was observed in the BSFL-fed groups.

What makes the presence of Negativibacillus especially compelling is that its abundance was higher in both BSFL-supplemented groups, but its positive correlation with propionic acid was strongest in the LL group. This might suggest that the physical form of BSFL—live vs. dehydrated—could influence microbial metabolic activity, perhaps by providing not just nutrients but also bioactive compounds like chitin in a more accessible form.

An equally intriguing aspect of the study is the relationship between VFAs and cardiovascular health indices, particularly the atherogenicity (AI) and thrombogenicity (TI) of chicken meat. The study found a positive correlation between butyric acid and TI, suggesting that higher levels of this SCFA may relate to increased cardiovascular risk indicators in meat. In contrast, lactic acid was negatively correlated with TI, potentially indicating that a gut environment favoring lactic acid production could be associated with healthier meat lipid profiles. While the exact physiological mechanisms behind these associations are still not fully understood, it is plausible that lactic acid bacteria (LAB), which are responsible for lactic acid production, influence fat metabolism or deposition in ways that reduce the prevalence of pro-atherogenic fatty acids.

LAB are also well known for their role in enhancing meat preservation and sensory quality, producing not only acids but also bacteriocins and other

metabolites that improve flavor, texture, and shelf-life. Their presence, promoted perhaps by the control diet, could therefore have both technological and health-related benefits, suggesting a trade-off between gut performance optimization (seen with BSFL diets) and certain consumer-focused meat qualities.

Another point worth noting is the absence of any negative immune or histological impacts associated with microbiota modulation by BSFL. Despite the observed shifts in microbial composition, intestinal histomorphometry, organ histopathology, and systemic immune markers remained stable across all groups. This reinforces the safety profile of BSFL supplementation and suggests that it does not provoke unwanted immune responses or gut inflammation—a crucial finding given the rising consumer interest in animal welfare and meat safety.

Beyond individual taxa, the broader implication of these findings is that BSFL inclusion—particularly in live form—can selectively enrich for microbial populations with functional benefits. This form of targeted microbiota modulation may represent a novel strategy for improving poultry productivity in agroecological systems. It supports a One Health approach, where animal welfare, environmental sustainability, and human health outcomes are intertwined.
From a broader perspective, these results contribute to the growing body of evidence supporting insects as functional feed ingredients, not only from a nutritional standpoint but also as microbiota modulators. While more research is needed to understand the long-term implications and the exact metabolic pathways involved, this study offers promising insights into how BSFL can help shape a healthier gut microbiome, which in turn supports sustainable and ethical poultry farming.

Conclusion

This study highlights the potential benefits of BSFL supplementation in enhancing the welfare and reducing the stress of slow-growing chickens. Both live and dehydrated BSFL resulted in similar overall benefits, though live larvae had a slightly better effect on reducing aggression and increasing exploratory behaviors. The results suggest that BSFL can serve as an effective enrichment strategy, particularly for chickens raised in organic systems. Further research with larger sample sizes is recommended to confirm these findings and explore additional benefits

UMU

INTRODUCTION

Facilities and animals

The study was conducted in the Animal Nutrition Experimental Facilities, located in the Veterinary Teaching Farm in Murcia, Spain. There were 15 floor pens with 8 chickens each. The pen size was 1 meter width and 3-meter length, having a hanging feeder, two water cups and a perch of 140 centimeters in length. Also, all the pens were provided with a nest. 120 Isazul breed hens were used, this breed is original from the South of Spain, adapted to mediterranean conditions. The age of the hens at the start of the experiment was 23 weeks old. The hens were distributed in homogeneous batches, the diets being distributed randomly among the pens.

Experimental diets

Three different diets were used: a control diet with usual ingredients (CON), which had 2730 kcal of AMEn/kg and 16.4% of crude protein, one alternative diet with source vegetables of low impact (ALT), and another alternative + insect diet that included a 5% of dry mater *Hermetia illucens* larvae (BSFL) (ALT+INSECT).

The larvae were placed in steel feeders. In addition, empty steel feeders were placed in the pens with the control and alternative diets, so all treatments had the same management. The hens were fed the experimental diets from weeks 23 to 38. The steel feeders with or without larvae were placed in the pens at 10:00 a.m. every day. The feeders were collected 3 hours later if they finished larval intake. If the larvae had not finished, the feeders were left until they were completely consumed.

WELFARE CONTROLS

The following information will be presented according to their methodology as well as the results obtained with respect to:

- Welfare check-up
- Tonic immobility test
- Behavior video recording
- Corticosterone in feathers
- Biochemical & hematological variables
- Digestibility test, histomorphometry and histopathology of tissues and organ weights

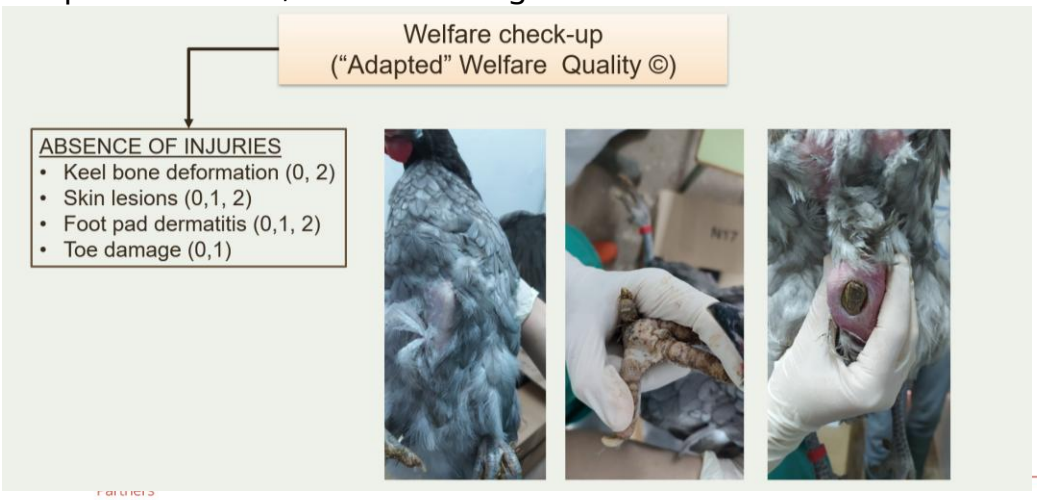
Welfare check-up (Welfare Quality®)

Materials and methods

When the hens were 27, 32 and 38 weeks old, welfare status checks were performed. The welfare check used was an adapted version of the welfare assessment protocols for birds (Welfare Quality®, 2009; Welfare Quality Network, 2019). The 120 hens were individually checked. Different parameters were evaluated: the absence of injuries, the absence of disease, and indicators of appropriate behavior.

For the absence of injuries, the presence of keel bone deformation, skin lesions, footpad dermatitis, and toe damage was evaluated. For each injury and hen, a score of two or three points will be assigned (the hens with the worst injuries had a higher score): keel bone deformation (0, 2), skin lesions (0, 1, 2), foot pad dermatitis (0, 1, 2), toe damage (0, 1) (Figure 2).

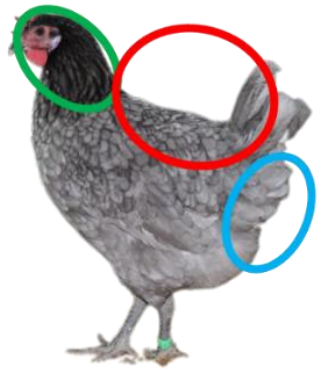
Figure 2. Study of the absence of injuries in keel bone deformation, skin lesions, footpad dermatitis, and toe damage.



To evaluate the absence of diseases, the appearance of enlarged crop, eye pathologies, respiratory infections, enteritis, parasites and comb abnormalities, such as pale comb and blue spots, were evaluated by palpation and observation. The presence of any of these symptoms was noted with a score of 1: enlarged crop (0, 1), eye pathologies (0, 1), respiratory infections (0, 1), enteritis (0, 1), parasites (0, 1) and comb abnormalities (0, 1).

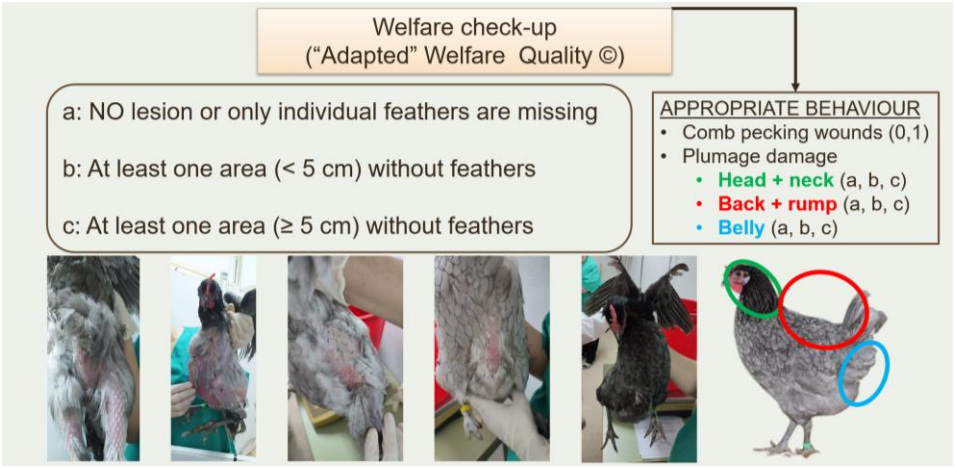
In addition, to study the appropriate behavior the comb peeking wounds, and the plumage damage were assessed. For comb peeking wounds a two-point score was used (0, 1); and for plumage damage, it was studied in 3 areas of the hen. The areas assigned were the head and the neck, the back and rump, and finally the belly (Figure 3).

Figure 3. Areas assigned: the head and the neck (green circle), the back and rump (red circle), and finally the belly (blue circle).



Thus, each area was scored depending on the feather status: “a” score was given to those hens with no lesions or with just a few feathers missing; “b” score was given to those hens with at least one area with without feathers less than 5 cm in diameter; and “c” score was given to those hens with at least one area higher than 5 cm without feathers (Figure 4). To achieve a single general score per bird, the scores of the 3 body parts were combined according to the following classification: 0 points were given to those hens that all body parts have scored ‘a’; 1 point was assigned to the birds with one or more body parts have scored ‘b’, but no body part has scored ‘c’; and 2 points was set when one or more body parts of the hens have scored ‘c’.

Figure 4. Study of appropriate behavior the comb peeking wounds and the plumage damage.



Statistical analysis

The data of Welfare check-up were analyzed using the statistical software SPSS, applying non-parametric test. A Chi-square test was performed on the dichotomous data set, providing that at least 80% of the cells have an expected frequency of 5 or greater, and any cell has an expected frequency smaller than 1. When these premises were not met, Fisher's exact test was applied. For the ordinal data set, the test Kruskal-Wallis was used. Significant differences were assumed when P-value was less than 0.05.

Results

Table 2 presents the results of welfare status related to the grade of injuries, not observing any negative effects between the treatments in any of the control periods studied (27, 32, and 38 weeks of age) ($p > 0.05$). Thus, inclusion of plant-based alternative ingredients or insects did not affect these parameters: keel bone deformation, skin lesions, foot pad dermatitis, and toe damage.

Table 2. Effect of diets on welfare status (grade of injuries) at 27, 32 and 38 weeks of age.

	CON	ALT	ALT+INSECT	Test	P-value
Keel bone deformation (0, 2)					
27 weeks					
0 (%)	97.4	100	97.5		
2 (%)	2.6	0	2.5	Fisher's exact	1.000
32 weeks					
0 (%)	94.9	97.3	97.4		
2 (%)	5.1	2.7	2.6	Fisher's exact	1.000
38 weeks					
0 (%)	100	100	94.7		
2 (%)	0	0	5.3	Fisher's exact	0.328
Skin lesions (0, 1, 2)					
27 weeks					
0 (%)	100	94.9	95		
1 (%)	0	2.6	2.5		
2 (%)	0	2.6	2.5	Kruskal-Wallis	0.608
32 weeks					
0 (%)	89.7	89.2	76.3		
1 (%)	10.3	10.8	21.1		
2 (%)	0	0	2.6	Kruskal-Wallis	0.168
38 weeks					
0 (%)	81.6	68.6	63.2		
1 (%)	10.5	25.7	18.4		
2 (%)	7.9	5.7	18.4	Kruskal-Wallis	0.183
Foot pad dermatitis (0, 1, 2)					
27 weeks					
0 (%)	94.9	97.4	97.5		
1 (%)	5.1	2.6	2.5		

2 (%)	0	0	0	Kruskal-Wallis	0.766
32 weeks					
0 (%)	100	100	100	-	-
1 (%)	0	0	0		
2 (%)	0	0	0		
38 weeks					
0 (%)	100	100	100	-	-
1 (%)	0	0	0		
2 (%)	0	0	0		
Toe damage (0, 1)					
27 weeks					
0 (%)	100	92.3	97.5		
1 (%)	0	7.7	2.5	Fisher's exact	0.222
32 weeks					
0 (%)	100	100	100	-	-
1 (%)	0	0	0		
38 weeks					
0 (%)	100	100	100	-	-
1 (%)	0	0	0		

Control diet (CON); Alternative diet (ALT); Alternative + insect diet (ALT+INSECT).

In Table 3 we can observe that, in general, for any animal of the experiment an alteration related to enlarged crops, eye pathologies, respiratory infections, enteritis or parasites were observed, in any of the periods analyzed (27, 32, and 38 weeks of age). However, comb abnormalities were affected by the dietary treatment ($p<0.05$). Thus, the dietary treatment including insect result in a higher percentage of hens without comb abnormalities (90%) in comparison with the other two groups of hens (71,8% and 66,7% for CON and ALT groups, respectively), during the first period of evaluation (27 weeks of age).

Table 3. Effect of diets of hens on welfare status (absence of disease) at 27, 32 and 38 weeks of age.

	CON	ALT	ALT+INSECT	Test	P-value
Enlarged crops (0,1)					
27 weeks					
0 (%)	100	100	100	-	-
1 (%)	0	0	0		
32 weeks					
0 (%)	100	100	100	-	-
1 (%)	0	0	0		
38 weeks					
0 (%)	100	100	100	-	-
1 (%)	0	0	0		
Eye pathologies (0, 1)					
27 weeks					
0 (%)	100	100	100	-	-
1 (%)	0	0	0		
32 weeks					
0 (%)	100	100	100	-	-
1 (%)	0	0	0		
38 weeks					
0 (%)	100	100	100		
1 (%)	0	0	0		
Respiratory infections (0,1)					
27 weeks					
0 (%)	100	100	100	-	-
1 (%)	0	0	0		
32 weeks					
0 (%)	100	100	100	-	-
1 (%)	0	0	0		
38 weeks					
0 (%)	100	100	100	-	-
1 (%)	0	0	0		
Enteritis (0, 1)					
27 weeks					
0 (%)	100	100	100	-	-

	CON	ALT	ALT+INSECT	Test	P-value
1 (%)	0	0	0		
32 weeks					
0 (%)	100	100	100	-	-
1 (%)	0	0	0		
38 weeks					
0 (%)	100	100	100	-	-
1 (%)	0	0	0		
Parasites (0, 1)					
27 weeks					
0 (%)	100	100	100	-	-
1 (%)	0	0	0		
32 weeks					
0 (%)	100	100	100	-	-
1 (%)	0	0	0		
38 weeks					
0 (%)	100	100	100	-	-
1 (%)	0	0	0		
Comb abnormalities (0, 1)					
27 weeks					
0 (%)	71.8	66.7	90.0		
1 (%)	28.2	33.3	10.0	Chi-square	0.037*
32 weeks					
0 (%)	89.7	91.9	100		
1 (%)	10.3	8.1	0	Fisher's exact	0.126
38 weeks					
0 (%)	100	100	100	-	-
1 (%)	0	0	0		

* Chi-square contrasts paired treatments for comb abnormalities: CON vs ALT, P=0.624; CON vs ALT+INSECT, P= 0.039; and ALT vs ALT+INSECT, P=0.012. Control diet (CON); Alternative diet (ALT); Alternative + insect diet (ALT+INS).

In Table 3, the results of appropriate behavior are presented. There were no statistical differences between the treatments in the parameter studied comb pecking wounds (p>0.05). However, the parameter plumage score was affected

by the diet during the first control period ($p<0.01$), observing the insect supplementation negatively affected the plumage of the animals. However, this situation was reverted in the two following controls conducted (32 and 38 weeks of age), without differences between the treatments ($p>0.05$).

Table 4. Effect of diets of hens on welfare status (appropriate behavior) at 27, 32 and 38 weeks of age.

	CON	ALT	ALT+INS	Test	P-value
Comb pecking wounds (0,1)					
27 weeks					
0 (%)	100	94.9	95.0		
1 (%)	0	5.1	5.0	Fisher's exact	0.544
32 weeks					
0 (%)	97.4	97.3	97.4		
1 (%)	2.6	2.7	2.6	Fisher's exact	1.000
38 weeks					
0 (%)	100	100	94.7		
1 (%)	0	0	5.3	Fisher's exact	0.328
Plumage score (0, 1, 2)					
27 weeks					
0 (%)	48.7	69.2	30.0		
1 (%)	38.5	5.1	32.5		
2 (%)	12.8	25.6	37.5	Kruskal-Wallis	0.009*
32 weeks					
0 (%)	30.8	37.8	18.4		
1 (%)	5.1	16.2	18.4		
2(%)	64.1	45.9	63.2	Kruskal-Wallis	0.194
38 weeks					
0 (%)	28.9	22.2	21.1		
1 (%)	7.9	16.7	7.9		
2 (%)	63.2	61.1	71.1	Kruskal-Wallis	0.690

* U de Mann-Whitney contrasts paired treatments for plumage score: CON vs ALT, P=0.330; CON vs ALT+INSECT, P= 0.018; and ALT vs ALT+INSECT, P=0.006. Control diet (CON); Alternative diet (ALT); Alternative + insect diet (ALT+INSECT).

Conclusions about Welfare check-out

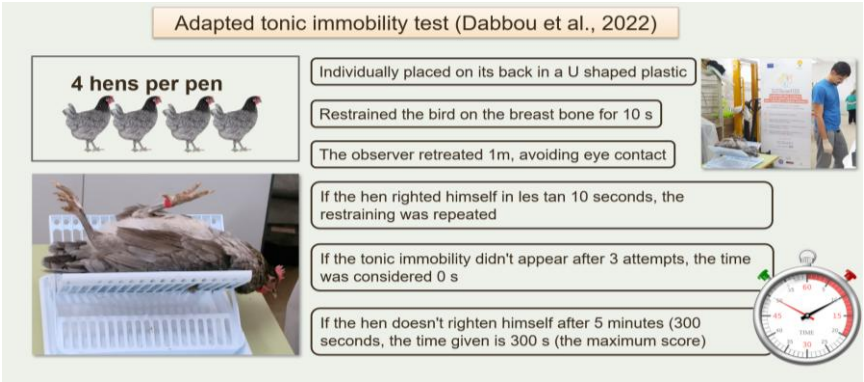
In general, the dietary treatments employed in the experimental animals did not show negative effects in the Welfare check-out of grade of injuries, absence of diseases or appropriate behavior, suggesting both experimental diets (ALT and ALT+INSECT) being appropriated to not negatively influence on these parameters.

Tonic immobility test

Material and methods

When hens were 27, 32 and 38 weeks of age an adapted method of the tonic immobility test of Dabbou et al. (2022) was performed, using 4 hens per pen (Figure 4). This test measures the time it takes the hen to righten itself after inducing the tonic-immobility effect. The longer it lasted the worse welfare status was recorded. The test started placing the hen in its back in a U-shaped bed, restraining the bird on the breastbone for 10 seconds. After that the observer moved back 1 m, avoiding eye contact. If the hen righten itself in less than 10 seconds, it will be placed again in the U-shaped bed. If this happened again after 3 attempts, the time was considered 0 seconds. If the hen does not righten itself after 5 minutes, the time recorded was 300 seconds (the higher score)

Figure 5. Study of tonic immobility using a method adapted from Dabbou et al. (2022).



Statistical analysis

The data of Tonic immobility test were analyzed using the statistical software SPSS, applying non-parametric test. Thus, the data was analyzed using the test Kruskal-Wallis. Significant differences were assumed when P-value was less than 0.05.

Results

Table 5 presents the results of the Tonic immobility test during the experimental period. There were no statistical differences between the groups ($p > 0.05$) in any of the control performed (27, 32 and 38 weeks). Thus, the hens did not show a higher immobility time when fed with alternative plant-based ingredients (ALT vs CON) or when this diet was supplemented with larvae (ALT+INSECT vs CON and ALT vs ALT+INS). A higher period of immobility indicates a higher stress level in the animals, an effect that wasn´t shown in any of the alternative groups in any control period (27, 32 and 38 weeks (Figure 6,7,8, respectively).

Table 5. Effect of diets of hens on Tonic immobility test at 27, 32 and 38 weeks of age. Kruskal-Wallis' test.

	CON	ALT	ALT+INS	P-value
Time (s)				
27 weeks	154.0 (58.50-244.75) *	178.0 (54.75-300.0)	186 (38.25-300.0)	0.892
32 weeks	253.0 (88.50-300.0)	207.0 (108.75-300.0)	237.0 (81.5-300.0)	0.681
38 weeks	200.0 (126.0-300.0)	222.0 (850.0-300.0)	232.0 (147.0-300.0)	0.701

* Median and interquartile range. Control diet (CON); Alternative diet (ALT); Alternative + insect diet (ALT+INSECT).

Figure 6. Box-and-whisker plot of effect of diets of hens on Tonic immobility test at 27 weeks. The box represents the interquartile range (25 and 75), the median line, the X the mean, and the whisker extremes the maximum and minimum. Control diet (CON); Alternative diet (ALT); Alternative + insect diet (ALT+INSECT).

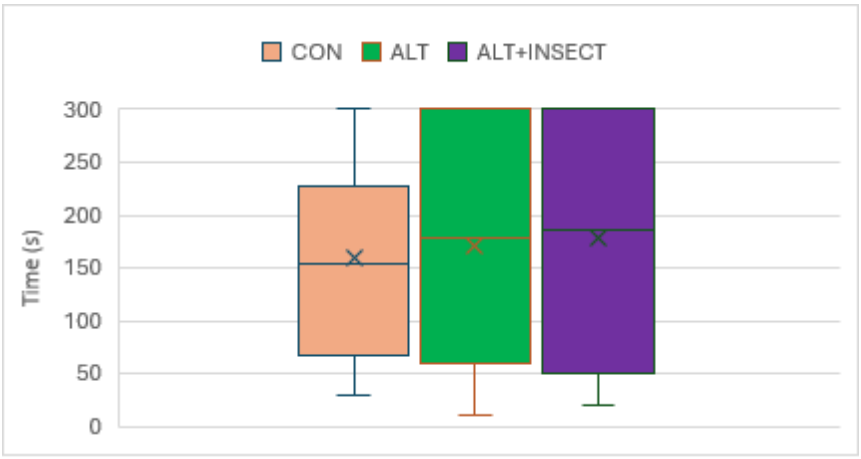


Figure 7 . Box-and-whisker plot of effect of diets of hens on Tonic immobility test at 32 weeks. The box represents the interquartile range (25 and 75), the median line, the X the mean, and the whisker extremes the maximum and minimum. Control diet (CON); Alternative diet (ALT); Alternative + insect diet (ALT+INSECT).

