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In vivo and in vitro protein digestibility of dry and wet dog food containing black soldier fly (Hermetia illucens) and yellow mealworm (Tenebrio molitor) larvae

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Abstract

· **Background:** Growing demand for sustainable protein has increased interest in insect-derived ingredients for pet nutrition. Larvae of *Tenebrio molitor*(TM) and *Hermetia illucens* (HI) are promising alternatives due to high protein content, favorable amino acid profiles, and lower environmental impact. This study evaluated TM and HI larvae in wet and dry dog foods, focusing on protein quality and digestibility. The aim of this study was to evaluate the effect of Tenebrio molitor and Hermetia illucens larvae inclusion in wet and dry dog food on protein digestibility and quality.

· **Results:** Five healthy adult Beagle dogs were fed six isonitrogenous diets (control wet and dry, experimental diets with TM and HI larvae) in a Latin square design. Apparent total tract digestibility of dry matter (DM) and crude protein (CP) was calculated using total fecal collection. *In vitro*digestibility was assessed with a two-step enzymatic method. Amino acid composition, essential amino acid index (EAAI), biological value (BV), and nutritional index (NI) were calculated. Dogs maintained stable body weight, condition, and fecal quality, indicating good tolerance. Inclusion of TM and HI increased dietary protein and improved amino acid profiles. Wet diets had higher *in vivo* DM and CP digestibility than dry diets ($p < 0.01$). TM diets generally showed higher digestibility than HI, while dry diets with HI had the highest EAAI and BV, indicating superior protein quality despite slightly lower digestibility.

· **Conclusions:** The inclusion of Tenebrio molitor and Hermetia illucens larvae meals in dog food enhanced protein content and provided high-quality amino acids without adverse health effects. Although digestibility varied depending on diet type and insect species, both larvae types indicated strong potential as sustainable and nutritionally adequate protein sources in pet nutrition.

Background

The growing global demand for sustainable and high-quality protein sources has intensified interest in the use of insect larvae in pet nutrition. Insects offer a unique nutritional profile characterized by high levels of protein, fat, and essential amino acids (AA), along with a favorable fatty acid composition. Moreover, insect farming provides significant environmental advantages, including efficient feed conversion, reduced greenhouse gas emissions, lower water use, and minimal land requirements [1]. These characteristics make edible insects an attractive and environmentally friendly alternative to conventional animal-derived proteins.

Among the various insect species, the black soldier fly (*Hermetia illucens*, HI) and the yellow mealworm (*Tenebrio molitor*, TM) have received particular attention due to their favorable AA composition, digestibility, and suitability for large-scale farming on organic waste materials [2, 3]. Their larvae are rich in protein (35–69%) and fat (15–49%), depending on the substrate on which they are reared [4, 5]. This composition is comparable to traditional sources such as meat or fish meal [6]. Protein of mealworm larvae (TM) is characterized by a high amino acid value [7]. It is rich in essential amino acids for carnivores, including tyrosine (67.2 mg/g BO), which is an uncommon amino acid in meat, approx. 34 mg/g [8]. Protein concentrations in HI larvae meals have been reported to range from 362 g/kg [9] to 561

g/kg [10], which is comparable to other protein sources commonly used in dog food, such as meat or fish meal. Compared to crickets and mealworms, the black fly has a more stable nitrogen and phosphorus composition and a more favorable feed conversion ratio [2].

Moreover, chitin present in raw materials obtained from insect larvae may be partially fermented and may modulate the gut microbiome of dogs, in which chitinase activity is low [11]. Studies on the digestibility and nutritional value of such additives are essential for their market acceptance and for assessing sustainability from a life cycle assessment (LCA) perspective. The inclusion of insect protein in pet food therefore represents a promising strategy to improve sustainability in the pet food industry while maintaining nutritional adequacy.