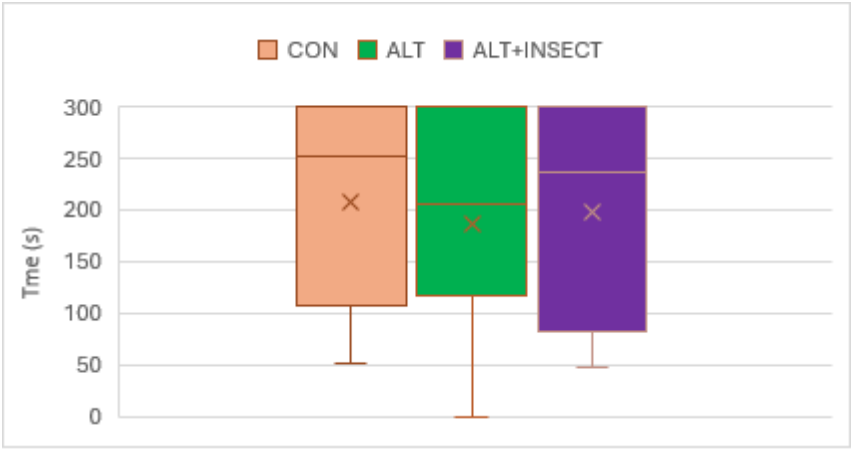
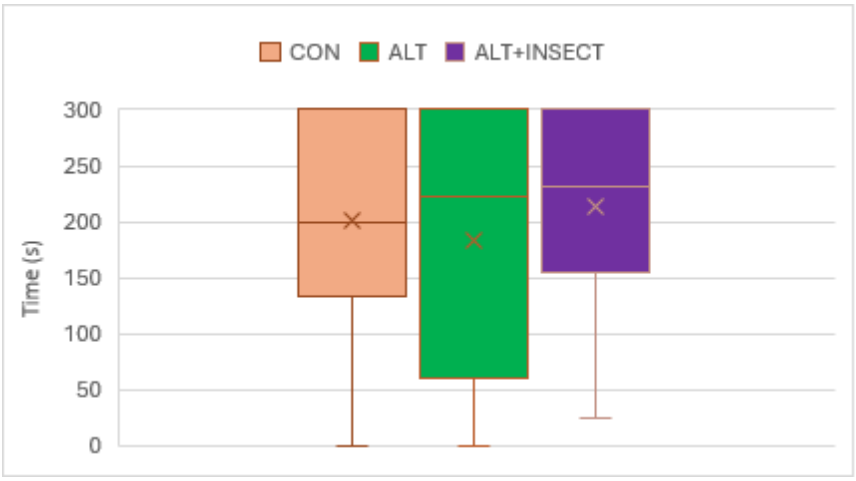


Figure 8. Box-and-whisker plot of effect of diets of hens on Tonic immobility test at 38 weeks. The box represents the interquartile range (25 and 75), the median line, the X the mean, and the whisker extremes the maximum and minimum. Control diet (CON); Alternative diet (ALT); Alternative + insect diet (ALT+INSECT).



Conclusion about immobility test

In conclusion, the dietary treatments used in the experimental hens did not show negative effects in the immobility test. Thus, both experimental diets (ALT and ALT+INSECT) seem to be appropriate since they did not increase the immobility time of the animals during the test performance.

Behavior video recording

Material and methods

Behavioral observations were conducted on one day during weeks 1, 5, 9, 12, and 15 of the experiment. Two cameras (Model CS-TY1-B0-1G2WF 2MP from EZVIZ Inc., USA) were installed in each pen to cover the entire pen area (3 m^2), recording three 10-minute intervals: one within the hour before the larvae/empty feeder placement (09:40–09:50 h), one within an hour after placement (10:40–10:50 h), and one five hours post-placement (15:40–15:50 h). Video streams were recorded in full HD quality (H264-MPEG-4 AVC codec) using EZVIZ PC Studio Software for Windows.

The ethogram was analyzed by a single observer, who quantified the frequency of behaviors (point events) observed during each 10-minute recording period. Behaviors related to feeding, water consumption, movement, socialization, resting, nesting, comfort, and stretching were recorded according to an adapted model from Fiorilla et al. (2024) (Table 5). Additionally, the time taken for the hens

to fully consume the larvae, from the moment of provision to complete consumption, was recorded.

Table 5. Ethogram of behaviors studied as a point even during hen experiment according to adapted proposal of Fiorilla et al. (2024).

Category	Behavior	Description
Feeding and water consumption	Scratching	Move the litter/dirt with claws (Biasato et al., 2022)
	Feeding (feed)	Eating feed from feeders (Jhetam et al., 2022)
	Grass feeding/object pecking	Eating grass in outdoor space/pecking object indoor (Veldkamp and Niekerk, 2019)
	Water	Drink water from the chicken waterer
Movement	Walking	Walking at slow speed (Rieke et al., 2021)
	Running	Running (Veldkamp and Niekerk, 2019)
	Wing flapping	Open wings wide and flapping up and down while walking or running (Liu et al., 2020)
	Perching	Laying hen with 2 feet on a perch for more than 3s, including standing, sitting, and walking (Wei et al., 2020)
	Jumping	Laying hen jumping up or down from the perch. (Wei et al., 2020)
Social	Menacing	Initiate fight with another chicken (Kayla et al., 2023)
	Fighting	Fighting between two or more chickens (Veldkamp and Niekerk, 2019)
	Pecking	Pecking movements directed at a pen mate (McCowan et al., 2006)
	Allo-preening	Social preening (Kenny et al., 2017)
Resting and nesting	Crouch	Sitting down (Webster, 2000)
	Stand	Holding standing position (Veldkamp and Niekerk, 2019)

Category	Behavior	Description
	Nesting	Including standing, sitting, laying, preening in the nest box (Wei et al., 2020)
Comfort and stretching	Sand bath	Rolling or moving around in dust, dry earth, or sand (Grebey et al., 2020)
	Self-preening	Self-feathers grooming by means of beak (McCowan et al., 2006)
	Leg stretching	Putting the leg in a certain position to lengthen and elongate the muscle (Biasato et al., 2022; Carvalho et al., 2022)
	Wing stretching	Putting the wing in a certain position to lengthen and elongate the muscle (Biasato et al., 2022; Carvalho et al., 2022)

Statistical analysis

A General Linear Model (GLM) was applied for the statistical analysis of behavioral data, with data normalization achieved through square root transformations. A three-way fixed-factor linear model was used to assess the effects of diet, time of day, and study week, as well as their interactions. Tukey's post-hoc test was utilized for mean comparisons. For the analysis of insect consumption time, a general linear model considering the week effect was applied. All statistical analyses were conducted using SPSS software, with a P-value <0.05 indicating significant differences.

Results

For the study of insect consumption, the time taken for the hens to consume the total amount was recorded weekly. Significant differences in insect consumption time were observed across the weeks (P<0.05) (Figure 9). The first week showed the highest mean consumption time (174.46 min), while intermediate values were recorded in weeks 5, 9, and 12 (152.66, 31.27 and 27.37 minutes, respectively). The shortest consumption time was registered in week 15, which was significantly

different from week 1 (10.26 versus 174.46 min), indicating an adaptation toward shorter consumption times as the experiment progressed. It is also worth noting that standard deviations were generally high, suggesting that although the insect portion were fully consumed each day, there was considerable variability in consumption rates among replicates.

Figure 9. Weekly insect consumption time during the experiment (mean± standard deviation).

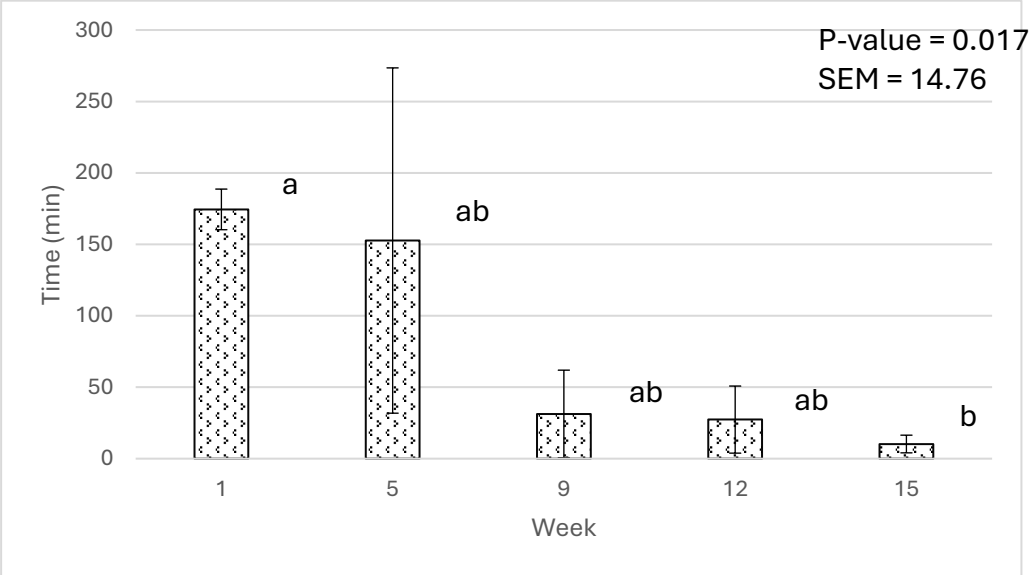


Table 7 presents the behavioral results of the laying hens in the "feeding and water consumption" category, analyzed by dietary treatment, time of day, and week of the experiment. Diet significantly affected ($P<0.01$) the frequency of certain behaviors, including grass/object pecking, and water consumption. The alternative diet and the alternative + insects (ALT+INS) diet showed the lowest percentages of grass/object pecking, with the insect-supplemented diet observing the quantitatively lowest values. Water consumption frequency was higher in hens on the control diet compared to those on the ALT+INS diet. It is important to note that the time spent studying did not significantly affect overall feed consumption frequency ($P>0.05$), although significant effects were observed in several subcategories ($P<0.05$), such as scratching, grass/object pecking, and water consumption. These behaviors generally occurred more frequently during the third observation period.

Regarding the effect of the week studied, differences were found in scratching and grass/object pecking behaviors (P<0.01). Scratching frequency peaked in week 9, showing higher values compared to weeks 1 and 15, with a generally consistent pattern throughout the experiment. Grass/object pecking frequency ranged from 10.17% in week 12 to 14.03% in week 15, with week 12 being significantly lower than the other weeks. In general, no significant interactions were found for the parameters of "feeding and water consumption" (P>0.05), except for time-of-day x week interaction (P<0.05) in relation to grass/object pecking.

Table 8 presents the behavior of laying hens (expressed as a percentage of total observations) in the movement category, analyzed by diet, time of day, and study week. For this subcategory, the dietary treatment did not show any effect on walking, running, wing flapping, or jumping (P>0.05), with the exception of perching (P<0.01). The insect-supplemented treatment resulted in the lowest perching frequency compared to CON and ALT diets. Regarding the time of day, significant differences were observed in the frequency of wing flapping and perching behaviors (P<0.05), although the quantitative differences between time periods were minimal. The effect of the week studied showed significant differences for running and wing flapping (P<0.05). Running frequency was higher in week 1 compared to week 12, with intermediate frequencies observed in the other weeks. Wing flapping reached its highest frequency in the final week of the study. In general, no interactions were found for these parameters (P>0.05), except the treatment x week interaction (P<0.05) for jumping behavior.

The results on the frequency of hens' behaviors in relation to the social activity parameters (menacing, fighting, pecking, and allo-preening), based on diet, time of day evaluated, and week of the experiment, are presented in Table 8. It was observed that none of the factors studied significantly affected these social behaviors (P>0.05), and no interactions were found between them either (P>0.05).

Table 9 presents the behavioral results related to resting and nesting activities, based on dietary treatment, time of day evaluated, and week of the experiment. The diet significantly affected the frequency of standing behavior (P<0.01), with the highest values observed in the insect-supplemented treatment compared to the control, although the alternative diet did not differ from the others. The time of day significantly impacted the frequency of standing and nesting behaviors

(P<0.001), with the lowest frequencies for both observed later in the day. In terms of the week studied, significant differences were found in crouching behavior (P<0.001), with a marked reduction from week 1 to week 5, as the frequency during the first week was five times higher than in week 5. Intermediate values were recorded in the other weeks, suggesting a stabilization of this behavior toward the end of the experiment. In contrast, the frequency of standing behavior did not show a clear trend correlating with the progression of the experiment, despite the variations found, which could be associated with other environmental factors linked to the week studied. Overall, no interactions were found for these parameters (P>0.05), except for the time-of-day x week interaction (P<0.05) for crouching behavior.

The results of the effects of diet, time of day evaluated, and week of the experiment on the frequency of hens' behaviors in relation to the comfort and stretching category (sand bathing, self-preening, leg stretching, and wing stretching) are presented in Table 10. None of the behaviors in this category were affected by the diet (P>0.05). Regarding the time of day, only the frequency of sand bathing was significantly affected (P<0.01), with higher frequencies observed during the third period compared to the start of the day, while the middle time period showed intermediate frequencies. Additionally, the week studied only affected the frequency of sand bathing (P<0.05); however, these changes between weeks did not indicate a clear effect related to the duration of the experiment, suggesting that other environmental factors specific to the week may have played a role. Additionally, a significant interaction between time-of-day x week was found (P<0.05) solely for sand bathing.

Table 7. Behavior of laying hens (frequency as a percentage of total observations) in relation to the feeding and water consumption category, based on dietary treatment, time of day evaluated, and week of the experiment.

	Treatment (Tr)[1]			**Time (Ti)[2]**			**Week (W)[3]**					**SEM[4]**	**P-value**						
	CON	**ALT**	**ALT+ INS**	**1**	**2**	**3**	**W1**	**W5**	**W9**	**W12**	**W15**		**Tr**	**Ti**	**W**	**TrxTi**	**TrxW**	**Tix W**	**TrxTixW**
Scratching	1.49	1.16	0.905	0.345 b	1.07 b	2.14 a	0.665 b	1.07 ab	2.25 a	1.09 ab	0.84 b	0.188	0.207	0.000	0.003	0.123	0.241	0.289	0.753
Feeding feed	6.82	6.32	5.37	6.03	5.92	6.56	6.38	5.99	5.93	7.30	5.24	0.319	0.198	0.379	0.457	0.378	0.727	0.507	0.166
Grass/Object pecking	14.76 a	12.34 b	10.92 b	10.27 b	11.61b	16.14a	12.88 a	13.16a	13.14a	10.17b	14.03a	0.317	0.000	0.000	0.001	0.616	0.089	0.036	0.749
Water	4.06a	2.87ab	2.53b	2.96b	2.71 b	3.79 a	3.14	2.79	2.80	3.91	3.13	0.179	0.009	0.011	0.318	0.601	0.745	0.341	0.209

[1] Dietary treatment (Tr): control diet (CON); alternative diet (ALT); alternative + insect (ALT+INS). [2] Time of day (Ti): from 09:40 to 09:50 h (1), from 10:40 to 10:50 h (2) and from 15:40 to 15:50 h (3). [3] Week of experiment (W): 1, 5, 9, 12, and 15. [4] SEM= standard error of mean (n= 3 by treatment x 3 by day x 5 by period). [a-b] Means with different letters indicate significant differences (P<0.05).

Table 8. Behavior of laying hens (frequency as a percentage of total observations) in relation to the movement category, based on dietary treatment, time of day evaluated, and week of the experiment.

	Treatment (Tr)[1]			Time (Ti)[2]			Week (W)[3]					SEM[4]	P-value						
	CON	ALT	ALT+INS	1	2	3	W1	W5	W9	W12	W15		Tr	Ti	W	Trx Ti	Trx W	Tix W	TrxTix W
Walking	39.89	40.07	42.63	41.85	40.33	40.41	38.78	41.07	42.53	41.06	40.88	0.567	0.123	0.658	0.414	0.720	0.287	0.553	0.401
Running	0.609	0.532	0.710	0.637	0.727	0.488	1.24a	0.416 ab	0.404 ab	0.345 b	0.682 ab	0.081	0.680	0.477	0.021	0.125	0.700	0.308	0.927
Wing flapping	0.55	0.574	0.673	0.402b	0.590 ab	0.803 a	0.632 b	0.374 cb	0.490 b	0.475 b	1.02a	0.060	0.234	0.020	0.048	0.229	0.923	0.439	0.406
Perching	1.02 a	1.21 a	0.527 b	0.799b	1.250 a	0.704 b	1.29	0.857	0.664	0.954	0.819	0.082	0.003	0.047	0.610	0.709	0.261	0.199	0.450
Jumping	0.781	0.762	0.462	0.587	0.746	0.672	0.929	0.821	0.543	0.525	0.523	0.065	0.156	0.422	0.369	0.881	0.042	0.509	0.338

[1] Dietary treatment (Tr): control diet (CON); alternative diet (ALT); alternative + insect (ALT+INS). [2] Time of day (Ti): from 09:40 to 09:50 h (1), from 10:40 to 10:50 h (2) and from 15:40 to 15:50 h (3). [3] Week of experiment (W): 1, 5, 9, 12, and 15. [4] SEM= standard error of mean (n= 3 by treatment x 3 by day x 5 by period). [a-c] Means with different letters indicate significant differences (P<0.05).

Table 9. Behavior of laying hens (frequency as a percentage of total observations) in relation to the social category, based on dietary treatment, time of day evaluated, and week of the experiment.

	Treatment (Tr)[1]			Time (Ti)[2]			Week (W)[3]					SEM[4]	P-value						
	CON	ALT	ALT+INS	1	2	3	W1	W5	W9	W12	W15		Tr	Ti	W	TrxTi	TrxW	TixW	TrxTixW
Menacing	0.842	0.735	0.872	0.841	1.03	0.578	1.09	0.953	0.632	0.794	0.616	0.085	0.519	0.083	0.242	0.443	0.283	0.367	0.974
Fighting	0.051	0.042	0.069	0.059	0.090	0.013	0.000	0.048	0.038	0.106	0.079	0.019	0.868	0.254	0.441	0.618	0.346	0.840	0.688
Pecking	3.50	4.20	3.48	3.24	4.17	3.77	4.43	4.16	3.40	2.76	3.89	0.236	0.701	0.239	0.258	0.181	0.374	0.797	0.669
Allo-preening	.392	0.610	0.309	0.440	0.507	0.363	0.384	0.305	0.119	0.952	0.426	0.093	0.330	0.767	0.095	0.865	0.239	0.175	0.888

[1] Dietary treatment (Tr): control diet (CON); alternative diet (ALT); alternative + insect (ALT+INS). [2] Time of day (Ti): from 09:40 to 09:50 h (1), from 10:40 to 10:50 h (2) and from 15:40 to 15:50 h (3). [3] Week of experiment (W): 1, 5, 9, 12, and 15. [4] SEM= standard error of mean (n= 3 by treatment x 3 by day x 5 by period). [a-b] Means with different letters indicate significant differences($P<0.05$).

Table 10. Behavior of laying hens (frequency as a percentage of total observations) in relation to the resting and nesting category, based on dietary treatment, time of day evaluated, and week of the experiment.

	Treatment (Tr)[1]			**Time (Ti)[2]**			**Week (W)[3]**					**SEM[4]**	**P-value**						
	CON	**ALT**	**ALT+INS**	**1**	**2**	**3**	**W1**	**W5**	**W9**	**W12**	**W15**		**Tr**	**Ti**	**W**	**TrxTi**	**TrxW**	**TixW**	**TrxTixW**
Crouch (Sitting down)	1.60	1.3	1.65	1.47	1.76	1.39	2.93 a	0.426c	1.53 abc	1.25 bc	1.54ab	0.165	0.856	0.740	0.000	0.059	0.200	0.014	0.636
Standing	16.88a	18.99 ab	20.74b	22.39a	18.53b	15.70c	15.45b	21.05a	17.06b	21.78a	19.02 ab	0.531	0.002	0.000	0.000	0.597	0.135	0.956	0.905
Nesting	0.550	0.690	0.835	1.02 a	0.866a	0.194b	0.712	0.578	0.602	0.717	0.849	0.081	0.078	0.000	0.801	0.807	0.493	0.933	0.671

[1] Dietary treatment (Tr): control diet (CON); alternative diet (ALT); alternative + insect (ALT+INS). [2] Time of day (Ti): from 09:40 to 09:50 h (1), from 10:40 to 10:50 h (2) and from 15:40 to 15:50 h (3). [3] Week of experiment (W): 1, 5, 9, 12, and 15. [4] SEM= standard error of mean (n= 3 by treatment x 3 by day x 5 by period). [a-c] Means with different letters indicate significant differences (P<0.05).

Table 11. Behavior of laying hens (frequency as a percentage of total observations) in relation to the comfort and stretching category, based on dietary treatment, time of day evaluated, and week of the experiment.

	Treatment (Tr)[1]			**Time (Ti)[2]**			**Week (W)[3]**					**SE M[4]**	**P-value**						
	CON	**ALT**	**ALT+INS**	**1**	**2**	**3**	**W1**	**W5**	**W9**	**W12**	**W15**		**Tr**	**Ti**	**W**	**TrxTi**	**TrxW**	**TixW**	**TrxTixW**
Sand bath	0.570	0.303	0.171	0.015b	0.250ab	0.779a	0.661ab	0.013c	0.936a	0.081bc	0.049c	0.132	0.725	0.006	0.028	0.226	0.755	0.015	0.744
Self-preening	5.31	6.90	6.42	6.31	7.02	5.31	7.78	5.50	6.41	5.30	6.07	0.335	0.076	0.070	0.060	0.590	0.772	0.414	0.188
Leg stretching	0.170	0.152	0.099	0.193	0.124	0.103	0.141	0.082	0.116	0.178	0.184	0.025	0.614	0.326	0.592	0.736	0.568	0.276	0.612
Win stretching	0.171	.166	0.085	0.150	0.169	0.102	0.205	0.026	0.165	0.183	0.123	0.025	0.423	0.605	0.129	0.740	0.454	0.170	0.357

[1] Dietary treatment (Tr): control diet (CON); alternative diet (ALT); alternative + insect (ALT+INS). [2] Time of day (Ti): from 09:40 to 09:50 h (1), from 10:40 to 10:50 h (2) and from 15:40 to 15:50 h (3). [3] Week of experiment (W): 1, 5, 9, 12, and 15. [4] SEM= standard error of mean (n= 3 by treatment x 3 by day x 5 by period). [a-c] Means with different letters indicate significant differences (P<0.0

Conclusion of the video recording evaluation

In summary, the effects of diet, time of day, and week studied on the hens' behavior were multifaceted. Alternative diets influenced some behaviors, such as reduced grass/object pecking and increased water consumption as well as standing frequency, with a specific reduction in perching frequency observed in the diet that included insects. Importantly, dietary treatments did not affect other social or movement-related behaviors, such as menacing, fighting, pecking, or allo-preening. Time of day and week studied also influenced a variety of activities, suggesting that the hens´ ethograms varied according to these factors, although social behaviors remained unaffected. As for insect consumption, hens consistently consumed the entire portion offered daily, with consumption periods shortening as the experiment lengthened.

Corticosterone in feather

Material and methods

On day of slaughter, feather samples were taken for corticosterone analysis as an indicator of stress. From two laying hens per replicate, primary feathers 2 and 8 were plucked from both wings according to the procedure of Nordquist et al. (2020) (Figure 10). In addition, three hens per replica were sampling interscapular feathers (Häffelin et al., 2021; Figure 11). The interscapular feathers (n = 3 – 5 / hen) were taken from the region on the back between the scapulae. All selected feathers had to be full-grown which were verified through the absence of blood and feather pulp in the calamus and the completeness of the vane. Interscapular feathers of each hen were processed and analyzed as a pool. Feathers were stored dark and dry at room temperature until processing.

Figure 10. Primary feather in hens. The feathers sampled are indicated in yellow.

Figure 11. Interscapular feather in hens.

- Corticosterone extraction and analysis

Feather corticosterone was extracted following a methanol-based technique and measured as blood corticosterone according to Bortolotti et al. (2008). A high sensitivity ELISA kit (Corticosterone HS (High Sensitivity) EIA, IDS® Immunodiagnostic Systems, Boldon, UK) was used for its determination following the manufacturer's instructions. The intra-assay and inter-assay variability of this kit was less than 10% and the limit of detection was 0.17 ng/ml.

Statistical analysis

The normality assumption was evaluated using the Shapiro-Wilk test. Since it was not possible to normal distribution, a non-parametric Kruskal Wallis analysis of the data was performed with dietary treatment as the main effect. In the case of differences between treatments, the U-Mann Witney test was used to study these differences. The animal was the experimental unit, and differences were considered for a p-value <0.05.

Results

The corticosterone results obtained in primary and interscapular feathers are shown in Table 12 and Figure 12 y 13. There were non statistical differences between the treatments in the feather corticosterone levels ($p > 0.05$), showing the dietary treatments did not increase this stress indicator. Thus, the incorporation of alternative ingredients and the supplementation with insects did not alter this parameter.

Table 12. Effect of diets on corticosterone concentration in feathers at the end of experiment.

	CON	**ALT**	**ALT+INSECT**	**P-value**
Corticosterone (ng/mL)*				
Primary feathers	0.36 (0.32-0.91)	1.06 (0.67-1.40)	0.64 (0.40-1.00)	0.082
Interscapular feathers	0.68 (0.49-1.12)	0.70 (0.49-0.90)	0.69 (0.60-1.24)	0.958

* Median and interquartile range. Control diet (CON); Alternative diet (ALT); Alternative + insect diet (ALT+INSECT).

Figure 12. Box-and-whisker plot of effect of diets of hens on corticosterone in 2 and 8 primary feathers at end of whole experimental period (105 days). The box represents the interquartile range (25 and 75), the median line, and the whisker extremes the maximum and minimum value. N=30. Control diet (CON); Alternative diet (ALT); Alternative + insect diet (ALT+INSECT).

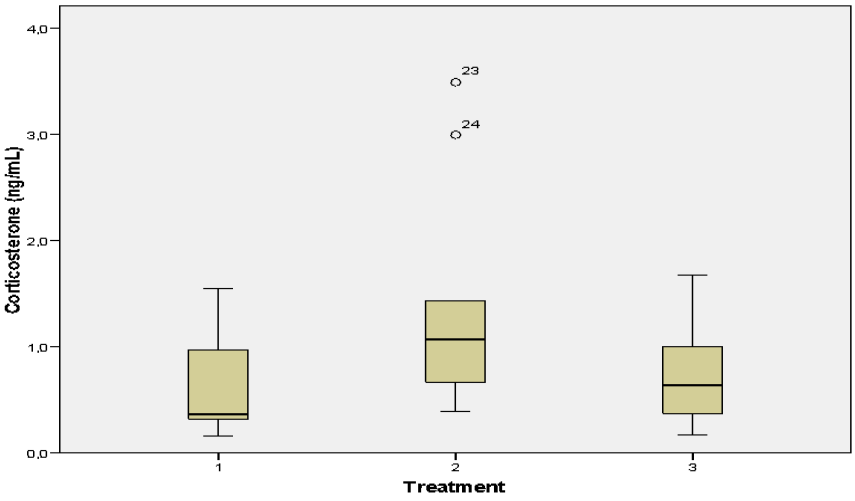
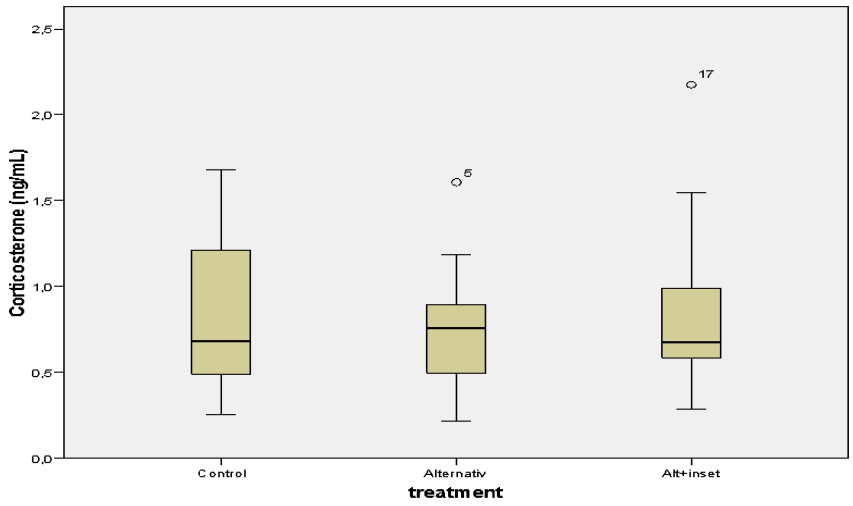


Figure 13. Box-and-whisker plot of effect of diets of hens on corticosterone in interscapular feather at end of whole experimental period (105 days). The box represents the interquartile range (25 and 75), the median line, and the whisker extremes the maximum and minimum value. N=45. Control diet (CON); Alternative diet (ALT); Alternative + insect diet (ALT+INSECT).



Conclusion of the feather corticosterone evaluation

In conclusion, the inclusion of alternative plant-based ingredients and the supplementation with larvae did not increase the corticosterone levels in the animal feather, suggesting the feasibility of the use of this new dietary treatment.

Biochemical and hematological variables

Materials and methods

Animals and sample collection

At the end of each control period (27, 32 and 38 weeks of aged), 2 hens per replicate were randomly sampled for blood sampling (10 samples per dietary treatment). Blood samples were obtained from the brachial vein into heparinized tubes using sterilized syringes and needles. Once the samples were obtained, they were transported as soon as possible to the Interdisciplinary Laboratory of Clinical Analysis of the University of Murcia (Interlab-UMU, Spain). where they were processed. An aliquot was directly used for hematological parameters determination. The rest was centrifuged at 3000 g for 10 min at 4ºC, and the

plasma was subsequently removed and stored at -20°C for determination of plasma albumin, cholesterol, total proteins, triglycerides, and uric acid. Plasma biochemical parameters were analyzed with an automated chemiluminescent immunoassay (Immulite System, Siemens Health Diagnostics, Deerfield, IL, USA).

Hematological parameters (erythrocytes, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration were measured using an automated hematology analyzer (ADVIA 120 Hematology System, Siemens Healthineers, Spain). Leukocytes, heterophil and lymphocyte were counted manually by examination of blood smears using a modification of the Wright-Giemsa stain. For each smear, 60 white blood cells were manually counted using a light microscope at 100x magnification according to Houshmand et al. (2012). After averaging the cells, the H/L ratios were then calculated.
In addition, plasma corticosterone was determined. Corticosterone was extracted following a methanol-based technique and measured as blood corticosterone according to Bortolotti et al. (2008). A high sensitivity ELISA kit (Corticosterone HS (High Sensitivity) EIA, IDS® Immunodiagnostic Systems, Boldon, UK) was used for its determination following the manufacturer's instructions. The intra-assay and inter-assay variability of this kit was less than 10% and the limit of detection was 0.17 ng/ml.

Statistical analysis

All statistical analyses were carried out using SPSS software for Windows (SPSS Inc., Chicago, IL, USA). Animal was considered the experimental unit. The Shapiro–Wilks test was first used to assess whether data were normally distributed. Albumin, cholesterol, total protein, triglycerides, uric acid, and corticosterone were not normally distributed and were analyzed with a Kruskal Wallis test. When differences were found, differences between treatment were investigated with U of Mann-Whitney test. Hematological data were analyzed using one-way ANOVA with dietary treatment effect using the General Linear Model (GLM) procedure. A p-value of less than 0.05 was considered significantly different.

Results

The results of the biochemical parameters and corticosterone concentration studied are shown in Table 12 and Table 13, respectively.

Blood chemical parameters are shown in Table 13. We did not observe significant differences in the blood biochemical parameters studied among the experimental diets ($p>0.05$), except in the second assessment conducted at 32 weeks of age in the hens. In this assessment, the animals on the ALT+INSECT diet had lower blood cholesterol levels and triglyceride levels. However, this difference disappeared in the assessment conducted at 38 weeks of age; while the values remained lower than those of the other experimental treatments, they were not significantly different.

Table 13. Serum biochemical parameters of laying hens at 27, 32 and 38 weeks of age with the different experimental diets. P-value Kruskal-Wallis, [a-b] U de Mann-Whitney. N=10 per treatment. (2 hens/replica).

	CON	ALT	ALT+INSECT	P-value
Albumin (g/dL) *				
27 weeks	1.64 (1.52-1.75)	1.56 (151-1.605)	1.60 (1.47-1.70)	0.546
32 weeks	1.68 (1.64-1.72)	1.67 (1.65-1.76)	1.63 (1.58-1.71)	0.439
38 weeks	1.85 (1.81-1.92)	1.82 (1.74-1.85)	1.84 (1.79-1.90)	0.490
Cholesterol (mg/dL) *				
27 weeks	102.1 (85.92-188.4)	110.7 (81.5-124.9)	97.66 (74.44-113-1)	0.568
32 weeks	143.1(113.2-160.1)a	145.6 (121.1-193.9)a	112.6 (88.0-120.9)b	0.028
38 weeks	159.1 (133.8-177.1)	165.6 (146.7-202.4)	128.2 (110.3-154.2)	0.112
Total protein (g/dL) *				
27 weeks	4.75 (4.46-5.28)	4.17 (3.96-4.49)	4.66 (4.19-4.97)	0.178
32 weeks	4.81 (4.70-4.95)	4.89 (4.70-5.12)	4.70 (4.56-4.78)	0.339
38 weeks	5.56 (5.38-5.73)	5.32 (5,12-5,53)	5.48 (5.26-5.58)	0.363
Triglycerides (mg/dL) *				

	CON	ALT	ALT+INSECT	P-value
27 weeks	1289 (847.9-1920)	1337 (954.6-1650)	1134 (789.6-1356)	0.278
32 weeks	1829 (1714-2076)a	1935 (1564-2173a)	1235 (967.1-1649)b	0.027
38 weeks	1786 (1676-1972)	1876 (1660-2048)	1692 (1253-1845)	0.412
Uric acid (mg/dL) *				
27 weeks	5.12 (4.79-6.20)	4.00 (2.94-5.84)	5.47 (4.15-6.03)	0.587
32 weeks	3.94 (1.99-4.62)	3.78 (2.14-5.74)	4.77 (3.61-6.13)	0.516
38 weeks	3.58 (1.08-6.81)	3.57 (1.60-5.32)	3.27 (2.178-5.67)	0.939

* Median and interquartile range. Control diet (CON); Alternative diet (ALT); Alternative + insect diet (ALT+INSECT).

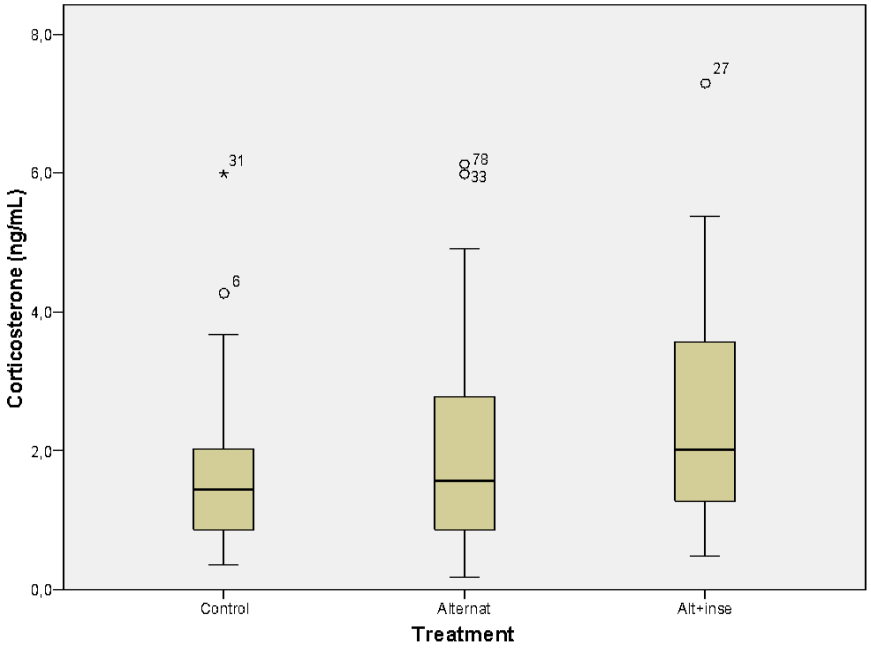
The blood corticosterone levels are shown in Table 14 and Figure 14. Regarding blood corticosterone levels, no significant differences were observed among the three diets studied ($p>0.05$) in any of the three assessments conducted.

Table 14. Effect of diets of hens on plasma corticosterone concentration at 27, 32 and 38 weeks of age. N=10 per treatment (2 hens/replica)

	CON	ALT	ALT+INSECT	P-value
Corticosterone (ng/mL) *				
27 weeks	1.38 (0.57-2.06)	1.05 (0.76-2.09)	2.49 (1.08-4.65)	0.124
32 weeks	1.80 (1.19-2.65)	1.81 (1.13-3.61)	2.40 (1.49-5.03)	0.588
38 weeks	1.34 (0.73-1.86)	1.82 (0.54-2.51)	2.01 (1.69-2.91)	0.217

* Median and interquartile range. Control diet (CON); Alternative diet (ALT); Alternative + insect diet (ALT+INSECT).

Figure 14. Box-and-whisker plot of effect of diets of hens on plasma corticosterone test in whole experimental period. The box represents the interquartile range (25 and 75), the median line, and the whisker extremes the maximum and minimum value. CONTROL: conventional diet; ALTER: Alternative experimental diet with fewer imported ingredients and new alternative ingredients incorporated; ALT + INSECT: ALTER diet plus 5% whole dehydrated Black Soldier Fly.



Hematological parameters are shown in Table 15. The hematological results obtained showed no statistical differences among the alternative experimental diets studied in relation to erythrocyte concentration, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, leukocytes, or the heterophil-to-lymphocyte ratio. Only hematocrit was lower with the ALT diet in the second control, although these differences disappeared in the third control.

Table 15. Hematological parameters of laying hens at 27, 32 and 38 weeks of age with the different experimental diets. N=10 per treatment (2 hens/replica)

	CON	ALT	ALT+INSECT	SEM	P-value
Erythrocytes (x106 cel/µL)					
27 weeks	2.45	2.40	2.47	0.043	0.827
32 weeks	2.52	2.45	2.63	0.031	0.069
38 weeks	2.82	2.80	2.87	0.038	0.360
Hemoglobin (g/dL)					
27 weeks	8.66	8.36	8.82	0.198	0.634
32 weeks	8.90	8.74	9.40	0.147	0.182
38 weeks	9.67	9.63	9.61	0.109	0.026
Hematocrit (%)					
27 weeks	18.50	18.38	17.93	0.371	0.805
32 weeks	19.34a	17.87b	19.93a	0.293	0.023
38 weeks	20.77	21.32	21.60	0.448	0.296
Mean corpuscular volume (fL)					
27 weeks	75.52	76.51	72.57	0.683	0.066
32 weeks	76.60	72.91	75.85	0.817	0.179
38 weeks	73.57	75.91	75.06	0.951	0.517
Mean corpuscular hemoglobin (pg)					
27 weeks	35.31	34.85	35.68	0.384	0.680
32 weeks	35.29	35.71	35.70	0.446	0.909
38 weeks	34.26	34.62	33.65	0.495	0.327
Mean corpuscular hemoglobin concentration (g/dL)					
27 weeks	46.92	45.53	49.38	0.757	0.129
32 weeks	46.14	49.33	47.39	0.896	0.356
38 weeks	46.82	46.17	44.98	1.148	0.220
Leukocytes (x10^3 cel/µL)					
27 weeks	11.46	11.51	11.12	0.278	0.824
32 weeks	11.53	11.65	11.10	0.244	0.631

	CON	ALT	ALT+INSECT	SEM	P-value
38 weeks	11.44	12.17	11.86	0.306	0.479
Heterophilic: lymphocyte ratio					
27 weeks	1.60	1.26	1.12	0.134	0.338
32 weeks	1.62	1.59	1.84	0.156	0.774
38 weeks	2.00	1.83	2.33	0.171	0.751

Data are shown as mean. SEM: Standard error of mean. 1CONTROL: conventional diet; ALTER: Alternative experimental diet with fewer imported ingredients and new alternative ingredients incorporated; ALT + INSECT: ALTER diet plus 5% whole dehydrated Black Soldier Fly

Conclusion of the Biochemical & hematological variables

In summary, the experimental diets did not significantly affect the blood biochemical, corticosterone, or hematological parameters of the hens in most assessments. Notably, at 32 weeks of age, the ALT+INSECT diet resulted in lower blood cholesterol and triglyceride levels, although this difference was not sustained at 38 weeks. Similarly, the ALT diet showed a temporary reduction in hematocrit levels during the second control, but no significant differences were observed in the final assessment. Overall, the alternative diets demonstrated minimal impact on the blood parameters studied.

Conclusion

The study demonstrated that the partial substitution of soybean meal with alternative plant-based ingredients and the inclusion of *Hermetia illucens* larvae in hens' diets did not negatively impact relative organ weights, digestive pH, or nutrient digestibility. Only the insect-supplemented diet slightly increased abdominal fat, with no significant effects on tissue histomorphology and organ histopathology. These findings suggest that incorporating *Hermetia illucens* larvae as a dietary supplement is a viable option without adverse impacts on poultry health or intestinal integrity, supporting its potential as an alternative protein source in poultry nutrition.

EGE

Animals

EGE used meat-type chickens from a slow-growing local strain (Anadolu-T) and a commercial strain (Cobb). A total of 252 day-old chicks were used from each strain. The chicks from each strain were randomly divided into 18 pens with 14 chicks in each. The standard brooding temperatures were used. The lighting regime was 23L:1D during the first 3 days, then gradually reduced to 18 L:6 D, and maintained until the end of the experiment, which was 40 d for Cobb50 and 55 d for Anadolu-T.

Diets

Birds from each strain divided into three dietary groups (each dietary group had 6 replicated pens) were fed Control, ALT, or ALT+BSFL diets. The diets were: A soybean and corn-based diet (CON), soybean and corn were partly substituted with sunflower meal, brewers dried grain, and wheat middlings (ALT), and 5 % dried BSFL meal was included in ALT (ALT+BSFL).

Methods

Behavior, welfare, blood parameters except leukocyte counts, organ weights, and fecal microbiota were determined in both local and commercial strain
Leukocyte counts, intestine histology, and cecal microbiota were determined only in local strain.

Behavior

There were 3 replicate pens per diet group. Scan sampling was used to record the number of birds performing one of the feeding, drinking, walking-standing, and sitting behaviors in each pen (Lehner, 1992). Scans were made at once every hour during the 18 h photoperiod on d 14, 35 and 49. Besides these behavioral categories, pecking (objects, equipment, or other chicks), preening, dustbathing, leg stretching, and wing flapping were also recorded in a minute at which scan sampling was done. Behavioral recordings were made by two observers at the

pen level. One of the observers recorded four basic behaviors (feeding, drinking, walking-standing, and sitting) while the second counted other behaviors in a minute whenever they were performed. Therefore, the same bird might have been recorded in different categories of ethogram.

Welfare: Tonic Immobility, Foot Pad Dermatitis, Feather Cleanliness

A tonic immobility test was conducted at 28 d of age and slaughter age. Two birds (one male and one female) from each replicate pen were randomly chosen (total 12) and placed on their backs on a table holding for 15 sec. Then hands were slowly removed from the bird and the timer was on to measure latency to the right with a maximum of 120 s (Archer, 2018). If the bird righted itself in less than 15 s the same procedure repeated up to 3 times. Birds were given a score of zero if all three attempts for TI induction failed. Longer tonic immobility durations were associated with higher fearfulness (Jones, 1986; Archer, 2018). On d 40 and 55, for commercial and local chickens, respectively, plumage cleanliness and footpad dermatitis (FPD) were scored on all birds. A four-scale scoring for plumage cleanliness (0: intact, 1: slight, 2 moderate, 3: severe soiling) (Welfare Quality, 2009) and a three-scale for footpad dermatitis (0:intact, 1: moderate FPD, 3: severe FPD) were used (Ekstrand et al., 1998).

Blood parameters and organ weights

At d 40 for commercial and d 55 for local chickens, 18 broilers (three birds/replicate, nine from each sex) from each treatment close to the average weight were randomly selected. After 8 h of feed withdrawal, blood samples were collected in a tube during the slaughtering process. Weights of the liver, whole intestine, spleen, and bursa of Fabricius were measured. The relative weights were calculated by dividing individual organ weights by the live body weight of birds.

The serum was separated after centrifugation at 4 ℃ and 2750 rpm for 10 minutes and stored at −80 ℃ until analysis of blood metabolites. Blood protein, triglycerides, uric acid, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), creatinine,

cholesterol, corticosterone, and blood minerals of Ca, P, and Mg concentrations were measured using commercial kits (Mindray, China) and autoanalyzer (Mindray BS-240 Vet, Nanshan, Shenzhen 518057, P. R. China). The ELISA kits (Chicken corticosterone ELISA kit; Bioassay Technology Lab, Shanghai, China) were used to assess and quantify the corticosterone concentration of blood samples following manufacturer instructions. The absorbance was measured at 450 nm and the concentration of corticosterone was determined relative to the absorbance of the calibration curve.

Blood samples from the local strain (18 birds/diet) with equal numbers of each sex were also collected for leucocytes counts and heterophil to lymphocytes ratio (H/L) measures. A blood smear was prepared, using one glass slide for each bird, from a drop of blood. The smears were stained using May-Grünwald and Giemsa stains and 60 leukocytes, including heterophils, lymphocytes, eosinophils, basophils, and monocytes were counted (Gross and Siegel, 1983). Heterophil to lymphocytes ratio was calculated.

Histomorphological analysis

About 2cm of samples from duodenum, jejunum, and ileum were collected for intestine histological analysis. The samples were rinsed with saline, fixed in formalin solution, and maintained in the formalin at room temperature until analysis. The samples were washed, dehydrated with alcohol, and cleared with xylene before paraffin wax. Tissue samples were cut by a microtome and stained using hematoxylin and eosin. Five villi and crypt were randomly selected under the light microscope and measurements of villi height and width and crypt depth were performed using the Sigma Scan Pro5 program.

Microbiota

16S rRNA gene amplicon sequencing by next-generation sequencing (NGS)

Amplicon sequencing analysis was performed with 8 chicken samples from each diet. PCR amplification of the targeted V3-V4 region was performed by using specific primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3'). The Nextera XT DNA Library Preparation Kit (Illumina, USA) was utilized to generate sequencing libraries after the

quantification and qualification of PCR products. The concentration of the libraries was normalized by diluting to 4 nM then libraries were sequenced on a paired-end Illumina NovaSeq 6000 platform to generate 250bp paired-end (2 × 250bp) raw reads. FastQC and QIIME2 were used to assess the raw data quality and read quality control, respectively. Effective tags were obtained by using DADA2 to remove primer and barcode sequences, chimeric reads, and reads with a Phred Score of less than 20, hence improving the accuracy and reliability of the results. QIIME2 was used for the taxonomic determination of each Operational Taxonomic Units (OTUs) representative sequence. OTUs were annotated to obtain the corresponding species information and the abundance distribution based on the species with ≥ 97% similarity against the SILVA (138.1) (Quast, C. et al, 2012). According to the results annotations of each sample, the species abundance tables at the level of kingdom, phyla, class, order, family, genus, and species were obtained. Since these abundance tables with annotation information were the core content of amplicon analysis; determination of relative abundance, and alpha and beta diversity analyses were carried out by selecting requested classification levels (e.g. phylum, genus). To clarify the richness and diversity of microbial communities in each sample, alpha diversity analyses were conducted. By using dimensionality reduction methods like PCoA in beta diversity analysis, the variations among several groups were investigated.