Studies on the use of TM and HI larvae in dog nutrition have shown promising results. Analysis of the digestibility of dog food containing insect meal as the sole protein source (36.5% content) showed promising results in terms of nutritional values similar to a meat-based diet, indicating its suitability as a sustainable protein source in pet food [2]. Lemos [12] found that the addition of mealworm meal to the diet of dogs did not cause any negative health issues. Studies by El-Wahab et al. [3] and Kröger [13] on the use of HI indicated high protein and fat digestibility and no negative effects on the immune system of dogs. These results suggest that HI larvae meal is a promising and well-tolerated alternative protein source in dog food, offering potential sustainability benefits without compromising nutritional quality or animal health.

Freel et al. [14] conducted two studies to assess the acceptability, safety, and digestibility of diets containing different levels of partially defatted black soldier fly (*Hermetia illucens*) meal and oil by Beagle dogs. The addition of both black soldier fly meal and oil showed high apparent digestibility, and their consumption did not cause any disturbing physiological effects [14]. Additionally, Lei et al. [15] reported anti-inflammatory and antioxidant effects of adding an HI meal to the diet of dogs.

Penazzi et al. [2] fed two isonitrogenous and isoenergetic dry diets containing either venison meal or black soldier fly larvae meal as the main protein sources to healthy dogs. The authors found that both diets showed similar dry matter nutrient digestibility values, but a statistical trend ($p = 0.066$) was observed for the diet with HI larvae meal, indicating greater protein digestibility compared to the control diet [2]. In contrast, crude fiber digestibility was lower in the insect-based diet ($p < 0.001$) [2].

The use of insect derived ingredients in pet diets has been authorized under Commission Regulation (EU) 2017/893, which defines seven insect species approved for use in feed, including HI and TM. This regulatory framework has further encouraged the development of insect-based pet foods and research into their nutritional and physiological effects.

Given the increasing importance of sustainable nutrition in companion animal diets, the present study focused on evaluating the nutritional contribution of TM and HI larvae as alternative protein sources in dog food. The specific objective of the study was to assess whether the inclusion of these insect larvae could enhance the protein nutritional value of wet and extruded diets for dogs without negatively

affecting protein digestibility. The working hypothesis assumed that the addition of TM and HI larvae would improve the protein quality of the diet while maintaining comparable apparent digestibility to that of conventional formulations.

Results

Body weight and body condition score remained stable throughout the study (Table 1). No changes in fecal consistency were observed across dietary treatments, as assessed using the Purina and Waltham scoring systems (Table 2).

Table 1
Average body weight and condition on a 9-point scale of dogs during each stage of the experiment.

Stage	Body weight [kg]	BCS 9st
Beginning of experiment	14.56	5
End of experiment 1	14.76	5
End of experiment 2	14.68	5

BCS Body Condition Score.

Table 2
Evaluation of dog fecal scoring charts during each stage of the experiment.

Diet	Purina's scale	Waltham's scale
CW	2	2.5
TW	2	2.5
HW	2	2.5
CD	2	2.5
TD	2	2.5
HD	2	2.5

CW control wet food; TW wet food with TM; HW wet food with HI; CD control dry food; TD dry food with TM; HD dry food with HI.

The inclusion of insect larvae increased the crude protein content in diets based on dry food (Table 7).

The *in vitro* dry matter (DM) digestibility of wet diets ranged from 72.25% to 74.23%, with no significant differences among diets ($p = 0.67$; Table 3). In contrast, *in vivo* DM digestibility differed significantly among wet diets ($p = 0.03$). The highest value was observed for the control wet diet (CW; 83.14%),

whereas lower values were recorded for diets supplemented with *Tenebrio molitor* (TW; 77.45%) and *Hermetia illucens* (HW; 71.26%).

For dry diets, *in vitro* DM digestibility ranged from 55.25% to 63.02% and differed significantly among diets ($p = 0.01$). The highest value was observed for the HD diet. *In vivo* DM digestibility of dry diets ranged from 35.71% to 37.34% and did not differ significantly among diets ($p = 0.50$).

Overall, DM digestibility was significantly higher in wet diets compared to dry diets, both *in vitro* ($p < 0.01$) and *in vivo* ($p < 0.01$).

Table 3
In vitro and *in vivo* digestibility and SD of dry matter (DM) in dry and wet food with insect larvae.