Statistical analysis

Several models were used to analyze the data.

Behavior and welfare: Nonparametric Wilcoxon Kruskall-Wallis test was used for statistics analysis of behavior and tonic immobility. Means of behavioral data were separated with nonparametric comparisons made for each pair using the Wilcoxon method. Data were expressed as a percentage (%) of the total number of birds that performed one specific behavior per replicate pen. $P \leq 0.05$ was considered significant while $P \leq 0.10$ was considered as a tendency.

Footpad dermatitis and feather cleanliness scores at slaughter age for each strain were analyzed with a chi-square test of independence. For footpad dermatitis scoring, there were very few birds with score 1 (mild) in Cobb therefore score 1

and score 2 were pooled resulting in a binomial scaling for intact and severely (score 1+ 2) affected birds. For plumage cleanliness of Cobb broilers, there were no birds with scored 3 and there were only a few birds that scored 2. Therefore, data was pooled as one-zero scaling as 0 indicates clean plumage and 1 indicates slight soiling. In the local line, there were no birds with scored 0 (clean) or 3 (severe soiling). Therefore, all birds from the local strain showed slight (1) or moderate (2) discoloration. When the chi-square test indicated a statistically significant effect, cell chi-squares and adjusted standardized residuals for each cell in the contingency table were calculated for post-hoc evaluation. The cells associated with adjusted standardized residuals greater than +/- 2 indicated a significant contribution of that specific cell to omnibus chi-square (Sharpe, 2015).

Blood parameters and intestine histology: A two-way factorial model including diet, strain, and their interaction was used to analyze blood parameters except the H:L ratio. Data of H:L ratio were subjected to one-way ANOVA analysis except with Eosinophil and Monocyte that did not meet the normality assumption. Those data were subjected to a non-parametric Kruskal Wallis test. One-way ANOVA including diets was used to analyze organ parameters. When there are significant interactions, means were separated using Student's t-test. P values < 0.05 indicate statistical significance.

Microbiota: The significance of variations in species composition and community structure of groups was tested using the T-test, Kruskal-Wallis, Anosim, and multiple response permutation process (MRPP) statistical tests. P values below 0.05 were considered statistically significant. All statistical analyses were performed with R software (Version 4.3.1) (https://www.r-project.org).

Results

Behavior

Local strain

Table 16 shows the effect of diets on feeding, drinking, walking-standing, and sitting behavior (%) of Anadolu-T broilers at different ages. There was no effect of diet on feeding, drinking, walking-standing, and sitting behaviors on d 14. The

only differences among the diets were observed for locomotor behavior on d 35. The percentage of birds walking-standing increased with the ALT+BSFL as compared to the Control. Drinking behavior tended to be higher in ALT+BSFL-fed Anadolu-T broilers than the Control ones on d 49.

The effect of diets on the mean percentage of broilers performing pecking, preening, wing-flapping and dustbathing behavior (%) has been presented in Table 17. On d 49, broilers fed with ALT had a lower tendency to perform leg stretching than the Control broilers.

Table 16. Diet effect on the percentage of broilers performing feeding, drinking, walking-standing, and sitting behavior (%)

Diet	Feeding(%)	Drinking(%)	Walking-Standing(%)	Sitting-Lying(%)
Day 14				
ALT	30.16±1.49	7.41±1.10	23.28±2.20	39.15±2.36
ALT+BSFL	30.69±1.78	6.08±0.99	24.21±2.41	39.02±2.88
Control	31.22±2.24	5.95±0.84	20.90±2.18	41.93±2.58
χ2	0.2901	0.8522	1.1662	0.7742
P-value	0.8650	0.6531	0.5582	0.6790
Day 35				
ALT	23.68±1.84	6.35±1.03	15.08±2.39^{ab}	54.89±3.20
ALT+BSFL	27.38±1.63	5.29±0.85	16.27±2.34^{a}	51.06±2.23
Control	28.02±1.81	5.80±0.99	8.40±1.42^{b}	57.77±2.28
χ2	4.3039	0.2871	6.6934	3.0303
P value	0.1163	0.8663	0.0352	0.2198
Day 49				
ALT	20.84±1.76	5.90±1.03^{b}	14.10±1.86	59.16±2.63
ALT+BSFL	19.57±1.79	9.70±1.32^{a}	15.16±2.39	55.58±3.28
Control	22.91±1.97	5.99±0.97^{ab}	13.06±2.24	58.03±2.69
χ2	2.5685	5.4030	0.8010	0.0667
P-value	0.2769	0.0671	0.6700	0.9672

[1]Diets: Control: corn-soybean based diet, ALT: soybean in the control diet was partially replaced by sunflower meal, brewer's dried grain, and wheat middlings, ALT+BSFL black soldier fly dried larvae were added to ALT.

[a,b]: Different superscript letters in columns indicate significant differences between the diets in the nonparametric comparisons for each pair using the Wilcoxon method (P<0.05).

Table 17. Diet effect on the percentage of broilers performing pecking, preening, leg stretching, wing-flapping, and dustbathing behavior (%)

Diet	Pecking(%)	Preening(%)	Leg stretching(%)	Wing flapping(%)	Dust bathing(%)
Day 14					
ALT	21.56±1.79	15.61±1.39	5.95±0.77	15.21±1.84	0.40±0.29
ALT+BSFL	22.62±1.61	15.48±1.52	8.47±1.15	15.74±1.64	1.06±0.51
Control	23.15±1.57	17.86±1.61	8.33±1.14	14.95±1.48	0.40±0.22
χ2	0.8601	1.7519	2.0377	0.1909	1.5184
P-value	0.6617	0.4165	0.3610	0.9089	0.4680
Day 35					
ALT	14.15±1.22	22.49±1.29	5.42±0.82	3.97±0.84	1.45±0.48
ALT+BSFL	13.62±1.33	23.41±1.48	7.14±0.96	2.91±0.81	1.06±0.44
Control	12.98±1.31	22.05±1.67	5.44±0.91	3.53±0.90	2.56±0.92
χ2	0.3552	0.3264	1.6205	1.6994	1.0232
P-value	0.8373	0.8494	0.4448	0.4275	0.5995
Day 49					
ALT	10.99±1.39	19.47±1.69	3.93±0.98[b]	1.87±0.66	1.53±0.58
ALT+BSFL	11.79±1.56	21.67±1.89	4.87±0.94[ab]	2.17±0.60	1.49±0.59
Control	11.55±1.71	17.43±1.82	5.97±1.02[a]	2.94±0.61	0.83±0.43
χ2	0.1376	1.5376	5.4859	4.2650	1.3741
P-value	0.9335	0.4636	0.0644	0.1185	0.5031

[1]Diets: Control: corn-soybean based diet, ALT: soybean in the control diet was partially replaced by sunflower meal, brewer's dried grain, and wheat middlings, ALT+BSFL black soldier fly dried larvae were added to ALT.

[a,b:] Different superscript letters in columns indicate significant differences between the diets in the nonparametric comparisons for each pair using the Wilcoxon method (P<0.05).

Commercial strain

Table 18 shows the effect of diets on feeding, drinking, walking-standing, and sitting behavior (%) of Cobb broilers at different ages. There was only a tendency for reduced feeding behavior in Control broilers on d 14 ($P<0.10$). The only difference among the diets was observed for locomotor behavior on d 35 is significantly higher in Cobb broilers fed with the ALT+BSFL diet as compared to the Control one ($P<0.05$).

The effect of diets on pecking, preening, wing-flapping, and dustbathing behavior (%) of Cobb broilers has been presented in Table 19. On d 14, broilers fed with the Control diet had more pecking behavior as compared to the Control ones ($P<0.05$). Control broilers also showed an increased tendency for dustbathing than the ALT ($P<0.10$). On d 35, broilers fed with ALT+BSFL had significantly lower legs than those Control and ALT-1-fed broilers ($P<0.05$).

Table 18. Diet effect on the percentage of commercial broilers performing feeding, drinking, walking-standing, and sitting behavior (%)

Diet	Feeding(%)	Drinking(%)	Walking-Standing(%)	Sitting-Lying(%)
Day 14				
ALT	28.57±2.34^{ab}	7.41±1.18	19.58±2.39	44.44±3.42
ALT+BSFL	29.10±2.04^{a}	7.14±1.12	22.35±2.04	41.40.2.43
Control	22.62±1.73^{b}	6.61±1.13	23.68±2.62	47.09±2.69
$\chi 2$	5.4612	0.3489	1.9086	2.3724
P-value	0.0652	0.8399	0.3851	0.3054
Day 35				
ALT	27.35±2.03	4.66±0.95	4.64±1.03^{b}	63.35±2.53
ALT+BSFL	23.96±2.02	5.58±0.91	9.90±1.77^{a}	60.56±2.64
Control	24.34±2.02	4.89±0.82	5.29±0.97^{b}	65.48±2.51
$\chi 2$	2.1699	0.9282	7.6636	2.1025
P value	0.3379	0.6287	0.0217	0.3495

[1]Diets: Control: corn-soybean based diet, ALT: soybean in the control diet was partially replaced by sunflower meal, brewer's dried grain, and wheat middlings, ALT+BSFL black soldier fly dried larvae were added to ALT.

[a,b]: Different superscript letters in columns indicate significant differences between the diets in the nonparametric comparisons for each pair using the Wilcoxon method (P<0.05).

Table 19. Diet effect on the percentage of commercial broilers performing pecking, preening, leg stretching, wing-flapping and dustbathing behavior (%)

Diet	Pecking (%)	Preening(%)	Leg stretching(%)	Wing flapping(%)	Dust bathing(%)
Day 14					
ALT	16.67±1.51[b]	17.46±1.63	10.05±1.42	16.27±2.19	0[b]
ALT+BSFL	18.78±1.67[ab]	21.56±1.62	10.58±1.48	18.52±1.90	0.13±0.13[ab]
Control	22.88±1.67[a]	20.63±1.60	8.86±1.02	16.93±2.14	0.79±0.41[a]
χ2	7.3465	4.3925	0.1168	1.6437	5.3770
P-value	0.0254	0.1112	0.9433	0.4396	0.0680
Day 35					
ALT	10.30±1.13	17.71±1.24	8.66±1.30[a]	2.28±0.73	0.94±0.50
ALT+BSFL	10.58±1.15	19.12±1.48	4.86±0.84[b]	3.75±1.28	1.50±0.78
Control	11.64±1.47	20.11±1.60	8.47±1.17[a]	2.38±0.63	0.66±0.34
χ2	0.0329	0.3531	5.8652	1.5864	0.2075
P value	0.3837	0.8381	0.0533	0.4524	0.9014

[1]Diets: Control: corn-soybean based diet, ALT: soybean in the control diet was partially replaced by sunflower meal, brewer's dried grain, and wheat middlings, ALT+BSFL black soldier fly dried larvae were added to ALT.

[a,b:] Different superscript letters in columns indicate significant difference between the diets in the nonparametric comparisons for each pair using Wilcoxon method (P<0.05).

Welfare

The effect of the diet on tonic immobility duration and induction numbers for each of the lines are presented in Table 5. Diet did not affect tonic immobility duration and number of inductions in both lines.

The severity of footpad dermatitis was independent of diets for both strains (P>0.05). The distribution of footpad scores of chickens at slaughter age is presented in Table 6. The percentage of birds with a score of "0" was 68.95% in the local strain and 78.48% in Cobb. The percentage of mildly affected birds was 19.35% in local strain and ranged between 29.17 and 39.58% within the dietary groups. The percentage of birds with a score of "2" was 11.69%. For commercial strain, footpad incidence was 21.52% and ranged between 27.08 and 43.75% within the diets.

The distribution of feather cleanliness scores among the diets for each strain is presented in Table 20. In the local strain, there were no birds with a score of "0" (clean) or "3" (severe dirtiness). All birds showed slight (score 1) or moderate (score 2) discoloration thus impaired cleanliness at the slaughter age. The 75.61% of birds examined had a score of "1", which indicated slight discoloration while the percentage of birds with moderate soiling (score "2") was 24.39 %. Chi-square analysis revealed that feather cleanliness scores of the birds were not dependent on diets in the local strain. However, in the commercial strain feather cleanliness scores depended on diet ($\chi 2 = 15.954$; P=0.0003; df:2). The percentage of clean birds was 20.9% for the commercial strain at slaughter age. The lowest number (%13.4) and the highest number (54.35%) of birds was found with a score of "0" within the birds fed ALT-1 and ALT-2 diets, respectively. Higher cell $\chi 2$ values of ALT-1 and ALT-2 diets by score "0" combinations revealed that ALT-1 reduced the number of clean birds which was indicated by greater adjusted standardized residual value (>-2). However, the ALT-2 diet resulted in a higher number of clean birds than that would be expected by chance with the highest contribution to total $\chi 2$ and higher adjusted standardized residual (>2).

Table 20. The effect of the diets[1] on tonic immobility (TI) test responses of local and commercial broilers on d 28 and slaughter age[2]

	Local strain					Commercial strain				
	Control	ALT	ALT+BSFL	SEM[3]	Diet (P-value)	Control	ALT	ALT+BSFL	SEM	Diet (P-value)
D28										
TI (s)	68.50	52.83	63.08	12.70	0.594	81.67	89.67	98.83	9.20	0.375
Induction number	2.17	1.83	1.58	0.26	0.249	1.08	1.33	1.42	0.15	0.346
Slaughter age										
TI n (s)	79.25	81.08	79.08	12.51	0.977	104.08	97.00	87.67	10.57	0.441
Induction number	2.08	1.83	1.67	0.24	0.489	1.42	1.17	1.17	0.15	0.738

[1]Diets: Control: corn-soybean based diet, ALT: soybean in the control diet was partially replaced by sunflower meal, brewer's dried grain, and wheat middlings, ALT+BSFL black soldier fly dried larvae were added to ALT.

[2] Slaughter age was 55 and 40d for local and commercial strains, respectively.

[3]SEM: Standard error of means.

Table 21. Distribution (%) of feather cleanliness scores of local and commercial broilers among the diets[1] at slaughter age[2] (observed numbers are given in parenthesis)

Diets	Feather Cleanliness, %			
	Local strain		Commercial	
Plumage Score	1(slight soiling)	2 (Moderate soiling)	0 (clean plumage)	1 (Slight soiling)
Control	35.48(n=66) Cell χ2= 0.502	23.33(n=14) Cell χ2= 1.557	32.61 (n=15) Cell χ2= 0.049	35.06 (n=61) Cell χ2= 0.013
ALT	32.80 (n=61) Cell χ2= 0.099	38.33(n=23) Cell χ2= 0.308	13.04 (n=6) Cell χ2= 5.622	38.51 (n=67) Cell χ2= 1.486
ALT+BSFL	31.72 (n=59) Cell χ2= 0.145	38.33(n=23) Cell χ2= 0.450	54.35 (n=25) Cell χ2= 6.946	26.44 (n=46) Cell χ2= 1.836
Total (%)	75.61 (n=186)	24.39 (n=60)	20.9 (n=46)	79.09 (n=174)
	Pearson χ2= 3.062; P=0.21		Pearson χ2 = 15.954; P=0.0003	

[1]Diets: Control: corn-soybean based diet, ALT: soybean in the control diet was partially replaced by sunflower meal, brewer's dried grain, and wheat middlings, ALT+BSFL: black soldier fly dried larvae were added to ALT1.
[2] Slaughter age was 55 and 40d for local and commercial strains, respectively.

Blood and organ weights

At slaughter age, all blood parameters measured, except corticosterone, were higher in commercial than those in local chickens. The local birds fed ALT+BSFL diets had the lowest glucose levels followed by ALT and Control diets (Table 8). ALT diet increased blood ALT levels of local chickens. Blood AST, GGT, protein, triglycerides, and cholesterol levels of local chickens were reduced by ALT+BSFL, whereas birds fed ALT and Control diets had similar levels. The diets had no significant effect on blood glucose, AST, triglycerides, total cholesterol, uric acid, and Ca levels of Cobb broilers (Table 22). ALT diet tended to reduce blood ALT and significantly increase blood GGT levels of Cobb broilers. While blood total protein levels of Cobb broilers decreased in birds fed ALT+BSFL, similar blood protein levels were obtained in birds fed control and ALT diets. The highest creatinine levels were observed for the Cobb birds fed the control diet. The diets had no significant effect on the blood corticosterone and blood uric acid levels of chickens from strains

Table 22. Effect of strains and diets[1] on corticosterone (CORT, ng/ml), glucose (GLU, mg/dl), alanine transaminase (ALT, U/L), aspartate aminotransferase (AST, U/L), gamma-glutamyl transferase (GGT, U/L), protein (PRO, g/L), triglycerides (TRIG, mg/dl), total cholesterol (HC, mg/dl), creatinine (CR, mg/dl), Uric acid (UA, mg/dl), magnesium (Mg, mg/dl), calcium(Ca, mg/dl), phosphorus (P, mg/dl)

Blood metabolits	Local strain					Commercial strain				
	Control	ALT	ALT+ BSFL	SEM[2]	P-value	Control	ALT	ALT+ BSFLK	SEM	P-value
CORT	8.1	7.44	8.18	0.592	0.222	8.77	7.59	8.31	0.740	0.531
GLU	183[a]	159[b]	110[c]	9.5	<0.001	212	226	221	7.0	0.354
ALT	1.57[b]	2.47[a]	1.40[b]	0.252	0.015	3.17	2.54	3.18	0.288	0.062
AST	239[a]	231[a]	146[b]	13.3	<0.001	411	396	386	28.1	0.837
GGT	15.82[a]	15.05[a]	9.21[b]	1.107	<0.001	18.39[b]	20.75[a]	17.44[b]	0.803	0.017
PRO	22.75[a]	24.11[a]	14.12[b]	1.463	<0.001	28.32[a]	29.49[a]	26.28[b]	0.555	<0.001

Blood metabolits	Local strain					Commercial strain				
	Control	ALT	ALT+ BSFL	SEM[2]	P-value	Control	ALT	ALT+ BSFLK	SEM	P-value
TRIG	14.51[a]	14.06[a]	8.55[b]	1.402	0.008	22.39	21.93	23.93	1.825	0.728
TC	88.39[a]	79.82[a]	55.43[b]	5.507	<0.001	133	134	126	4.1	0.304
CR	0.183	0.197	0.153	0.0163	0.175	0.259[a]	0.224[b]	0.221[b]	0.0111	0.044
UA	1.71	1.64	1.17	0.187	0.094	2.24	1.96	1.94	0.158	0.365
Mg	2.32[a]	2.06[a]	1.32[b]	0.114	<0.001	2.52[ab]	2.74[a]	2.43[b]	0.089	0.051
Ca	7.80[a]	7.55[a]	4.92[b]	0.415	<0.001	8.33	8.66	8.22	0.169	0.187
P	6.53[a]	5.85[a]	4.06[b]	0.323	<0.001	7.49[a]	7.50[a]	6.61[b]	0.171	<0.001

[1]Diets: Control: corn-soybean based diet, ALT: soybean in the control diet was partially replaced by sunflower meal, brewer's dried grain, and wheat middlings, ALT+BSFL: black soldier fly dried larvae were added to ALT1.

[2]SEM: Standard error of means

[a,b] Means within each row/strain/diet with different superscripts differ significantly.

Diet effect was not significant on any of the leucocytes measured (Table 9) except the eosinophil percentage (P<0.05). Alt-2 diet increased eosinophil counts as compared to Alt-1. The control diet was in between.

Table 22. The effect of the diets[1] on white blood cells (%) and H/L of local chickens on slaughter age of 55 d.

Diets[1]	Control	ALT	ALT+BSFL	P value
H/L ratio	0.28±0.03	0.31±0.03	0.29±0.03	0.830
Heterophil, %	14.11±1.74	16.56±1.66	15.98±1.82	0.582
Lymphocyte, %	52.81±3.15	53.12±3.02	56.44±3.31	0.668
Basophil, %	26.40±3.48	25.1±3.33	20.10±3.65	0.418

Eosinophil, %	2.86±0.69 [ab]	1.14±0.52 [b]	4.36±1.39[a]	0.027, χ2=7.183
Monocyte, %	3.80±1.06	3.75±0.70	2.99±0.81	0.629, χ2=0.926

[1]Diets: Control: corn-soybean based diet, ALT: soybean in the control diet was partially replaced by sunflower meal, brewer's dried grain, and wheat middlings, ALT+BSFL: black soldier fly dried larvae were added to ALT+BSFL.

[a,b] Means within each row with different superscripts differ significantly.

ALT and ALT+BSFL diets reduced relative liver weights in both strains. The relative weights of the intestine, spleen, and bursa of Fabricius were not influenced by diets (Table 10)

Table 23. Effect of strain and diet[1] on organ weights (%)

	Local strain					Commercial strain			
	Control	ALT	ALT +BSFL	SEM[3]	P-value	ALT	ALT +BSFL	SEM	P-value
Liver	1.62[a]	1.49[b]	1.52[b]	0.043	0.007	1.48	1.46	0.033	0.853
Intestine	4.25	4.62	4.09	0.17	0.122	3.64	3.71	0.126	0.891
Spleen	0.112	0.116	0.112	0.006	0.922	0.099	0.083	0.0055	0.104
Bursa of Fabricius	0.0516	0.0467	0.0506	0.0417	0.701	0.0484	0.0543	0.0044	0.476

[1]Diets: Control: corn-soybean based diet, ALT: soybean in the control diet was partially replaced by sunflower meal, brewer's dried grain, and wheat middlings, ALT+BSFL: black soldier fly dried larvae were added to ALT+BSFL.

[a,b] Means within each row with different superscripts differ significantly.

Alternative diets reduced villus length and width in the duodenum (Table 11). ALT diet decreased villus length in the jejunum but increased in the ileum. Jejunum and ileum villi lengths were similar in chickens fed control and ALT+BSFL diet. The villus length/crypt depth ratio was not influenced by diets.

Table 24. Effect of diets on villus length (VL), villus width (VW), crypt depth (CD) and ratio between VL/CD in local chickens

	Duodenum				Jejunum				Ileum			
Diet[1]	VL	VW	CD	VL/CD	VL	VW	CD	VL/CD	VL	VW	CD	VL/CD
Control	0.668a	0.077a	0.099a	7.01	0.643a	0.065	0.095	6.82	0.402b	0.063a	0.082	4.95
ALT	0.514b	0.069b	0.095ab	6.54	0.574b	0.061	0.096	6.29	0.441a	0.054b	0.083	5.27
ALT+BSFL	0.616b	0.059c	0.086b	7.62	0.658a	0.059	0.098	7.09	0.403b	0.060a	0.080	4.73
SEM	0.016	0.001	0.003	0.35	0.001	0.001	0.002	0.26	0.001	0.001	0.002	0.17
P	0.018	<0.001	0.043	0.121	<0.001	0.066	0.648	0.092	0.003	<0.001	0.477	0.103

[1]Diets: Control: corn-soybean based diet, ALT: soybean in the control diet was partially replaced by sunflower meal, brewer's dried grain, and wheat middlings, ALT+BSFL: black soldier fly dried larvae were added to ALT+BSFL.

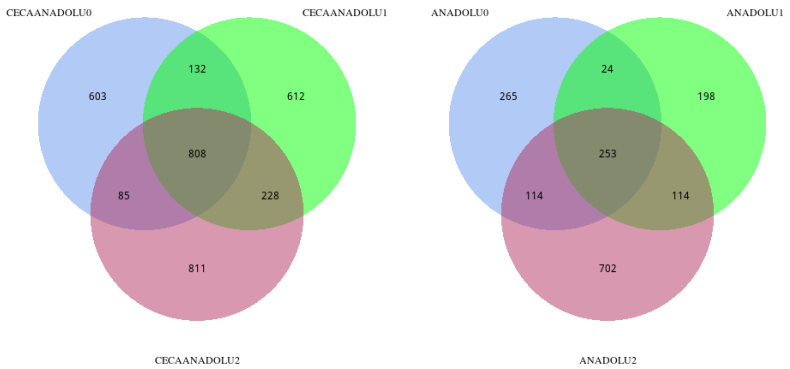
[a,b,c] Means within each row with different superscripts differ significantly

Microbiota

Cecal and fecal microbiota in local chickens

Venn diagrams illustrating the shared and unshared bacteria among the cecal and fecal samples of local chickens fed different diets are given in Figure 15. There was a total of 808 shared Operational Taxonomic Units (OUTs) in cecal samples. Chickens fed control and ALT diets had 132 shared OTUs while chickens fed ALT and ALT+BSFL diets had 228 shared OTUs in ceca. There were 603, 612, and 811 unshared OTUs in chickens fed control, ALT, and ALT+BSFL diets, respectively (Figure 15). Venn diagram illustrating fecal microbiota among the dietary groups showed that a total of 253 bacteria were shared in all groups (Figure 2A). The numbers of individual microbiota in control, ALT, and ALT+BSFL diets were 265, 198, and 702, respectively.

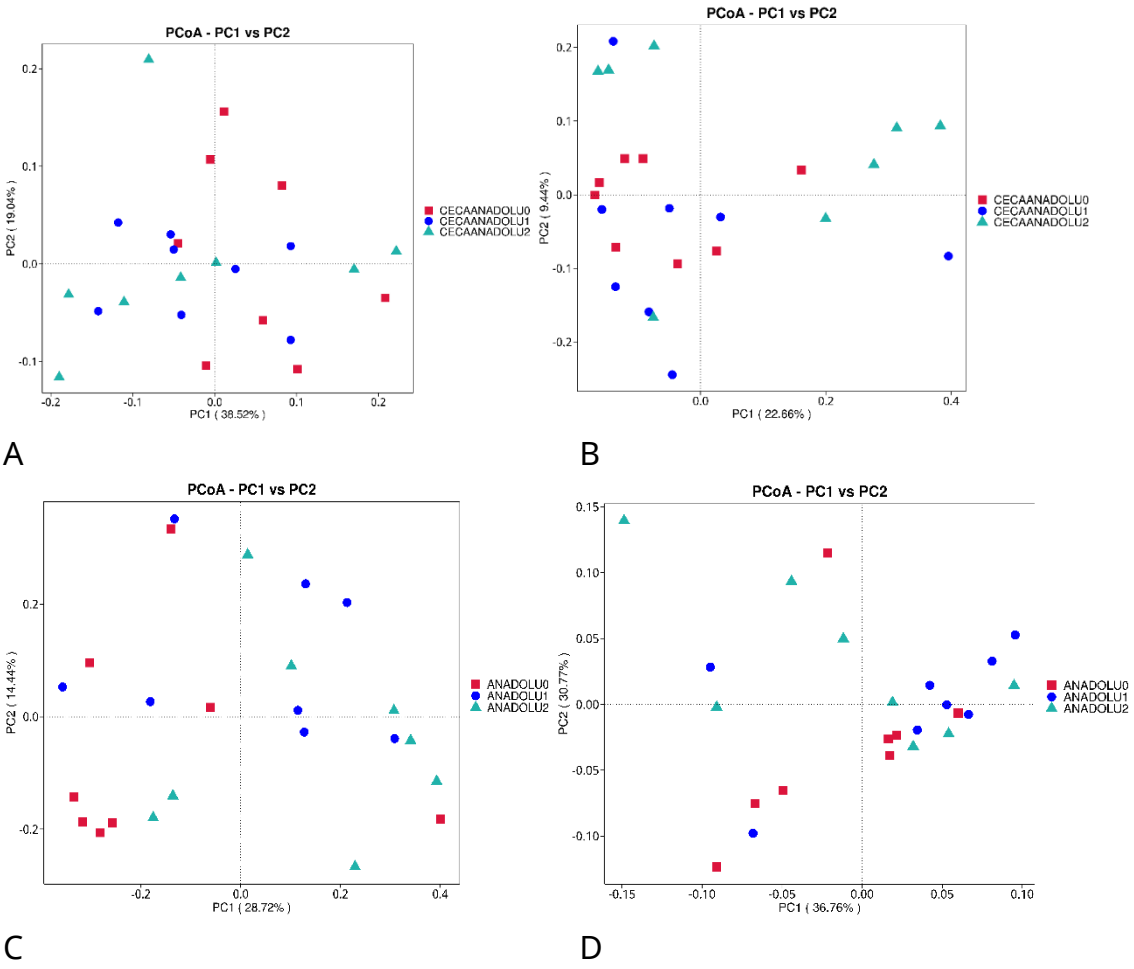
Figure 15. Venn diagram illustrating the shared and unshared bacteria in cecal (A) and fecal (B) samples of local chickens fed different diets (Anadolu0: chickens fed a control diet, Anadolu1: chickens fed an ALT diet, and Anadolu2: chickens fed an ALT+BSFL diet)



The PCoA, based on the UniFrac distance including unweighted and weighted values in ceca and fecal samples of local chickens is presented in Figure 16. The dietary

groups showed no obvious differences in the composition of cecal microbiota in the PCoA distribution (Figure 17 A&B). Similarly, the PCoA plots of weighted and unweighted UniFrac distances did not demonstrate a clear distinction among the chickens fed different diets (Figure 17 C&D).

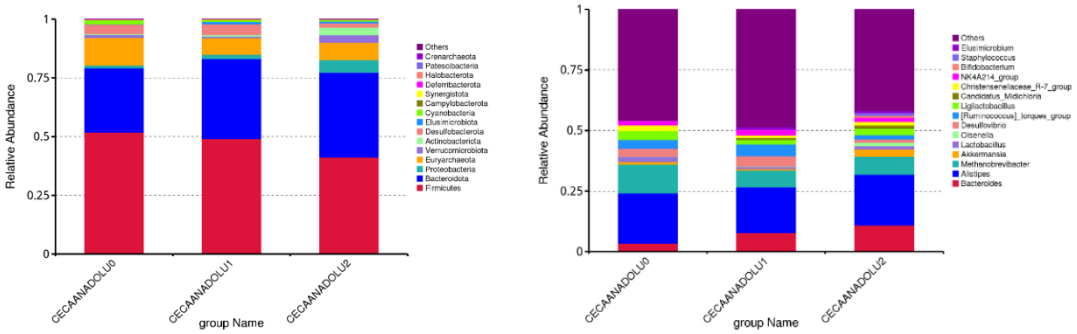
Figure 16. Unweighted and weighted UniFrac PCoA plot of cecal (A&B, respectively) and fecal (C&D, respectively) microbiome composition of local chickens (Anadolu0: chickens fed a control diet, Anadolu1: chickens fed an ALT diet, and Anadolu2: chickens fed an ALT+BSFL diet)



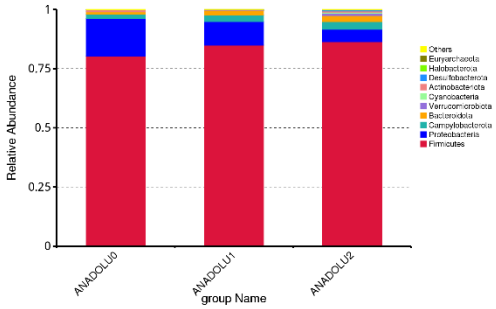
The most abundant phyla and genus with high average relative abundance are presented in Figure 3. At the phylum level, *Firmicutes and Bacteriodota* were the most

dominant phyla in the cecal samples of local chickens (Figure 3A). The relative abundance of Firmicutes and Bacteroides was 51.7 and 27.4 % for chickens fed the Control diet, 49.1 and 34.1% for chickens fed the Alt -1 diet, and 41.1 and 36.1% for chickens fed the Alt-2 diet, respectively (Figure 3A). At the genus level, Bacteroides, Alistipes, and Methanobrevibacter had the highest relative abundance (Figure 3B).
The relative microbial abundances of the fecal samples at phylum indicated that Firmicutes was the most abundant phyla in all dietary treatments (Figure 3C). At the genus level, Ligilactobacillus and Lactobacillus were the most abundant in all groups (Figure 3D).

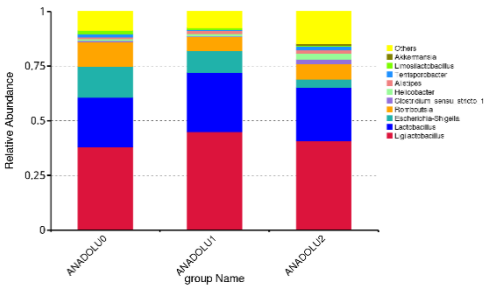
Figure 17. The relative abundance of the microbial composition at the phylum and genus level in cecal (A&B, respectively) and fecal (C&D, respectively) content of local chickens (Anadolu0: chickens fed a control diet, Anadolu1: chickens fed an ALT diet, and Anadolu2: chickens fed an ALT+BSFL)



A B


Relative Abundance
1
0.75
0.5
0.25
0
ANADOLU0
ANADOLU1
ANADOLU2
group Name
Others
Euryarchaeota
Halobacteroia
Desulfobacterota
Actinobacteriota
Cyanobacteria
Verrucomicrobiota
Bacteroidota
Campylobacterota
Proteobacteria
Firmicutes

C


Relative Abundance
1
0.75
0.5
0.25
0
ANADOLU0
ANADOLU1
ANADOLU2
group Name
Others
Akkermansia
Limosilactobacillus
Terrisporobacter
Acetitomaculum
Helicobacter
Clostridium sensu stricto 1
Romboutsia
Escherichia-Shigella
Lactobacillus
Ligilactobacillus

D

Fecal microbiota in commercial chickens

The Venn diagram illustrated the overlap of bacterial communities in fecal samples (Figure 18). A total of 1347 Operational Taxonomic Units (OTUs) clustered and 259, 261, and 312 unique OTUs belonged to the chickens fed control, ALT, and ALT+BSFL diets.

Figure 18. Venn diagram illustrating the shared and unshared bacteria in fecal samples of commercial chickens fed different diets (COBB0: chickens fed a control diet, COBB1: chickens fed an ALT diet, and COBB2: chickens fed an ALT+BSFL diet)

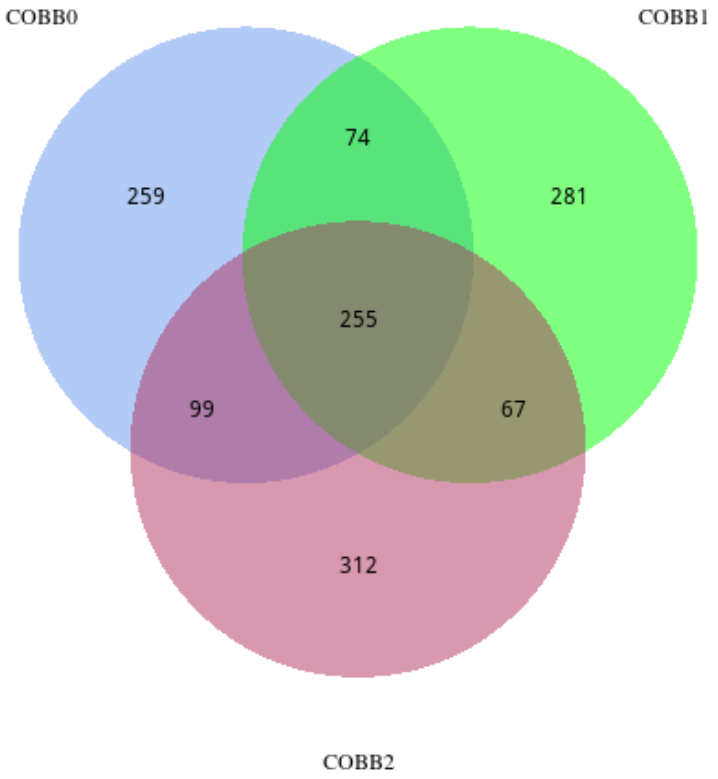
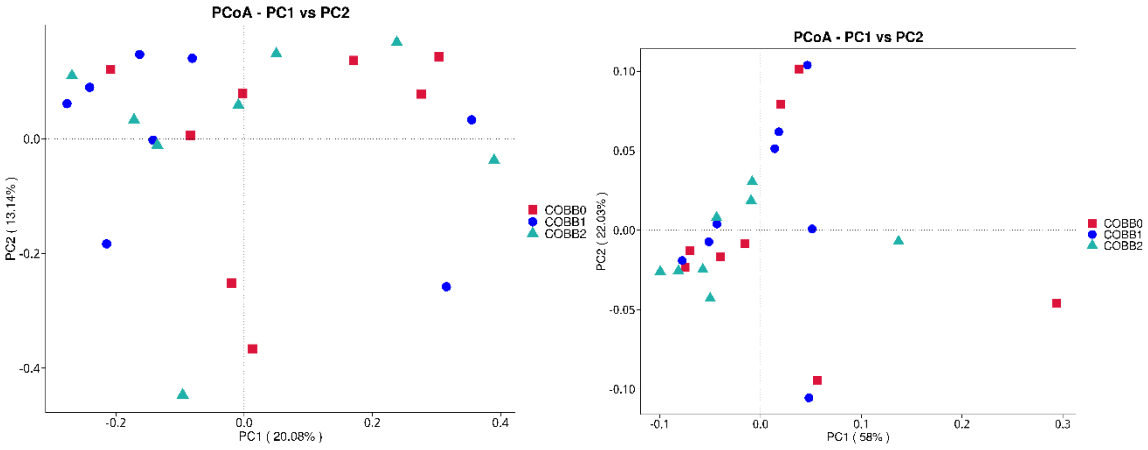


Figure 19 presents an unweighed and weighed UniFrac PCoA plot of the microbiome composition in the fecal content of commercial chickens. The fecal samples of chickens fed different diets were similar to each other.

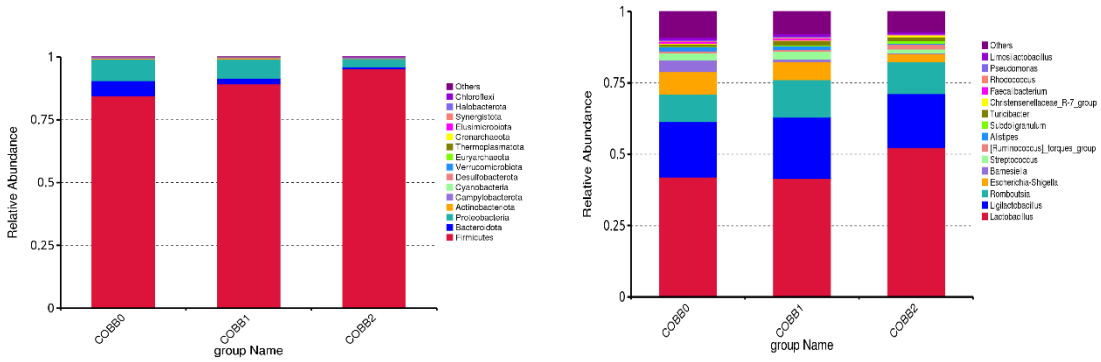
Figure 19. Unweighted (A) and weighted (B) UniFrac PCoA plot of fecal microbiome composition of commercial chickens (COBB0: chickens fed a control diet, COBB1: chickens fed an ALT diet, and COBB2: chickens fed and ALT+BSFL diet)



A B

The most abundant phyla found in the fecal content of commercial chickens were Firmicutes and Proteobacteria in all groups (Figure 20).

Figure 20. The microbial composition of fecal content of commercial chickens representing the relative abundance at the phylum (A) and genus (B) level (COBB0: chickens fed a control diet, COBB1: chickens fed an ALT diet, and COBB2: chickens fed and ALT+BSFL diet)



Conclusion

In conclusion, the significant effect of diets was observed on the percentage of broilers performing walking-standing behavior and thus locomotor behavior which was significantly higher in ALT+BSFL in which insect larvae were added into the diet. This effect was consistent across the strains and increased locomotor activity could be associated with better leg health and welfare. However, in earlier studies, increased activity-related behavior has been associated with the insect larvae provision separately from the diets as an enrichment. For Cobb strain, reduced pecking behavior in ALT fed birds which was similar to ALT+BSFL fed birds on d 14, and increased leg stretching in Control and ALT fed broilers than those ALT+BSFL ones at 35 d old age.

As far as the authors' knowledge, this is the first study on the effect of dry larvae meal inclusion into the diet on the behavioral characteristics of broilers. Therefore, results need to be interpreted with caution and need further studies. Overall, it is clear that ALT and ALT+BSFL did not result in any negative effect on the behavior of broilers under the experimental conditions.

The blood parameters showed that the response of chicken strains to the diets differed which may be related to the feeding period, which was longer for the local chickens

compared to the commercial chickens, and/or their different growth rates and genetic backgrounds.

The ratio between the villi length and crypt depth is one of the most important parameters showing intestinal health. The alternative diets reduced villi lengths in different intestine parts, however, there was no effect of diets on the ratio between villi length and crypt depth showing a sufficiently matured intestinal development. The diets did not introduce significant changes in the microbiota in the cecal and fecal samples at the phyla and genus levels.

ISA-CM

Fattening trial (Meat-type chickens)

Material and methods

Larvae consumption time

The daily calculated quantity of dried larvae was provided at 09:00 am in two identical plates for Alter 2 (including 5% supplemented BSFL) group pens. Simultaneously, two empty plates were also provided to the C and Alter 1 groups to have the same operator interaction in all treatments. The larvae consumption time was recorded daily using a stopwatch, starting from the moment when the plates were placed in the pen and ending when the larvae was totally consumed. The cut-off time was 30 min (Bellezza Oddon et al. 2020).

Tonic immobility

A tonic immobility test (**TI**) was carried out on days 52, 63, 73, and 81 of age on 3 selected identified birds in each replicate pen (45 birds/diet). The test was performed following the method described by Campo et al. (2007) and by the same operator during the whole experimental period. The chickens were placed gently but quickly on their backs, in a V-form wooden stand and the operator immobilized them by placing one hand on their sternum for 15seconds and holding the head with the other hand. As the animal stopped moving, the operator moved away and maintained visual contact with the animal throughout the test. The time recording began when the chicken became immobilized and stopped as soon as the chicken attempted to stand. If the animal moved within the first ten seconds, the test was cancelled and repeated up to three times. After the third attempt, the test is considered invalid and the latency was recorded as 0 seconds. The maximal duration of the TI was 600 s.
After each test, birds were kept in wire-mesh cages placed inside their respective pens for 1 h. Fresh excrement samples were then collected in sterile containers and immediately frozen at -20°C until corticosterone analysis (Biasato et al. 2022).

Avoidance distance test

The avoidance distance test (ADT) was performed on days 53, 67, 74, 82 of age using Welfare Quality® (2009) method. For this test, the same operator approached a group of chickens inside the pen before crouching for 10 seconds. Then, the number of

animals that reduced one-arm length distance to the operator (approx. 1 m) was recorded. There again, all the chickens in the enclosure had the opportunity to interact with the human. This procedure was conducted at selected locations, called observation points, inside each enclosure of the 3 dietary treatment groups. The allocation of observation points was identical between the different feeding groups (Welfare Quality®, 2009; Meuser et al., 2021).

Animal behavior

Chickens' behaviors over 15 pens (5 pens /treatment) were controlled at pen level by video recordings according to the ethogram developed by Ipema et al. (2020) as shown in the table 25. Recordings were performed at 50, 64, 71, 80 days of age 3 times per day. On the recording day, the video cameras were scanned for 10 consecutive minutes at 7am, 9 am (at larvae distribution) and 4 pm (in the afternoon) throughout the light period.

Table 25: Experimental ethogram (Ipema et al.,2020)

Behaviors	**Description**
Feeding	Chickens pecking in feeders
Drinking	Chickens at drinkers
Standing	Stand up
Sitting	Sitting on the floor
Walking/Running	Walking or running with no other discernible activity
Pecking the floor	Pecking the floor
Pecking other birds	Pecking any body part of other birds
Comfort	Other comfort behaviors, such as preening, scratching or wing stretching

Feather cleanliness, hock burn, footpad dermatitis and skin lesions

All broilers were visually assessed at d51, d65, d74 and d83 age of birds and received scores for the following health indicators (Welfare Quality®, 2009; Welfare Quality Network, 2019).

- Feathering scores for back, chest, wing and tail. Feathering scores were from:

  Score 0: fully feathered
  Score 1: rough
  Score 2: some broken feathers
  Score 3: heavily broken feathers

Score 4: almost bald
Score 5: bald

- Feather cleanliness was rated on four levels, from

Score 0: absent
Score 1 and 2: exist
Score 3: severe

- The incidence of skin lesions was also assessed. The whole body was considered for this evaluation. Each chicken was evaluated according to the following scoring system:

Score 0: no lesions, only single (<3) pecks or scratches;
Score 1: at least one lesion < 2 cm diameter at a widest extent or ≥ 3 pecks or scratches;
Score 2: at least one lesion ≥ 2 cm diameter at a widest extent.

- The footpad dermatitis (**FPD**) was scored for both feet as follow:

Score 0: No signs of FPD. The skin of the foot pad is soft to the touch and no swelling or necrosis is clear.
Score 1: The skin of the foot pad is harder and denser than an unaffected foot's.
Score 2: Marked swelling of the foot pad. The reticulated scales are black and form scale-like necrotic areas.
Score 3: Swelling is clear and the total size of the footpad is extended. The reticulated scales are marked, more numerous, and separated from each other. Necrosis spreads to half the plantar pad.
Score 4: Same as score 3, but with more than half the plantar pad covered with necrotic cells.

- Hock burn:

Score 0: no evidence of hock burn
Score 1 and 2: Minimal evidence of hock burn
Score 3 and 4: Evidence of hock burn

Corticosterone analysis

Feather samples were collected from the wings and the back of broilers (2 birds/ replicate pen) at the end of the trial and stored until feather corticosterone determination (Ataallahi et al. 2020).

Hematological and biochemical blood traits

At day 84, fresh blood samples were collected from the wing vein of 10 birds per treatment group (2 birds * 5 replicate pen). A volume of 2.5 mL was placed in an K_3EDTA vacutainer tube and analyzed within 2 hours for hematological parameters, using an automated analyzer (Celtac ES MEK-7300K).
The total white blood cells (leukocytes, 10^3, cell/µL) and red blood cell (erythrocytes, 10^6, cell/µL) counts were enumerated. Granular (heterophils, eosinophils, and basophils) and non-granular (lymphocytes and monocytes) leukocytes were differentiated and counted. Hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and platelets) were also determined. The heterophil to lymphocyte ratio (H/L) was subsequently calculated.
For serum biochemical analysis, blood samples were collected from the jugular vein of 10 birds per treatment group (2 birds * 5 replicate pen), during the slaughtering procedure. A total volume of 2,5 ml was placed in serum-separating tubes and centrifuged at 3000 rpm for 15 minutes to obtain serum, which was then stored at −20°C until further analysis. The serum was assessed for biochemical indices, including glucose, total cholesterol, triglycerides, creatinine, uric acid, urea, total protein (TP) and serum electrolytes, including sodium (Na), chloride (Cl), potassium (K) and alkaline reserves. Additionally, liver activity was evaluated through analysis of aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), and gamma-glutamyl transferase (GGT) using specific commercial kits.