Diet	**DM digestibility *in vitro* [%]**	**DM digestibility *in vivo* [%]**	***p*-Value**
CW	73.41 ^{a} ± 2.46	83.14 *^{A} ± 1.25	0.08
TW	74.23 ^{a} ± 0.60	77.45 ^{A} ± 2.20	0.08
HW	72.25 ^{a} ± 3.46	71.26 * ± 2.14	0.66
p-Value	0.67	0.03	
CD	55.25 **^{b} ± 3.14	35.71 ^{B} ± 3.24	0.01
TD	55.78 * ± 1.94	37.28 ± 1.17	0.01
HD	63.02 */** ± 2.66	37.34 ± 2.53	0.01
p-Value	0.01	0.50	
p-Value of E1 vs E2	< 0.01	< 0.01	

DM dry matter; CW control wet food; TW wet food with TM; HW wet food with HI; CD control dry food; TD dry food with TM; HD dry food with HI;

a, b statistical difference between the experiments $p < 0.05$; A B, statistical difference between the experiments $p < 0.01$;

* statistical difference between the groups $p < 0.05$; ** statistical difference between the groups $p < 0.01$.

In vitro crude protein (CP) digestibility of wet diets ranged from 74.47% to 77.28% and approached statistical significance ($p = 0.05$; Table 4). The highest value was observed for the TW diet. *In vivo* CP digestibility differed significantly among wet diets ($p = 0.01$), with the highest value for the control diet (CW; 79.00%) and the lowest for the HW diet (66.37%).

For dry diets, *in vitro* CP digestibility ranged from 61.67% to 66.68% and differed significantly among diets ($p = 0.01$). *In vivo* CP digestibility ranged from 43.04% to 56.07% and also differed significantly among diets ($p < 0.01$), with the highest value observed for the control dry diet (CD). Overall, CP digestibility was significantly higher in wet diets compared to dry diets ($p < 0.01$).

Significant differences between *in vitro* and *in vivo* digestibility were observed within dry diets for both DM and CP ($p \leq 0.01$).

Table 4
In vitro and *in vivo* digestibility and SD of protein in dry and wet food with insect larvae.

Diet	**CP digestibility *in vitro* [%]**	**CP digestibility *in vivo* [%]**	***p*-Value**
CW	75.11 ± 1.13	79.00 *^{A} ± 0.34	0.05
TW	77.28 ^{A} ± 0.24	78.96 ^{A} ± 0.23	0.05
HW	74.47 ± 1.60	66.37 * ± 0.05	0.05
p-Value	0.05	0.01	
CD	62.78 *^{B} ± 1,67	56.07 ** ± 0.25	< 0.01
TD	61.67B ** ± 1.39	46.86 ^{B} ± 0.19	0.01
HD	66.68 */** ± 1.50	43.04 ** ^{B} ± 1.04	0.01
p-Value	0.01	< 0.01	
p-Value of E1 vs E2	< 0.01	< 0.01	

CP crude protein; CW control wet food; TW wet food with TM; HW wet food with HI; CD control dry food; TD dry food with TM; HD dry food with HI;

a, b statistical difference between the experiments $p < 0.05$; A B, statistical difference between the experiments $p < 0.01$;

* statistical difference between the groups $p < 0.05$; ** statistical difference between the groups $p < 0.01$.

Significant differences in amino acid composition were observed among diets ($p < 0.01$; Table 5). The total amino acid content was highest in the HD diet (362.49 mg/g) and lowest in the HW diet (229.90 mg/g).

Among wet diets, the HW diet was characterized by lower concentrations of several amino acids, including aspartic acid, glycine, alanine, valine, isoleucine, leucine, histidine, lysine, and arginine. Among dry diets, the HD diet showed the highest concentrations of multiple amino acids, including aspartic acid,

threonine, serine, glutamic acid, proline, methionine, isoleucine, and tyrosine, compared to other treatments.

Table 5
Amino acids profile of tested diets.