Histomorphometryand histopathology and organs weight

At 86 days of age, 10 birds per dietary treatment group (2 birds per replicate pen) were slaughtered and submitted for anatomopathological investigations. Samples of intestinal segments (approximately 1 cm in length) from the mid-duodenum, mid-jejunum, and mid-ileum were collected and rinsed with 0.9% saline to remove all contents. The liver, spleen, thymus, and bursa of Fabricius were also sampled. Gut segments and organ samples were fixed in a 10% buffered formalin solution for 24 hours for morphometric analysis (gut segments) and histopathological examination (organ samples) and were subsequently transferred to distilled water until analysis (Dabbou et al. 2018).
Formalin-fixed, paraffin-embedded intestinal segments and bursa of Fabricius sections were subjected to hematoxylin and eosin staining (H&E) and examined under a light microscope (Leica DM2000 microscope).

The evaluated morphometric indices included villus height (Vh: from the tip of the villus to the crypt), crypt depth (Cd: from the base of the villi to the submucosa), and their ratio (Vh/Cd) (Laudadio et al., 2012).
A semi-quantitative score was assigned to each sample for the bursa of Fabricius sections, based on a scoring system.

Cecal microbiota analysis

Cecal samples (10 samples per dietary group) underwent DNA extraction using the QIAamp Fast DNA Stool Mini Kit, followed by quality control using capillary electrophoresis, spectrophotometry, and fluorometry. Libraries were then prepared with the QIAseq FX DNA Library Kit and sequenced on the Illumina NextSeq 550 platform. Raw sequencing reads were assessed for quality using FastQCand MultiQC (Ewels et al., 2016), with taxonomic classification performed via Kraken (Wood &Salzberg, 2014) and abundance refined with Bracken (Lu et al., 2017).Pavian(Breitwieser&Salzberg, 2019)was used for visualization. Diversity analysis, encompassing both alpha and beta diversity measures, was conducted using R's vegan package (McMurdie and Holmes, 2013; Oksanen, et al. 2017) and weighted UniFRAC, respectively, with visualization throughPrincipal Coordinates Analysis (PCoA),Non-Metric Multidimensional Scaling (NMDS), and hierarchical clustering.

Results

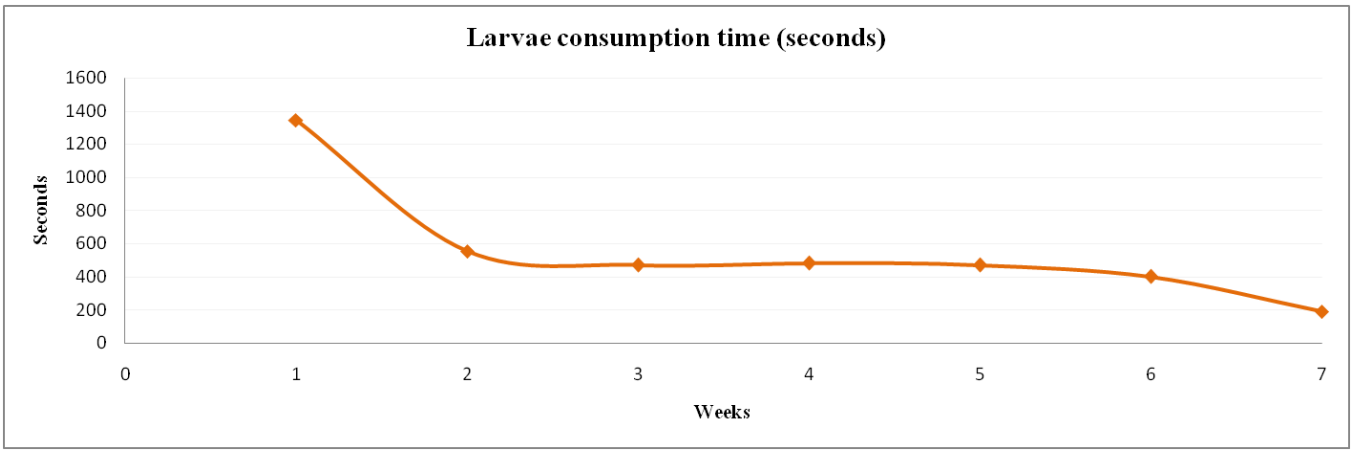
Larvae consumption time

The duration of larvae consumption per week in Alter 2 lot is presented in Table 26. Throughout the 7 weeks, the consumption time displayed a distinct trend of decreasing as the birds matured (Figure 21). The peak value was noted in week 1 (1344.63 s). By week 7, the consumption time had decreased to 190.80 s.

Table 26: Effect of the period on the larvae consumption time of chickens

	Week 1 (D37-D43)	Week 2 (D44-D50)	Week 3 (D51-D57)	Week 4 (D58-D64)	Week 5 (D65-D71)	Week 6 (D72-D78)	Week 7 (D79-D85)	SEM	p-value
Larvae consumption time (seconds)	1344.63 ± 436[a]	554.59 ± 411[b]	470.39 ± 216[b]	480.63 ± 339[b]	470.32 ± 194[b]	399.15 ± 218[b]	190.80 ± 14[b]	186.31	<0.001

Figure 21: Evolution of the larvae consumption time of the chickens



Tonic immobility test

The nonparametric Kruskal-Wallis test was used for statistical analysis of TI data (Table 27). Significant differences were considered when P<0.05.

Diet did not influence the duration of tonic immobility or the number of inductions across the four control days. However, within the control group, chickens required more time to stand as they aged (p=0.0165).

Table 27: Effects of diets and period on tonic immobility (TI) at 51, 65, 74, and 82 days of chickens' age.

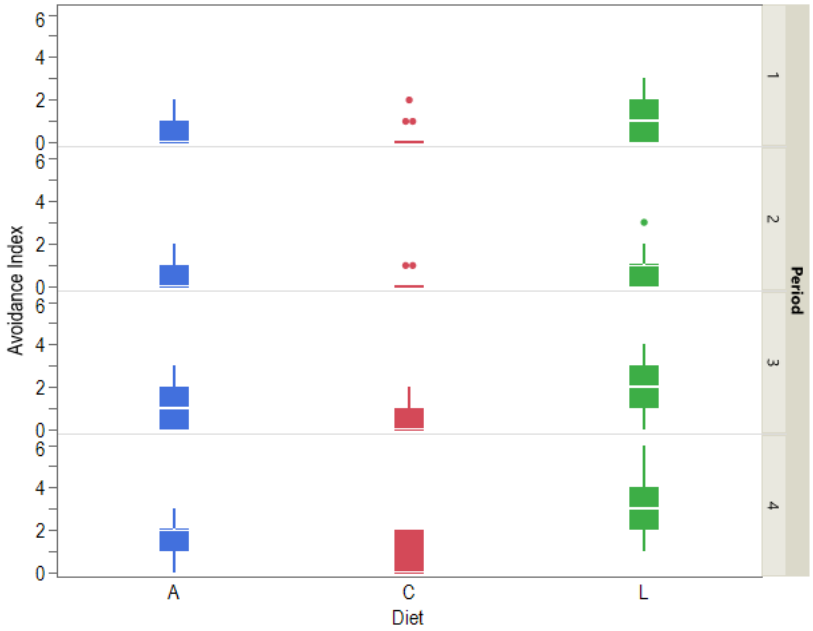
		Diets			
	Parameters	**Control**	**Alter 1**	**Alter 2 (BSFL)**	**p-value**
51 days	Duration (s)	358.2 (375.0-412.2)*	371.8 (334.2-241.8)	309.4 (265.8-477.6)	0.5858
	Induction number	1.06 (0.25)**	1.06 (0.25)	1.06 (0.25)	1.000
65 days	Duration (s)	412.1 (432.0-394.8)	358.2 (387.0-395.4)	346.0 (301.2-381.0)	0.5917
	Induction number	1.46 (0.51)	1.46 (0.74)	1.33 (0.61)	0.6616
74 days	Duration (s)	504.9 (600.0-108.0)	369.8 (375.0-349.8)	368.28 (320.4-295.8)	0.0628
	Induction number	1.46 (0.51)	1.20 (0.56)	1.33 (0.48)	0.2039
82 days	Duration (s)	546.8 (600.0-0.0)	441.7 (562.2-337.2)	425.2 (551.4-345.0)	0.1178
	Induction number	1.46 (0.51)	1.35 (0.49)	1.33 (0.48)	0.7699
p-value		0.0165	0.5658	0.3433	

* Median and interquartile range, ** Standard deviation

Avoidance distance test

Results of the avoidance distance test are represented in figure 1 and summarized in table 28.

Figure 22. Box-and-whisker plot of effect of diets of hens on Avoidance index at 51 (1), 65 (2), 74 (3) and 82 (4) weeks. The box represents the interquartile range (25 and 75), the median line, the X the mean, and the whisker extremes the maximum and minimum. A: Alter 1; C: Control; L: Alter 2.



Results indicated a significant effect of diet on the avoidance index from 65 to 82 weeks. The number of birds approaching the operator was higher in the groups fed alternative diets. Additionally, a significant period effect on AD was observed in these groups, with the avoidance index increasing as the chickens aged.

Table 28: Effects diets and period on avoidance distance test at 51, 65, 74 and 82 days of chickens' age (Kruskal-Wallis test)

	Control	**Alter 1**	**Alter 2**	**p-value**
51 days	0.26 (0.59)	0.40 (0.63)	0.93 (0.96)	0.0559
65 days	0.13 (0.35)	0.46 (0.74)	0.93 (0.88)	0.0101
74 days	0.66 (0.81)	1.06 (0.96)	1.86 (1.24)	0.0180
82 days	0.66 (0.89)	1.53 (0.91)	2.93 (1.57)	0.0020
p-value	0.1105	0.0018	0.0030	

Welfare evaluation

The results of the welfare check-up are shown in Table 29. No significant differences between groups were observed for the body scoring parameters except for hock burn scores.
Between 74 and 82 days of age, the control group exhibited a higher percentage of birds with minimal hock burn (score 1) compared to the other groups, with 13.33% at 74 days and 10% at 82 days.

Table 29: Effect diets on the percentage of feather cleanliness, feather condition, skin lesions, hock burn, and footpad dermatitis (% of score)

Feather cleanliness (0,1)	**Control**	**Alter 1**	**Alter 2**	**Fisher's exact**	***P value***
Day 51					
0 (%)	96.67	100.00	98.33	0.1110	0.7740
1 (%)	3,33	0	1,67		
Day 65					
0 (%)	96.67	100.00	98.33	0.1110	0.7740
1 (%)	3,33	0	1,67		
Day 74					
0 (%)	98.33	100.00	98.33	0.2234	1.0000

1 (%)	1,67	0	1,67		
Day 82					
0 (%)	98.33	100.00	96.67	0.1110	0.7740
1 (%)	1,67	0	3,33		
Feather condition (0,1,2)					
Day 51					
0 (%)	90.00	96.67	98.33	0.0079	0.1785
1 (%)	8,33	3,33	1,67		
2 (%)	1,67	0	0		
Day 65					
0 (%)	88.33	96.67	96.67	0.0065	0.1819
1 (%)	10	3,33	3,33		
2 (%)	1,67	0	0		
Day 74					
0 (%)	93.33	95.00	96.67	0.0294	0.9102
1 (%)	5	5	3,33		
2 (%)	1,67	0	0		
Day 82					
0 (%)	93.33	100.00	96.67	0.0132	0.2084
1 (%)	5	0	3,33		
2 (%)	1,67	0	0		
Skin lesion (0,1)					
Day 51					
0 (%)	98.33	100.00	100.00	0.3333	1.0000
1 (%)	1,67	0	0		
Day 65					
0 (%)	98.33	100.00	100.00	0.3333	1.0000
1 (%)	1,67	0	0		
Day 74					
0 (%)	98.33	100.00	100.00	0.3333	1.0000
1 (%)	1,67	0	0		
Day 82					
0 (%)	98.33	98.33	100.00	0.2234	1.0000
1 (%)	1,67	1,67	0		
Hock burn (0,1)					

Day 51					
0 (%)	100.00	100.00	100.00	-	-
1 (%)	0	0	0		
Day 65					
0 (%)	100.00	98.33	98.33	0.2234	1.0000
1 (%)	0	1,67	1,67		
Day 74					
0 (%)	86.67	95.00	98.33	0.0031	0.0442
1 (%)	13,33	5	1,67		
Day 82					
0 (%)	90.00	100.00	100.00	0.0011	0.0035
1 (%)	10	0	0		
Footpad dermatitis (0,1)					
Day 51					
0 (%)	98.33	100.00	98.33	0.2234	1.0000
1 (%)	1,67	0	1,67		
Day 65					
0 (%)	96.67	98.33	98.33	0.1506	1.0000
1 (%)	3,33	1,67	1,67		
Day 74					
0 (%)	93.33	98.33	98.33	0.0404	0.3702
1 (%)	6,67	1,67	1,67		
Day 82					
0 (%)	96.67	98.33	96.67	0.1262	1.000
1 (%)	3,33	1,67	3,33		

Hematological and biochemical blood parameters

The hematological traits of meat-type chickens are summarized in Table 30. The total red blood cell count was unaffected by diet (p = 0.485). In contrast, total white blood cell (leukocyte) counts were significantly influenced by dietary variations, with the highest counts observed in the groups fed alternative diets. Furthermore, the levels of heterophils and eosinophils were diet-dependent, being more prevalent in the control group, while lymphocytes were more abundant in the groups fed on the Alter 1 and **Alter 2(+BSFL)** diets (p = 0.018).

The H/L ratio was significantly reduced in the sustainable diet groups (p = 0.012). The control group recorded a ratio of 0.09, whereas the Alter 1 and **Alter 2** groups had ratios of 0.05 and 0.06, respectively.
The mean corpuscular volume (MCV) was significantly higher in the **Alter 2** group (p = 0.002). Conversely, the mean corpuscular hemoglobin concentration (MCHC) was highest in the control group, with a level of 28.12 g/dl.

Table 30: Effect of diets on the hematological traits of chickens

	Diets				
	C	**Alter 1**	**Alter 2**	**SEM**	***P value***
Erythrocytes (10^6/µl)	2.78±0.21	2.71±0.17	2.69±0.17	0.083	0.485
Leukocytes (10^3/µl)	58.12±9.18^{b}	74.64±7.39^{a}	70.41±7.03^{a}	3.544	<0.001
Heterophils (%)	8.67±4.03^{a}	4.22±1.55^{b}	5.33±2.45^{b}	1.281	0.005
Eosinophils (%)	1.20±1.03^{a}	0.20±0.42^{b}	0.50±0.97^{ab}	0.382	0.041
Lymphocytes (%)	86.75±4.21^{b}	91.20±2.74^{a}	90.10±3.03^{ab}	1.515	0.018
Monocytes	4.25±2.19	3.7±1.16	3.4±2.07	0.834	0.592
Platelets (10^3/µl)	2±1.41	2.2±1.23	1.56±0.68	0.515	0.452
H/L Ratio	0.09±0.04^{a}	0.05±0.02^{b}	0.06±0.03^{b}	0.015	0.012
Hemoglobin (g/dl)	9.84±0.73	9.39±0.55	9.63±0.51	0.270	0.259
Hematocrit (%)	35±2.05	34.8±2.04	35.4±2.01	0.911	0.800
MCV (fl)	125.5±1.94^{b}	128.5±3.27^{ab}	130.33±2.75^{a}	1.213	0.002
MCHC (g/dl)	28.12±0.61^{a}	27.01±0.97^{b}	27.16±0.31^{b}	0.307	0.002
MCH (pg)	35.24±0.81	34.77±1.66	35.72±1.31	0.585	0.284

Analysis of the biochemical serum parameters (Table 31) indicated that the diet significantly influenced certain traits while leaving others unchanged. Urea concentrations were significantly reduced in the groups fed sustainable diets (ALTER 1 and **Alter 2(+BSFL)**) with levels of 0.03 g/l and 0.028 g/l, respectively, compared to the

control group (0.047 g/l). In contrast, levels of key electrolytes, including sodium, chloride, and alkaline reserve, showed a marked increase in these groups compared to the control. Regarding liver enzymes, only the ALT level was influenced by the diets, which was higher in the ALT1 group than in the other groups (P<0.001).

Table 31: Effect of diets on biochemical parameters of meat-type chickens' serum

	Diets				
	C	**Alter 1**	**Alter 2**	**SEM**	***P value***
Glucose (g/l)	2.9±0.44	2.69±0.56	2.85±0.60	0.240	0.676
Total cholesterol (g/l)	1.66±0.51	1.38±0.20	1.57±0.43	0.180	0.279
Triglycerides (g/l)	0.25±0.08	0.33±0.10	0.34±0.14	0.049	0.138
Creatinine (mg/l)	2.32±0.64	2.34±0.63	2.72±0.73	0.297	0.329
Uric acid (mg/l)	41.8±13.21	42.19±7.37	44.57±15.23	4.995	0.865
Urea (g/l)	0.047±0.01 **a**	0.03±0.01 **b**	0.028±0.01 **b**	0.004	<0.001
Total protein (g/l)	39±11.54	39.80±9.13	40.22±8.94	4.445	0.962
Sodium (mmol/l)	82.56±6.18 **b**	117.11±33.4 **a**	119.78±31.05 **a**	11.882	0.006
Chloride (mmol/l)	67.4±6.96 **b**	86.56±24.14 **ab**	89.89±21.82 **a**	8.593	0.030
Potassium (mmol/l)	4.12±0.73	4.10±1.20	4.61±1.50	0.530	0.564
Alkaline reserve (mmol/l)	11.90±1.10 **b**	16.90±3.67 **a**	15±3.83 **ab**	1.398	0.005
AST (UI/L)	349.20±103.69	321.25±32.60	277.11±75.55	34.178	0.124
ALT (UI/L)	10.33±0.94 **b**	14.4±3.56 **a**	10.25±0.62 **b**	0.966	<0.001
GGT (UI/L)	26.33±8.11	28.56±8.71	29.11±4.01	3.242	0.667

Organs weight,histomorphometry and histopathology

The dietary treatments did not affect the edible giblets' relative weight (%SW), the spleen, thymus, bursa of Fabricius, or abdominal fat relative weight (%SW).

Table 32. Effect of diets on Organs relative weights of slow meat chickens (n=10 birds/diet)

Relative weight of organs and abdominal fat (%SW)					
	C	**Alter 1**	**Alter 2**	**SEM**	***P value***
Spleen	**0,19±0,048**	**0,17±0,046**	**0,20±0,04**	**0,020**	**0,294**
Thymus	**0,225±0,032**	**0,262±0,075**	**0,260±0,038**	**0,023**	**0,222**
Bursa of Fabricius	**0,128±0,77**	**0,103±0,40**	**0,119±0,05**	**0,026**	**0,563**
Heart	**0,57±0,063**	**0,58±0,062**	**0,55±0,022**	**0,023**	**0,331**
Liver	**1,59±0,14**	**1,64±0,15**	**1,75±0,24**	**0,083**	**0,166**
Empty gizzard	**1,54±0,25**	**1,40±0,25**	**1,53±0,39**	**0,138**	**0,525**
Abdominal fat relative	**3,39±0,73**	**3,59±0,67**	**3,10±1,31**	**0,425**	**0,521**

SW: slaughter weight

Alternative diets supplemented or not with dried full-fat BSF larvae had no significant impact on the proportions of internal organs (%SW) and abdominal fat relative weight (%SW) of meat chickens. Relative weights of organs all the groups were similar. Similar findings were reported by Acar et al. (2024) for commercial broiler chickens. Their study revealed that diets with BSFL meal added to a diet in which soybean was partially replaced with agri-industrial by-products (brewers' dried grain, wheat middling, and

sunflower meal) did not affect internal organ weights. However, a previous study on broilers fed diets containing 200 g/kg faba bean demonstrated a heavier relative gizzard weight, which was explained by the contribution of dietary non-starch polysaccharides (Nalle et al., 2010). Similar to our findings, the offal relative weights of Barbary partridges fed BSFL meal were unaffected (Loponte et al. 2017).

The intestinal histomorphometryanalysis indicated that the diet influenced the intestinal morphometric indices. The jejunum exhibited higher Vh in the Alter 2 group, as well as increased Vh/Cd in both the Alter 1and Alter 2 groups, with a higher Cd observed in the C group. The ileum demonstrated elevated Vh and Vh/Cd in the Alter 1and Aletr 2 groups compared to the C group.

Table 32: Effect of diet on the intestinal morphometric indices of the broiler chickens

	Jejunum			Ileum		
Diet	**Vh (µm)**	**Cd (µm)**	**Vh/Cd**	**Vh (µm)**	**Cd (µm)**	**Vh/Cd**
Control	**1157.91±124.29b**	**238.06±15.46a**	**4.86±0.45b**	**759.31±192.13b**	**164.85±10.20**	**4.36±0.91b**
Alter 1	**1288.88±167.66ab**	**159.86±39.60b**	**8.43±1.93a**	**1126.55±93.16a**	**151.23±6.11**	**7.46±0.68a**
Alter 2	**1417.93±79.60a**	**165.15±12.40b**	**8.62±0.76a**	**1189.07±78.08a**	**163.40±34.18**	**7.54±1.50a**
P value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.294**	**<0.001**
SEM	**57.676**	**11.436**	**0.550**	**58.703**	**9.346**	**0.487**

Vh: villus height from the tip of the villus to the crypt); Cd: crypt depth from the base of the villi to the submucosa; and their ratio (Vh/Cd); (n = 10 per dieatary group)

In the current study, alternative diets, specifically the one supplemented with BSFL (Alter 2), have been shown to influence the intestinal morphology of broiler chickens. Indeed, longer villi, shorter crypt depth (Cd), and increased villus height to crypt depth ratio (Vh/Cd) were observed in the jejunum of birds fed the Alter 2 diet. Dabbou et al.

(2021) found no effects on the intestinal morphology of broilers fed BSF larvae fat. In contrast, shorter villi, deeper crypts, and reduced Vh/Cd were noted in broilers fed 15% BSFL (Dabbou et al., 2018). The presence of both short villi and deep crypts indicates negative intestinal development.

The histopathological analysis revealed mild to moderate lymphoid depletion in the bursa of Fabricius across all groups (Table 33). However, as shown in Table 33, the diet did not influence the severity of the observed histopathological changes in this organ ($p > 0.05$). This observation aligns with the findings of Schiavone et al. (2017), who noted that the inclusion of dietary BSF larva fat did **not significantly impact (P>0.05)** the severity of histopathological changes in the bursa of Fabricius in broiler chickens.

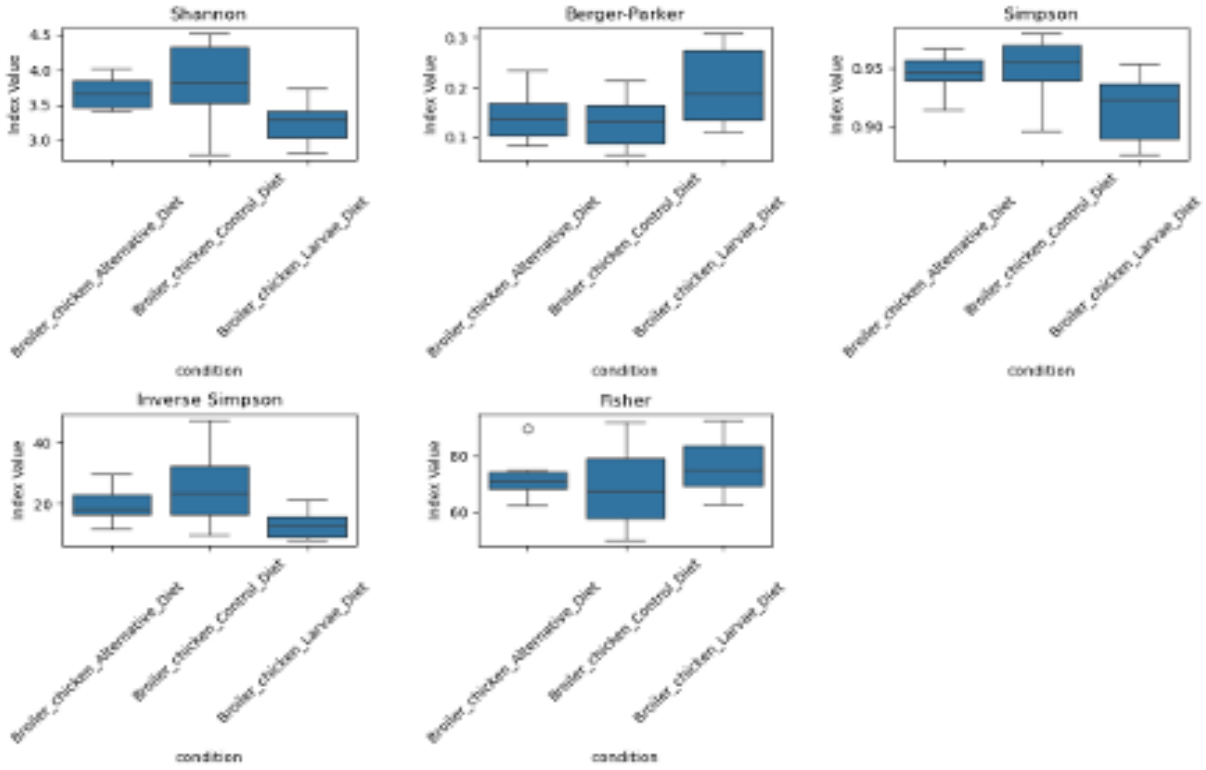
Table 33: Effect of diet on the histopathological scores of the broiler chickens' bursa of Fabricius

Diet	Median	Standard Deviation	P value	X2 (Kruskal-Wallis H)
C	2.20	1.229	0.830	3.001
Alter 1	3.00	0.667		
Alter 2	2.80	0.919		

Cecal microbiota analysis

Alpha diversity indices (Shannon Diversity Index, Berger-Parker Dominance Index, Simpson's Diversity Index, Inverse Simpson Index, and Fisher's Alpha) were computed to assess species richness (the number of taxa) and evenness (how evenly taxa are distributed) within each sample.Alpha diversity analysis (Figure 21) revealed distinct microbial diversities between the control and alternative diets groups, indicating that gut microbial community structure was significantly impacted by dietary treatments ($p=0.01$ for all indices).

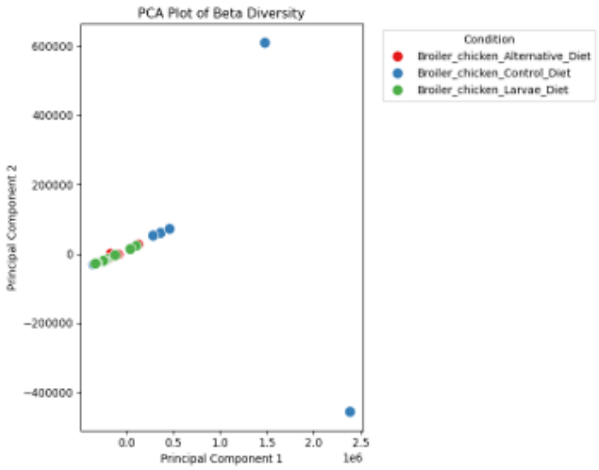
Figure 23. Alpha diversity analysis



Beta diversity was assessed, via pairwise dissimilarities, to compare microbial community composition across samples.

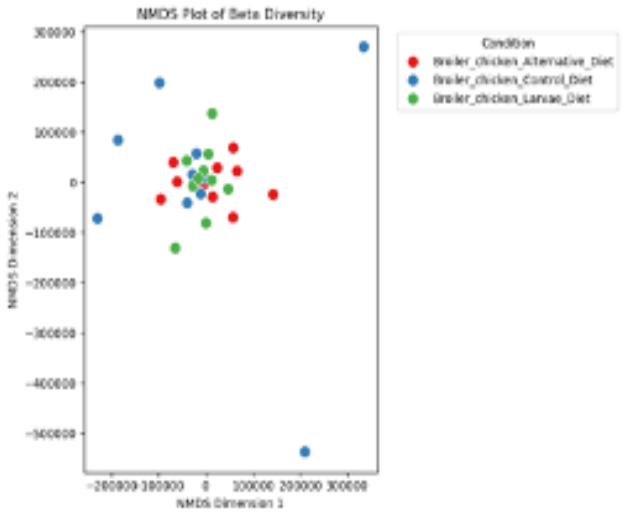
Results visualized through a Principal Coordinates Analysis (PCA)plot (Figure 22) revealed higher variability within the Control diet samples, suggesting key microbial differences driven by the diet.

Figure 24. Principal Coordinates Analysis (PCoA) plot visualizing beta diversity distances among the dietary treatments



NMDS plot (Figure 23) reinforced these findings, demonstrating that Control diet samples were more dispersed, indicating higher variability and the presence of significant outliers, while Alter 1 and Alter 2 diets samples clustered closely, suggesting more homogeneous microbiota.

Figure 25. Non-Metric Multidimensional Scaling (NMDS) plot visualizing the differences in microbial community composition across the dietary treatments



Hierarchical clustering further supported these observations, identifying two major clusters associated with dietary treatment and highlighting the presence of outlier samples within the dataset.

It can be concluded that dietary treatment significantly influenced microbial diversity and community structure in broiler chickens. The Control diet showed high variability, while the Alter 1 and Alter 2 diets resulted in more uniform microbial communities.

Ethogram evaluationEthogram data collected from video recording is still being explored.

Conclusion

In conclusion, the findings highlight the significant effects of dietary variations on the physiological and behavioral aspects of meat-type chickens. Larvae consumption time decreased with age, indicating improved efficiency as chickens grew. Although the diet did not influence tonic immobility responses, a significant age-related increase in the time taken by chickens to stand after induction was observed, suggesting that chickens may become less responsive as they age. In the avoidance distance test, diet significantly influenced the avoidance index, with chickens fed alternative diets showing greater approach behavior towards the operator, and an age-related increase in the avoidance index was observed, indicating a greater tendency for approach as chickens matured.

Regarding welfare and body scoring, no major diet differences were found, except the control group having fewer minimal hock burns. Hematological and biochemical results demonstrated that the diet influenced immune and metabolic markers, with sustainable diets enhancing immune responses and metabolic stability, including lower urea levels and a healthier H/L ratio. Overall, while some welfare aspects were not significantly impacted by diet, the results suggest that alternative diets incorporating sustainable ingredients, especially the diet containing BSFL, can enhance immune function, hematological parameters, and metabolic balance, contributing to improved overall chickens' health and welfare.

ISA-CM

Laying trial (Egg-type chickens)

Larvae consumption time

Dried black soldier fly larvae were provided every day at 9 am to Alter 2 birds. Larva consumption time was recorded every day for all the pens from 30 to 39 weeksof age. For this evaluation, the operator stood in front of the pen and the stopwatch was started when the two plates touched the floor of the pen and stopped when the hens consumed all the distributed larvae. Then, the plates were immediately removed from each pen. Control birds received two empty plates to avoid differences between treatments. If the hens do not consume all the quantity of larvae provided within the first 30 minutes, the operator pours the remaining quantity into the feeders, (Bellezza Oddon et al. 2020).

Animal behavior

Behavioral observations were performed five times over the 10-weeks experimental period (d18, d32, d47, d60 and d69). For each observation day, video recordings were performed for 10 minutes during the hour before, within and after larva provision.
The data will be analyzed with BORIS (Behavioral Observation Research Interactive Software)version 7.13.8 according to the following ethogram:

BEHAVIOR CODE	Description
EATING	*Animal engaged in picking feeder*
DRINKING	*Animal engages in picking drinkers*
STANDING	*Standing up*
SITTING	*Sitting down*
WALKING	*Walking or running with no other discernible activity*
PECKING FLOOR	*picking the floor*
GENTIL PECKING FEATHER	*gently pecking other bird feather*
SEVERE FEATHER PEAKING	*Severe pecking on feathers of other chicken. Can result in wounding the other bird; large head movement*

AGRESSIVE PEAKING	*Pecking on head, fighting, sparring*
FORAGE	*Scraping over floor with foot*
GROOMING	*Cleaning itself with beak or feet, feather ruffling, preening*
WING FLAPPING	*Making movements/flapping with the wings*
VOCAL GAKEL	*Making pre-laying sound*
VOCAL ALARM	*Making alarm call*
LAYING	*Laying*
DUSTBATHING	*Lay down in substrate and make fluttering movements*
PERCHING	*Perch use*

Novel object test

For this test, the hens were visited 4 times during the whole experimental period (d16, d33, d48, and d62) according the WelfareQuality® (2009) protocol. The novel object (NO) used in this test was a 50 cm long stick covered with colored stripes. At each visit, the same operator introduced the NO into the pen, moved about 1.5 m away from the NO in order to do not influence on hens' behavior. Immediately, the number of hens gathering around the NO (in a radius of a hen body length) was counted every 10 seconds for a total time of 2 minutes.

Avoidance distance test

The avoidance test was performed at d10, d30, d47and d63 of the experiment following Meuser et al. (2021) and Welfare Quality® Assessment Protocols for Poultry (2009) indications. For this test, the operator came into the pen, crouched down and waited for 10 seconds. Then the total number of birds located 1m far away from the operator was counted. The test was carried out at similar observation points between the feeding groups.

Body scoring

All birds were subjected to a body scoring following Welfare Quality® Assessment protocol for poultry and Welfare Quality Network, Assessment protocol for laying hens (2019). The scoring was carried out 3 times during the trial: at the beginning (d1), the middle (d37), and the end of the experiment (d70) after body weight recording.

Scores for plumage cleanliness

Score 0 = Absent
Score 1 and 2=exist
Score 3 = Severe

Scores for foot pad dermatitis

Score 0 = no evidence of foot pad dermatitis
Score 1 and 2 = Minimal evidence of foot pad dermatitis
Score 3 and 4 = Evidence of foot pad dermatitis

Scores for hock burn

Hock burn is a contact dermatitis found on the skin of the caudal (back) part of the hock joint. The skin is turned dark by contact with litter and consequently skin lesions can result. The scoring scale allows assessment of the severity of these lesions.
Score 0 = no evidence of hock burn
Score 1 and 2 = Minimal evidence of hock burn
Score 3 and 4 = Evidence of hock burn

Scores for feather condition

Feathering scores for back, chest, wing and tail. Feathering scores were from:
Score 0: fully feathered
Score 1: rough
Score 2: some broken feathers
Score 3: heavily broken feathers
Score 4: almost bald
Score 5: bald

Scores for skin lesion

Score 0 = No lesions, only single (<3) pecks (punctiform damage <0.5 cm diameter) or scratches.
Score 1 = At least one lesion <2 cm diameter at largest extent or ≥3 pecks or scratches.
Score 2 = At least one lesion ≥2 cm diameter at largest extent.

Scores for comb pecking wounds

The number of peck wounds on the comb was scored

0 – No evidence of pecking wounds

1 – Less than 3 pecking wounds

2 – Starting from 3 pecking wounds and more.

Blood analyses

At day 69 of the experimental period, fresh blood samples were collected from the wing vein of 10 birds per treatment group (2 hen/replicate pen). A volume of 2.5 mL was placed in an K_3EDTA vacutainer tube and analyzed within 2 hours for hematological parameters, using an automated analyzer (Celtac ES MEK-7300K).
The total white blood cells (leukocytes, 10^3, cell/µL) and red blood cell (erythrocytes, 10^6, cell/µL) counts were enumerated. Granular (heterophils, eosinophils, and

basophils) and non-granular (lymphocytes and monocytes) leukocytes were differentiated and counted. Hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and platelets) were also determined. The heterophil to lymphocyte ratio (H/L) was subsequently calculated.

For serum biochemical analysis, blood samples were collected from the jugular vein of 10 hens per treatment group (2 hens * 5 replicate pen), during the slaughtering procedure. A total volume of 2,5 ml was placed in serum-separating tubes and centrifuged at 3000 rpm for 15 minutes to obtain serum, which was then stored at −20°C until further analysis. The serum was assessed for biochemical indices, including glucose, total cholesterol, triglycerides, creatinine, uric acid, urea, total protein (TP) and serum electrolytes, including sodium (Na), chloride (Cl), potassium (K) and alkaline reserves. Additionally, liver activity was evaluated through analysis of aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), and gamma-glutamyl transferase (GGT) using specific commercial kits.

Microbiota

DNA Extraction

DNA was extracted from fecal samples using the QIAamp Fast DNA Stool Mini Kit (QIAGEN).

Quality Control of DNA samples

Qualitative control of extracted DNA was assessed by capillary electrophoresis using QIAxcel Connect (QIAGEN). Quantitative control of DNA sample was carried out by spectrophotometry on QIAxpert (QIAGEN) as well as by fluorometry using QUBIT (Thermo Fisher). Libraries were prepared using the QIAseq FX DNA Library Kit (QIAGEN). Qualitative control of prepared libraries was carried out by capillary electrophoresis on QIAxcel Connect (QIAGEN). Quantitative control was carried out by real-time PCR using the QIAseq Library Quant kit (QIAGEN). Libraries were then pooled together and sequenced on NextSeq 550 (illumina) using the High Output kit (paired-end sequencing 2 x 150 bp).

Quality control of raw sequencing reads was performed using **FastQC**[1] to assess the overall quality of the sequencing data. To facilitate the visualization and comparison of multiple FastQC reports, **MultiQC**[2] was used to aggregate the results into a single comprehensive report.

Taxonomic classification of shotgun metagenomic sequencing data was performed using **Kraken2**[3], a k-mer-based classifier that assigns reads to microbial taxa with high precision using the Standard plus PFP database. **Bracken**[4] was then used to refine

abundance estimates by statistically adjusting read assignments at different taxonomic levels, providing more accurate microbial community profiling. The results were visualized using **Pavian**[5], an interactive tool that facilitates exploration of taxonomic classifications, allowing for better interpretation of microbial composition and potential functional implications.
Alpha and beta diversity analyses were performed to assess within- and between-sample microbial community diversity.

Alpha diversity indices were computed to assess species richness and evenness within each sample. The following diversity metrics were calculated using the vegan package in R :

- **Shannon Diversity Index (H')** Measures both species richness and evenness. A higher value indicates a more diverse community.
- **Berger-Parker Dominance Index** Measures dominance by quantifying the proportion of the most abundant species. A higher value indicates lower diversity.
- **Simpson's Diversity Index (D)** Represents the probability that two randomly selected individuals belong to the same species. Lower values indicate higher diversity.
- **Inverse Simpson Index** The reciprocal of Simpson's Index, providing a more intuitive interpretation where higher values correspond to greater diversity.
- **Fisher's Alpha** Measures species richness using a logarithmic model.

To compare alpha diversity metrics across multiple groups (e.g., treatment vs. control), **Dunn's test** (a non-parametric post-hoc test) was used following **Kruskal-Wallis analysis**. Dunn's test was performed using **R** with **Bonferroni correction** to adjust for multiple comparisons.
Beta diversity was assessed to compare microbial community composition across samples. Pairwise dissimilarities between samples were computed using weighted UniFRAC which incorporates phylogenetic relationships and accounts for species abundance, providing a quantitative measure of community divergence.
After computing beta diversity, the results are often visualized using ordination plots:

- Principal Coordinates Analysis (PCoA): Reduces dimensionality to visualize differences between samples.
- Non-Metric Multidimensional Scaling (NMDS): Another method that represents beta diversity distances in a lower-dimensional space.
- Hierarchical Clustering & Heatmaps: Grouping samples based on similarity.

All statistical analyses were performed in **R (vegan[7], ggplot2[8], phyloseq[6])**.

Results

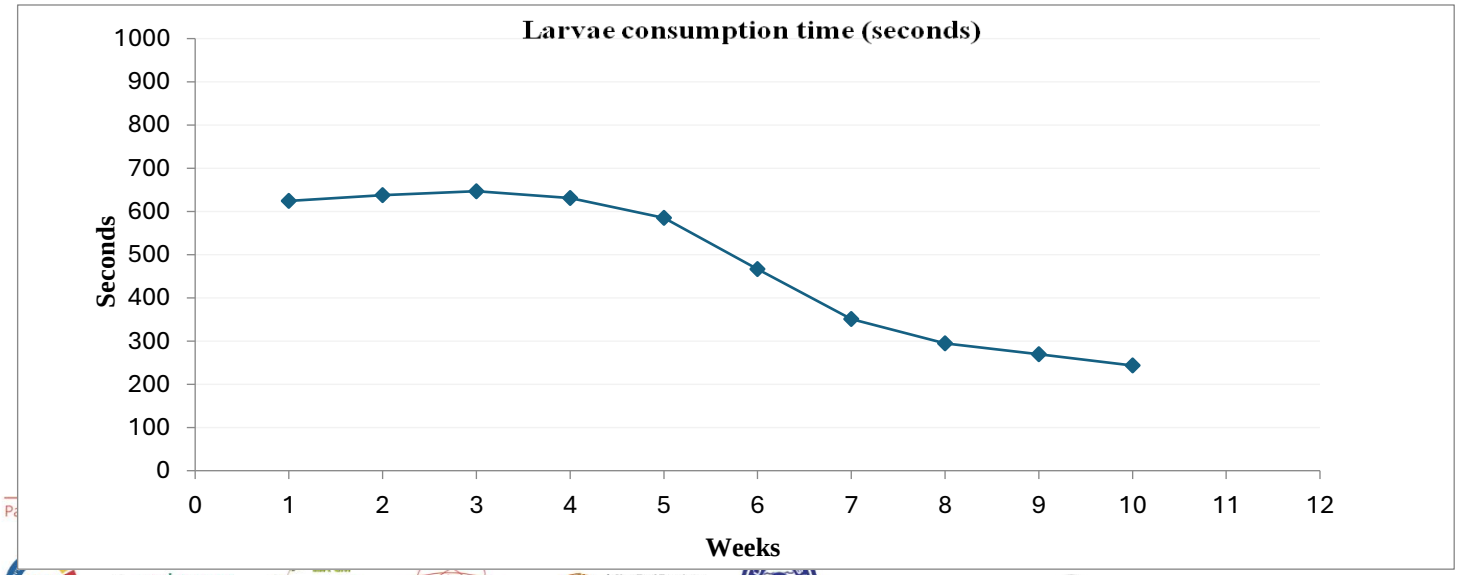
Larvae consumption time

Data on larvae consumption time of laying hens are presented in Table 32 and Figure 23. Overall, the results indicated a clear decline in larvae consumption time by laying hens over the 10-week trial ($p<0.001$). During the first four weeks, consumption time remained stable, ranging from 623 to 646 seconds. However, from week 5 onward, a steady decrease in consumption time was observed, dropping sharply starting in week 6 (466.66 seconds) and continuing to decline through week 10, reaching 243.40 seconds.

Table 34: Effect of the period on the larvae consumption time of laying hens

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	SEM	p-value
Larvae consumption time (seconds)	623.93 ± 111.02^{a}	637.83 ± 106.28^{a}	646.80 ± 111.85^{a}	630.91 ± 101.7^{a}	585.29 ± 98.89^{ab}	466.66 ± 91.82^{bc}	351.16 ± 51.82^{cd}	295.02 ±67.04^{d}	269.27 ±71.28^{d}	243.40 ±24.50^{d}	47.072	<0.001

Figure 26: Evolution of the larvae consumption time of the laying hens



Novel Object test

Table 35 presents the data from the novel object test. The average percentage of hens gathering around the novel object introduced in their pen was significantly influenced by diet (p=0.001). Birds in the ALTER2 group exhibited the highest approach rate (35.21%) compared to the Control and ALTER1 groups which recorded 24.37% and 26.92%, respectively. The period had no significant effect on the approach behavior (p=0.679). However, the interaction between diet and period was highly significant (p<0.001), indicating that the effect of diet on the approach rate was not consistent across different time points. No differences were observed between groups during the first (d16) and second (d33) visits. However, during the third (d48) and fourth (d62) visits, the ALTER2 group consistently showed the highest approach rate, while the control group had the lowest one.

Table 35: Effects diets and period on novel object test at 16, 33, 48 and 62 days of the experiment period.

Control days	**Diets**				**p-value**		
	Control	**ALT1**	**ALT2**	**SEM**	**Diet**	**Period**	**D*P**
16 days (%)	19±10.33	29.33±15.39	33.17±13.58	5.934	0.001	0.679	< 0.001
33 days (%)	41±14.13	25±7.66	27.17±8.79	4.731			
48 days (%)	17.83±1.51^{c}	25.66±1.60^{b}	41.83±5.57^{a}	1.547			
62 days (%)	19.66±2.17^{c}	27.67±1.49^{b}	38.69±5.32^{a}	1.533			
TOTAL	24.37±12.78 ^{b}	26.92±8.14 ^{b}	35.21±10.02 ^{a}	2.791			

Avoidance distance test

Results of the avoidance distance test are presented in table 36.

Table 36: Effects diets and period on avoidance distance test at 10, 30, 47 and 63 days of the experiment period.

Control days	Diets				p-value		
	Control	Alter 1	ALTER2	SEM	Diet	Period	D*P
10 days (%)	30±15.81	40±10	42±13.04	8.327	0.004	0.079	0.773
30 days (%)	32±13.04	36±18.17	42±8.37	8.718			
47 days (%)	20±7.07	26±8.94	34±11.40	5.888			
63 days (%)	28±8.37	26±8.94	46±16.73	7.572			
TOTAL	27.5±11.64^{b}	32±12.81^{ab}	41±12.52^{a}	4.133			

The percentage of hens that approached the operator was significantly influenced by the diet (p = 0.004). Hens from the Alter2 including BSFL group showed a higher approach rate (41%) compared to the other groups, with Control and ALT1 groups showing lower approach rates of 27.5% and 32%, respectively. However, the period did not significantly affect the hens' behavior (p=0.079). The interaction between diet and period was also not significant (p=0.773), indicating that the effect of diet on the avoidance distance remained consistent across the different time points.

Body scoring

The assessment of the hens' body condition was conducted by examining various parameters such as plumage cleanliness, feather condition, skin lesions, hock burns, footpad dermatitis, and comb pecking wounds. A chi-square test was initially applied to assess the overall impact of diet and time period on the distribution of scores for each body part. When significant effects were detected, the frequencies of the scores were further analyzed using the non-parametric Kruskal-Wallis test to evaluate differences between dietary and age groups. Post-hoc pairwise comparisons were

performed using the Mann-Whitney U test, with p-values adjusted for multiple comparisons using the Bonferroni correction to identify significantly different groups. The distribution of scores among the diets for each date is presented in Table 34. Neither diet nor date had an effect on skin lesions, hock burns and feather conditions as all birds received a (score 0) for these parameters at all three time points.

The dietary treatments had a significant effect on the distribution of scores of plumage cleanliness (p=0.002), becoming more evident towards the end of the experiment (d70). Birds in the Alter 1 group were notably cleaner than those in the other groups, with all individuals in this group receiving a score of 0 (indicating no dirtiness, 100%). However, 16% of the examined birds in Alter 2 and 2% of the control group showed slight dirtiness, receiving a score 1. Additionally, 2% of the control group had severe soiling (score 3). Furthermore, only the Alter 2 group's cleanliness scores were dependent on the date, with a decreasing percentage of hens receiving a score of "0" and an increasing percentage of hens receiving a score of "1" as they aged. As for footpad dermatitis and comb pecking wounds, these scores were only dependent on the date. No evidence of footpad dermatitis was observed until day 70 of the experiment (d70), at which point the scores increased, reaching a score of "4" (indicating that more than half of the plantar pad was covered with necrotic cells).