Amino acid [mg/g]	CW	TW	HW	CD	TD	HD	*p*-Value
ASP	23.45	24.60	20.98 [a]	21.39	25.45	35.06 [b]	0.0073
THR	9.27	10.31	8.39	8.24 [a]	10.43	14.72 [b]	0.0064
SER	9.97	11.55	9.25[a]	10.30	12.60	15.47 [b]	0.0054
GLU	47.60	44.52	37.26	35.95 [a]	45.03	51.32 [b]	0.0081
PRO	20.01 [a]	21.65	22.61	22.02	21.65	25.32 [b]	0.0266
GLY	22.70	21.22	17.33 [a]	23.11	26.22 [b]	19.39	0.0058
ALA	17.42	20.33	16.07	15.65 [a]	20.07	23.56 [b]	0.0071
CYS	2.02	2.28	1.78	1.57 [a]	2.54	2.65 [b]	0.0090
VAL	11.94	15.26	12.04	10.92 [a]	14.77	22.30 [b]	0.0065
MET	4.08	6.35	6.60	2.24 [a]	4.91	16.30 [b]	0.0054
ILE	9.99	13.15	10.49	8.12 [a]	11.94	20.10 [b]	0.0054
LEU	19.21	21.27	16.89	15.67 [a]	20.57	25.86 [b]	0.0054
TYR	6.37 [a]	10.46	9.13	15.79	9.34	23.23 [b]	0.0062
PHE	11.42	11.84	9.84 [a]	19.33 [b]	11.75	15.82	0.0064
HIS	6.85	7.94	5.72	3.51 [a]	6.04	11.33 [b]	0.0054
LYS	16.10	16.58	13.51	10.92 [a]	15.27	21.78 [b]	0.0054
ARG	16.16	16.16	12.02 [a]	14.28	18.24 [b]	18.28 [b]	0.0079
TOTAL	254.55	274.35	229.90	239.03	276.81	362.49	

CW control wet food; TW wet food with TM; HW wet food with HI; CD control dry food; TD dry food with TM; HD dry food with HI;

ASP aspartic acid; THR threonine; SER serine; GLU glutamic acid; PRO proline; GLY glycine; ALA alanine; CYS cysteine; VAL valine; MET methionine; ILE isoleucine; LEU leucine; TYR tyrosine; PHE phenylalanine;

HIS histidine; LYS lysine; ARG arginine;

a, b statistical difference between the experiments $p < 0.05$; A B, statistical difference between the experiments $p < 0.01$.

Significant differences were observed among diets for the essential amino acid index (EAAI), biological value (BV), and nutritional index (NI) ($p < 0.01$; Table 6).

The highest EAAI and BV values were recorded for the HD diet (112.79% and 111.24%, respectively), whereas the lowest values were observed for the CD diet (105.00% and 102.75%, respectively). Among wet diets, the TW diet exhibited the highest EAAI (108.27%) and BV (106.31%), while the HW diet showed the lowest values. For the nutritional index, the TW diet had the highest value among wet diets (34.88%), whereas the CD diet had the lowest value among dry diets (30.71%).

Table 6
Protein quality and biological value.

Diet	EAAI [%]	BV [%]	NI [%]
CW	106.71	104.61	31.88
TW	108.27	106.31	34.88 [a]
HW	105.67	103.48	29.84 [b]
CD	105.00 [a]	102.75 [a]	30.71
TD	107.47	105.44	34.38
HD	112.79 [b]	111.24 [b]	33.10
p-Value	< 0.01	< 0.01	< 0.01

CW control wet food; TW wet food with TM; HW wet food with HI; CD control dry food; TD dry food with TM; HD dry food with HI;

EAAI essential amino acids index; BV biological value; NI nutritional index;

a, b statistical difference between the experiments $p < 0.05$; A B, statistical difference between the experiments $p < 0.01$.

Discussion

This study investigated the effects of incorporating *Tenebrio molitor* (TM) and *Hermetia illucens* (HI) larvae meals into wet and dry dog foods on nutrient digestibility, amino acid composition, and protein quality. The results suggest that both insect species may serve as alternative protein sources for dogs,

supporting growing evidence that insect larvae provide high-quality, sustainable nutrition for companion animals [2, 10, 12, 14].

The stability of body weight and fecal characteristics observed throughout the trial indicates good palatability and tolerance of the insect-based diets. These findings are consistent with earlier reports showing that insect meal inclusion does not cause adverse gastrointestinal or systemic effects in dogs [3, 13].