In contrast, pecking wounds were evident from the beginning of the experiment. In all groups, the percentage of birds receiving score "1" (indicating minor pecking wounds) decreased over time. Consequently, the number of birds with a score of "0" (indicating no visible wounds) increased from d1 to d70, suggesting that the wounds were healing.

Table 37: Effect of the diet on the percentage of plumage cleanliness, Footpad dermatitis, and Comb pecking wounds

	Control	**Alter 1**	**Alter 2**	***Kruskall Wallis p-value***	**(χ^2) *p- value***	
					Diet	***Date***
Plumage cleanliness (0,1,2,3) (%)						
Day 1					0.002	0.242
0 (%)	100	98	92	0.291		
1 (%)	0	2	8	0291		
Day 37						
0 (%)	98	98	98	1.000		

1 (%)	2	2	2	1.000		
Day 70						
0 (%)	96^{ab}	100^{a}	84^{b}	0.009		
1 (%)	2^{b}	0^{b}	16^{a}	0.003		
3(%)	2	0	0	0.368		
p-value	0.551	0.602	0.044			
Footpad Dermatitis (0,1,2,3,4) (%)						
Day 1						
0 (%)	100	100	100	1.000		
Day 37						
0 (%)	100	100	100	1.000		
Day 70						
0 (%)	40	60	36	0.447	0.116	<0.001
1 (%)	4	8	2	0.703		
2 (%)	12	10	26	0.672		
3 (%)	30	18	20	0.414		
4 (%)	14	4	16	0.500		
p-value	<0.001	<0.001	<0.001			
Comb pecking wounds (0,1,2) (%)						
Day 1						
0 (%)	62	58	66	0.603		
1 (%)	34	32	30	0.878		
2 (%)	4	10	4	0.421		
Day 37						
0 (%)	84	94	92	0.315		
1 (%)	12	0	4	0.114		
2 (%)	4	6	4	0.891	0.499	<0.001
Day 70						
0 (%)	88	94	86	0.811		
1 (%)	10	6	4	0.679		
2 (%)	2	0	10	0.099		
p-value	0.015	<0.001	<0.001			

Hematological and biochemical blood parameters

The hematological analysis of laying hens (Table 38) revealed that the diet had a significant effect on most of the measured parameters. However, no statistically

significant changes were observed for platelet (p=0.077), hemoglobin concentration (p=0.05), mean corpuscular volume (MCV) (p=0.196) and mean hemoglobin concentration (MCH) (p=0.884). The inclusion of BSF larvae in the Alter 2 group, increased the counts of erythrocytes, leucocytes and lymphocytes compared to Alter1 and Control groups. However, the level of heterophils was the lowest in groups fed Alter 2 diet (including 5% BSFL added on top) . Eosinophils were completely absent in the control group. Interestingly, the H/L ratio was reduced by the sustainable diets, with a more pronounced decrease observed with larvae supplementation.

Table 38: Effect of diets on hematological parameters of laying hens

	Diets				
	C	**Alter 1**	**Alter 2**	**SEM**	***P value***
Erythrocytes (10^6/µl)	2.53±0.31^{ab}	2.14±0.71^{b}	2.68±0.13^{a}	0.204	0.04
Leukocytes (10^3/µl)	213.38±5.12^{b}	35.44±2.92^{c}	233.38±14.2^{a}	3.971	<0.001
Heterophils (%)	87.60±4.60^{a}	63.88±8.10^{b}	7.62±2.54^{c}	2.493	<0.001
Eosinophils (%)	0^{c}	11.22±4.51^{a}	5.22±4.73^{b}	1.687	<0.001
Lymphocytes (%)	13.22±3.79^{c}	21.62±8.27^{b}	87.93±7.16^{a}	2.989	<0.001
Platelets (10^3/µl)	13.22±2.39	12.36±3.07	17.03±7.10	2.090	0.077
H/L Ratio	7.13±2.00^{a}	3.05±0.97^{b}	0.09±0.04^{c}	0.574	<0.001
Hemoglobin (g/dl)	10.56±1.57	10.83±1.33	11.89±0.29	0.536	0.05
Hematocrit (%)	31.86±3.51^{a}	26.13±6.71^{b}	32.36±2.52^{a}	2.061	0.009
MCV (fl)	121.50±7.81	126.23±5.91	121.83±4.99	2.839	0.196
MCHC (g/dl)	34.25±1.29^{b}	35.47±1.86^{ab}	36.47±2.20^{a}	0.814	0.037
MCH (pg)	43.35±6.16	43.96±4.58	44.35±1.64	2.027	0.884

Analysis of the biochemical serum parameters (Table 6) revealed significant dietary effects on several parameters. Triglyceride levels were highest in the Alter 1 group

(14.63 ± 6.55 g/l), significantly exceeding those in the Control and Alter 2 groups (9.79 and 7.79 g/l, respectively, p = 0.003). Sodium levels were also significantly elevated in the Alter 1 group (157 ± 6.57 mmol/l) compared to the other groups. Potassium levels decreased progressively across the groups, with the Control group having the highest concentration (4.64 ± 0.40 mmol/l), followed by Alter 1 (4.34 ± 0.30 mmol/l), and the lowest in Alter 2 (4.07 ± 0.28 mmol/l, p = 0.003). Gamma-Glutamyl Transferase activity was significantly higher in Alter 1 (26.05 ± 7.27 UI/L) compared to both the Control and Alter 2 groups (14.29 ± 4.98 and 14.50 ± 4.71 UI/L, respectively, p < 0.001).

Table 39: Effect of diets on biochemical parameters of laying hens serum

	Diets				
	C	**Alter 1**	**Alter 2**	**SEM**	***P value***
Glucose (g/l)	2.36±0.18	2.34±0.17	2.46±0.10	0.069	0.184
Total cholesterol (g/l)	0.95±0.15	1.10±0.32	0.90±0.12	0.096	0.117
Triglycerides (g/l)	9.79±0.97^{b}	14.63±6.55^{a}	7.79±2.67^{b}	1.843	0.003
Creatinine (mg/l)	2.25±0.37	1.76±0.68	2.18±0.38	0.221	0.076
Uric acid (mg/l)	40.58±8.67	36.93±3.23	44.06±6.72	2.952	0.071
Urea (g/l)	0.013±0.004	0.014±0.009	0.020±0.008	0.003	0.128
Total protein (g/l)	49.90±5.49	50.30±5.08	50.30±7.07	2.657	0.985
Sodium (mmol/l)	151.11±2.02 ^{b}	157±6.57^{a}	153.5±2.49^{ab}	1.887	0.015
Chloride (mmol/l)	113.33±3.81	116.5±4.35	115.44±3.89	1.779	0.218
Potassium (mmol/l)	4.64±0.40 ^{a}	4.34±0.30 ^{ab}	4.07±0.28 ^{b}	0.149	0.003
Alkaline reserve (mmol/l)	21±0.67	20.44±0.50	21.90±3.28	0.874	0.261
AST (UI/L)	237±34.26	226.70±24.27	222±36.27	14.326	0.570
ALT (UI/L)	16.22±6.03	12.90±3.51	15.80±4.10	2.091	0.242
GGT (UI/L)	14.29±4.98^{b}	26.05±7.27^{a}	14.50±4.71^{b}	2.570	<0.001

Organs weight and histomorphology

Table 40 shows the organ weights of hens across three different diet groups: ALT 1, Control, and ALT 2, along with the standard deviations and p-values for each organ's weight comparison. Organs weights and relative weight were not influenced the diets tested (ALT 1, Control, ALT 2) do not have a statistically significant impact on the lengths of the small intestine, caeca, or rectum in hens. These results suggest that, in terms of organ length, the different dietary interventions do not lead to major physiological changes.

Table 40: Effect of diets on organs relative weight of laying hens

Organs relative weight (%SW)	Diets	Mean	SEM	*P-value*
Intestine	C	5 .10	0 .21	
	ALT1	4 .73	0 .12	0 .271
	ALT2	5 .05	0 .17	
Spleen	C	0 .09	0 .00	
	ALT1	0 .09	0 .01	0 .819
	ALT2	0 .09	0 .01	
Thymus	C	0 .13	0 .02	
	ALT1	0 .09	0 .01	0 .129
	ALT2	0 .08	0 .01	
Bursa of Fabricius	C	0 .09	0 .02	
	ALT1	0 .12	0 .02	0 .476
	ALT2	0 .11	0 .01	
Gizzard	C	1 .33	0 .09	
	ALT1	1 .33	0 .09	0 .603
	ALT2	1 .24	0 .04	

Liver	C	2 .39	0 .08	
	ALT1	2 .19	0 .09	0 .303
	ALT2	2 .22	0 .12	
Abdominal fat	C	1 .90	0 .22	
	ALT1	2 .35	0 .21	0 .093
	ALT2	2 .57	0 .21	
Ovary and oviduct	C	8 .97	0 .44	
	ALT1	8 .51	0 .62	0 .328
	ALT2	9 .55	0 .35	
	C	0 .42	0 .01	
Heart	ALT1	0 .41	0 .02	0 .730
	ALT2	0 .40	0 .02	

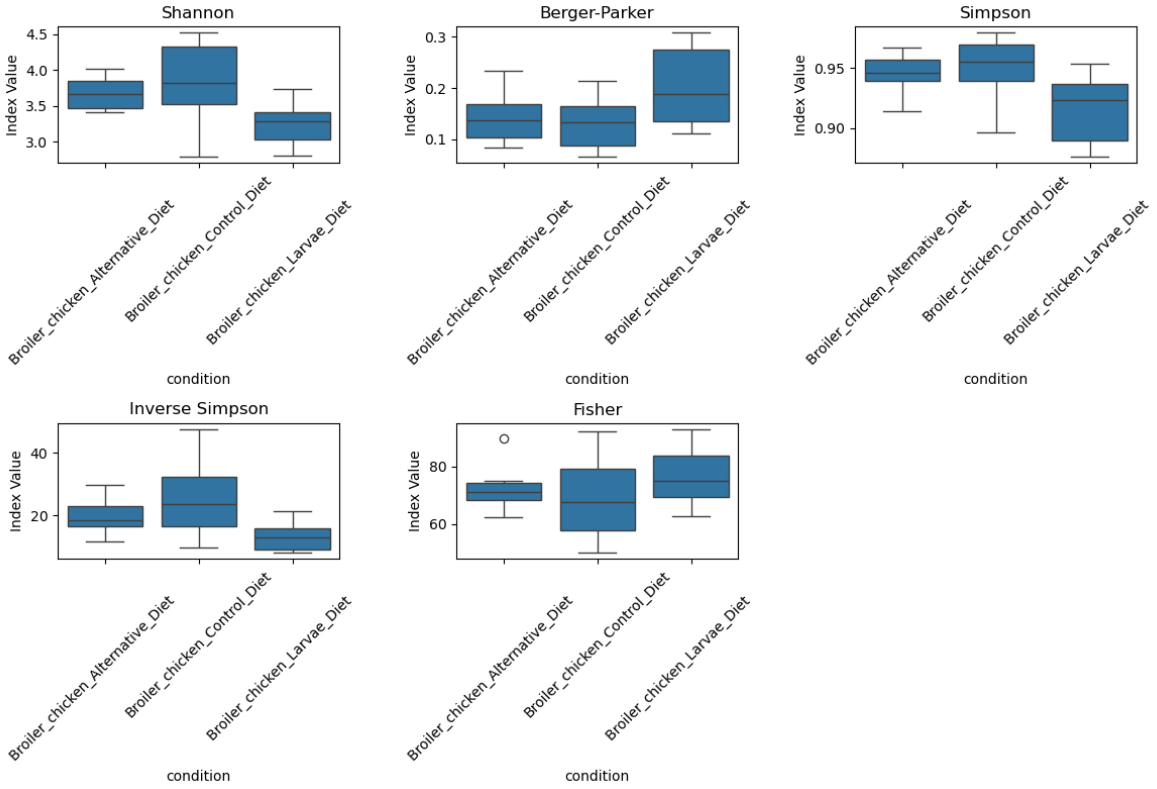
Histomorphology evaluationresults were not collected, since the in vivo essays of ISA-CM were delayed and this analysis is still in progress.

Microbiota

Cecal microbiota analysis

Alpha diversity indices (Shannon Diversity Index, Berger-Parker Dominance Index, Simpson's Diversity Index, Inverse Simpson Index, and Fisher's Alpha) were computed to assess species richness (the number of taxa) and evenness (how evenly taxa are distributed)within each sample(Figure 25).The Simpson Index indicated significant differences in microbial community diversity between control and alternative diet groups (p=0.04). The alternative diets groups (Alter 1 & Alter 2) demonstrated a lower Simpson Index, suggesting increased microbial diversity and a more balanced ecosystem. The Inverse Simpson Index corroborated these findings with significant differences (p=0.04), indicating that the alternative diets led to a more uneven microbial community dominated by fewer taxa.

Figure 27. Alpha diversity indexes



Beta diversity was assessed,via pairwise dissimilarities,to compare microbial community composition across samples.Results revealed that the Alter 2 diet samples exhibited higher distances from both control and Alter 1 diets. The Principal Coordinates Analysis (PCoA) (Figure 26) showed that while the Alter 2 diet samples were widely dispersed, as seen by its dispersed clustering pattern and extreme outliers, control and Alter 1 diet samples clustered closely together, indicating similar microbial compositions. The Non-Metric Multidimensional Scaling (NMDS) plot (Figure 27) further supported these observations, highlighting that the Alter 2 diet induced greater variability in microbial compositions compared to the other diets.

Figure 28. Principal Coordinates Analysis (PCoA) plot visualizing beta diversity distances among the three diet groups

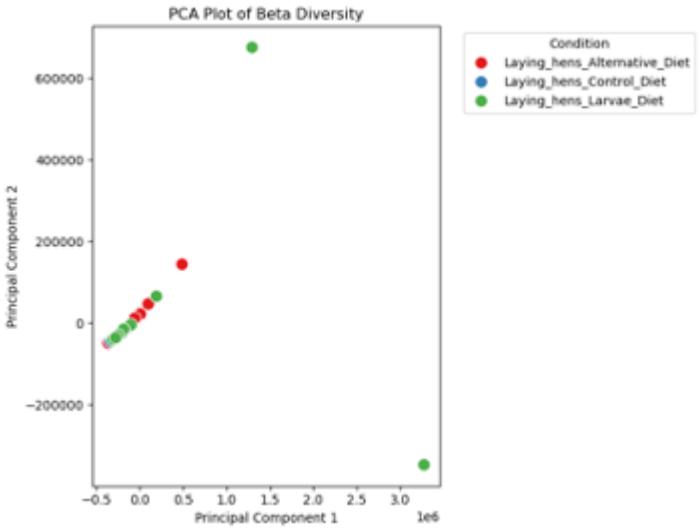
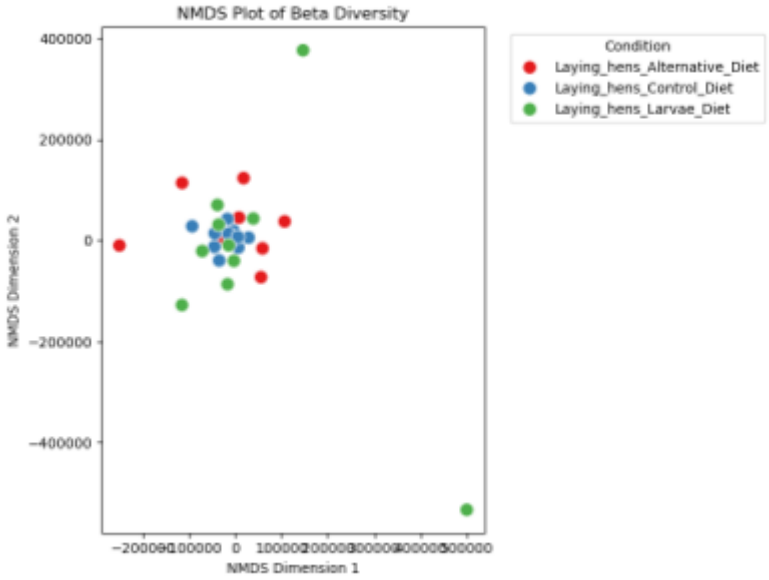


Figure 29. Non-Metric Multidimensional Scaling (NMDS) plot visualizing the differences in microbial community composition across the three diet groups:



Ethogram Evaluation

Ethogram evaluation data are still being explored.

Conclusion

In conclusion, the results demonstrate that dietary variations have a notable influence on the behavioral, welfare, and physiological characteristics of laying hens. Over the 10-week trial, a clear decline in larvae consumption time was observed, indicating improved feeding efficiency as hens matured. As for the novel object test, the diet had a significant effect on hens' approach behavior, with the Alter 2 group consistently showing the highest approach rate, particularly in later visits, indicating increased curiosity or engagement with the novel object. Similarly, the Alter 2 diet also promoted more sociable behavior toward humans, with hens displaying higher approach rates, suggesting reduced fearfulness. Furthermore,dietary treatments impacted plumage cleanliness, with the Alter 1 group maintaining the cleanest plumage. Moreover, while footpad dermatitis increased with age, there was a noticeable decrease in the incidence of pecking wounds over time, indicating healing. Hematological and biochemical analysis demonstrated that sustainable diets resulted in improved immune function, as indicated by higher erythrocyte, leucocyte, and lymphocyte counts, and a reduced H/L ratio. Additionally, significant differences were observed in triglycerides, sodium, potassium, and gamma-glutamyl transferase activity, with dietary changes reflecting metabolic adjustments. Results of organs showed that there is no incidence of diets on their relative weights. Findings from the fecal microbiota analysis suggest that dietary treatment significantly impact microbial diversity in hens. The BSFL supplemented diet notably alters microbial composition, leading to increased variability among individuals. In contrast, the control and Alter 1 diets maintain more stable microbiome structures, indicating that these diets do not significantly affect microbial diversity.

On the whole, these implications suggest that diets with sustainable ingredients, particularly BSF larvae, can enhance laying hens' behavior, welfare, immune function, and metabolic health, promoting better overall health and well-being.

General Conclusions

The integration of Black Soldier Fly Larvae (BSFL) in poultry diets has demonstrated considerable potential as a sustainable and welfare-enhancing strategy. This research, involving contributions from the University of Turin (UNITO), the University of Murcia (UMU), and EGE University (EGE), provides comprehensive evidence of the multiple benefits of BSFL supplementation, including improved growth performance, enhanced behavioral outcomes, reduced stress, and better overall welfare in both slow-growing and commercial chicken breeds.

The combined studies highlight that BSFL, whether live or dehydrated, offer an innovative approach to addressing the challenges of organic and sustainable farming systems. The findings underscore the significant impact of BSFL on welfare indicators, such as reduced aggression, enriched exploratory behavior, and lower stress markers, which are essential for optimizing poultry well-being in low-input, environmentally conscious farming practices.

Key Findings from Each Partner

University of Turin (UNITO)

The research conducted at UNITO demonstrated that slow-growing chickens supplemented with BSFL, particularly live larvae, exhibited marked improvements in natural behaviors such as foraging and exploration. The study highlighted the role of BSFL in reducing aggressive interactions within the flock, particularly in birds supplemented with live larvae, where the movement of the larvae acted as a natural stimulant for foraging. Additionally, stress levels were reduced, as evidenced by lower corticosterone metabolite levels in BSFL-supplemented groups. These results strongly suggest that BSFL can serve as both a nutritional and behavioral enrichment tool, contributing to the welfare and stress reduction in poultry under organic farming systems.

University of Murcia (UMU)

The study conducted by the University of Murcia provides robust evidence that the partial replacement of soybean meal with alternative ingredients, either of plant origin or through supplementation with dried black soldier fly larvae, can be implemented in the diets of laying hens without compromising their welfare, physiological health, or

behavior. The results confirm that alternative diets are viable from a sustainability perspective, maintaining key indicators of animal welfare such as the absence of injuries and diseases, appropriate behaviors, and specific health parameters, and emotional stability. Furthermore, the analysis of physiological and digestive parameters, alongside histological studies, supports the suitability of these diets in preserving the functionality and health of digestive organs and tissues.
In conclusion, the incorporation of alternative ingredients to partially replace soybean meal in hen diets may be an effective strategy to move towards more sustainable poultry farming without compromising hen health and welfare. It should be noted that the use of both non-conventional plant-based ingredients and the use of insect larvae could represent a particularly promising avenue, combining nutritional adequacy with important environmental benefits.

EGE University (EGE)

The findings from EGE University further supported the role of BSFL in enhancing poultry welfare, with a specific focus on microbial health, behavior, and physiological outcomes. EGE's analysis of gut health revealed that BSFL supplementation had a beneficial impact on gut microbiota, which is crucial for long-term immune function and disease resistance in poultry. Moreover, the behavioral data from EGE's research showed that chickens supplemented with BSFL exhibited more natural behaviors, including increased foraging, reduced aggression, and lower stress levels. The histological analysis of intestinal health also provided evidence that BSFL supplementation supports better digestive health, further contributing to the overall well-being of the birds.

ISA-CM

The ISA-CM pilot program provided valuable insights into the effects of BSFL supplementation in both meat-type chickens and layers. For meat-type chickens, results demonstrated significant enhancements in behavioral parameters, such as reduced fear responses during tonic immobility and increased sociability during avoidance distance tests. Improvements in immune and metabolic markers were observed, including a healthier heterophil-to-lymphocyte ratio and optimized liver enzyme activity.
In layers, ISA-CM found that BSFL supplementation significantly influenced behavioral traits, with increased curiosity and approach behavior toward novel objects and humans. Welfare outcomes such as improved plumage cleanliness and reduced fear responses were notable, alongside enhancements in immune function and metabolic

stability. These findings emphasize BSFL's potential to improve welfare, behavior, and overall health in layers, making it a viable option for sustainable egg production systems.

Implications for Sustainable Poultry Farming

The combined results of this study highlight the significant advantages of incorporating BSFL into poultry diets, particularly in organic and low-input farming systems. BSFL represent a sustainable alternative to conventional protein sources, such as soybean meal and fishmeal, which have significant environmental impacts. By utilizing organic waste as feed for BSFL, poultry farmers can reduce their environmental footprint, while simultaneously providing a high-quality, protein-rich supplement that enhances animal welfare.

The behavioral enrichment provided by live BSFL is particularly valuable for slow-growing breeds, which are more sensitive to environmental stimuli and require greater levels of engagement to reduce stress and improve welfare outcomes. As consumer demand for ethically produced and sustainable food products increases, the use of BSFL aligns with the growing trend toward eco-friendly and welfare-focused farming practices.

Future Research Directions

While this study provides compelling evidence of the benefits of BSFL supplementation, further research is needed to fully understand the long-term effects of BSFL on poultry health and production. Specifically, future studies should focus on:

1. **Long-term welfare and health outcomes**: Investigating the effects of prolonged BSFL supplementation on gut health, immune function, and overall productivity in both slow-growing and fast-growing commercial breeds.
2. **Economic viability**: Conducting cost-benefit analyses to determine the economic sustainability of large-scale BSFL production and supplementation, particularly in organic and free-range farming systems.
3. **Nutritional optimization**: Exploring the ideal inclusion rates of BSFL in poultry diets to maximize both welfare and productivity without compromising growth performance or feed efficiency.

4. **Environmental impact assessments**: Quantifying the environmental benefits of BSFL supplementation in terms of reduced greenhouse gas emissions, land use, and water consumption compared to conventional protein sources.

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