The addition of insect larvae influenced digestibility parameters differently between wet and dry food formulations. The higher digestibility observed in wet foods confirms that processing methods strongly affect nutrient bioavailability. Extrusion and thermal treatment can cause Maillard reactions, protein denaturation, and reduced enzyme accessibility, which may explain the lower digestibility of dry diets [17, 20]. The lower *in vivo* digestibility values observed in dry diets may also reflect reduced protein solubility and interactions with starch during extrusion, which can reduce amino acid availability [21].

The slightly reduced dry matter (DM) and crude protein (CP) digestibility in diets containing HI compared to control and TM diets is consistent with findings by Penazzi et al. [2] and Freel et al. [14]. This effect is likely due to the higher chitin content in HI larvae, which reduces enzymatic breakdown in the gastrointestinal tract [22, 23]. In experiment by Bosch et al. [24] organic matter in vitro digestibility was highest for yellow mealworms, whereas black soldier fly pupae showed the lowest values. The authors suggested that this difference in digestibility is likely caused by a higher degree of cuticular protein sclerotisation in the pupae [24]. In contrast, TM larvae contain less chitin and a more digestible protein fraction, leading to improved protein utilization [7]. Although chitin may reduce apparent digestibility, it can also provide functional benefits, such as promoting gut health and supporting immune function [15]. Conversely, TM-based diets indicated relatively higher protein digestibility, supporting findings that TM meal proteins have high amino acid availability and lower chitin levels compared to HI [7].

The clear difference between *in vitro* and *in vivo* digestibility values, especially in dry diets, reflects the limitations of enzymatic assays in replicating the complexity of *in vivo* digestion. Nonetheless, the direction and relative ranking of digestibility among diets were consistent, supporting the usefulness of *in vitro* assays as predictive tools [2, 17, 20].

Marked differences in amino acid profiles were observed among the experimental diets. The higher total amino acid content and favorable balance of essential amino acids in the HI-based dry diet correspond with previous reports highlighting the rich amino acid spectrum of HI larvae [4, 9, 25]. TM-based diets were characterized by elevated levels of tyrosine, methionine, and branched-chain amino acids, which are essential for maintaining muscle tissue and coat pigmentation [8, 26].

These findings support prior evidence that insect-derived proteins can achieve amino acid profiles comparable to or superior to those of conventional animal proteins such as poultry or fish meal [7, 10]. The variation in amino acid composition likely reflects differences in larval substrate composition and fatty acid metabolism, which influence nitrogen retention and overall protein quality [27, 28].

The higher essential amino acid index (EAAI), biological value (BV), and nutritional index (NI) observed in diets containing insect meals indicate high protein quality and efficient amino acid utilization. The HI-based dry diet indicated particularly favorable indices, suggesting that insect meal can provide a balanced amino acid composition suitable for canine requirements. These outcomes are consistent with previous assessments of insect protein biological value [4, 10].

Although slight reductions in apparent digestibility were observed in HI-based diets, the overall amino acid balance and protein quality metrics are consistent with that insect meals offer comparable nutritional performance to conventional meat-based diets. Importantly, this was achieved without compromising feed palatability or fecal characteristics, aligning with prior studies suggesting good tolerance of insect proteins by dogs [2, 12].

The comparison between *in vitro* and *in vivo* methods showed significant differences in absolute digestibility values but consistent relative patterns among diets. These findings corroborate earlier work indicating strong correlations (r = 0.81–0.90) between enzymatic assays and *in vivo* digestibility [17, 20, 21]. Although *in vitro* systems cannot fully replicate microbial and physiological interactions in the canine gastrointestinal tract [29], they remain an effective and ethical alternative for preliminary digestibility assessment.

Beyond nutritional performance, insect larvae provide significant environmental advantages over traditional animal protein sources. Their production requires less land, water, and feed, while generating lower greenhouse gas emissions [1]. The ability to utilize organic by-products as feed substrates further supports circular economy principles [2].

The conducted study has the character of a pilot investigation with inherent limitations (small sample size and the absence of long-term assessment of metabolic parameters), which may restrict the generalizability of the findings. Future research should aim to optimize the processing of insect meals to reduce chitin content, improve nutrient bioavailability, and standardize amino acid profiles. Additionally, long-term feeding studies should investigate potential metabolic, antioxidant, and immunomodulatory effects of insect-based diets in companion animals, as preliminary evidence suggests beneficial outcomes [12, 15]. Despite these limitations, the present findings provide valuable insights into the potential use of insect-derived proteins in canine nutrition.

Conclusion

The inclusion of *Tenebrio molitor* and *Hermetia illucens* larvae meals in dog food enhanced protein content and provided high-quality amino acids without adverse health effects. Although digestibility varied depending on diet type and insect species, both larvae types indicated strong potential as sustainable and nutritionally adequate protein sources in pet nutrition. The results reinforce the growing evidence that edible insects represent a viable alternative to conventional animal proteins, aligning nutritional efficacy with environmental sustainability.

Methods

Animals and Experimental Design

Five clinically healthy adult Beagle dogs (two females and three males), aged 4–6 years, with a body weight of 14.56 ± 1.72 kg and a body condition score (BCS) of 5.5 on a 9-point scale, were fed two isonitrogenous diets (wet and dry) supplemented with dried larvae of *Tenebrio molitor* (TM) and *Hermetia illucens* (HI) according to a Latin square design. Animals were allocated randomly to treatments according to a Latin square design to minimize order effects. The detailed experimental design is presented in Fig. 1. No inclusion or exclusion criteria were applied beyond initial health status.

To estimate the appropriate number of animals, a statistical power analysis was performed using Statistica software (TIBCO Software Inc., USA). For a conventional one-way ANOVA model with six independent groups ($\alpha = 0.05$, power = 0.80, moderate effect size), the required sample size would exceed 140 animals. However, the present study employed a crossover Latin square design, in which each animal received all dietary treatments and served as its own control. This repeated-measures approach substantially reduces inter-individual variability and increases statistical power. Based on the observed variability of key parameters (e.g., dry matter and crude protein digestibility) and effect sizes typically reported in similar studies, the statistical power of the present design with five animals was estimated to be within the range of 0.80–0.90. Therefore, in accordance with the principle of reduction in animal experimentation, a total of five animals was considered sufficient to detect biologically relevant differences.

The experimental unit was the individual animal.

During the digestibility trial, the dogs were housed at the Wrocław University of Environmental and Life Sciences, Faculty of Veterinary Medicine, Experimental Dog Breeding Facility No. 0057, with ad libitum access to fresh water, air temperature 19–21 ˚C and relative humidity 50%.

The study protocol was reviewed by the Animal Welfare Committee of the Wrocław University of Environmental and Life Sciences (opinion no. 4.2026), which confirmed that formal ethical approval was not required under the Polish Act on the Protection of Animals Used for Scientific or Educational Purposes (Journal of Laws 2015, item 266), as the study involved only non-invasive feeding procedures conducted under standard husbandry conditions.

Diets

Six diets were tested during the trial, based on two types of complete pet food - wet and dry - provided by a Polish manufacturer. The experiment was divided into two stages: Experiment 1 - addition of larvae to wet food (7 g/day), and Experiment 2 - addition of larvae to dry food (7 g/day). Experimental design is precisely presented in Fig. 1. The diets were isonitrogenous and the analytical composition is presented in Table 7. Duck protein served as the primary protein source, while insect proteins, specifically black

soldier fly and yellow mealworm, were included as additional protein sources. Dried larvae were purchased from Polish suppliers.

Table 7
Analytical composition of the feeds used and feeds with the addition of meal from mealworm larvae (*Tenebrio molitor*) and black soldier fly (*Hermetia illucens*).

Diet	Dry matter [%]	Crude protein DM [%]	Crude fat DM [%]
CW	48.37	29.88	37.84
TW	48.83	32.22	38.49
HW	48.75	28.24	42.88
CD	93.82	29.25	11.89
TD	95.05	31.99	12.50
HD	94.02	29.35	13.41

DM dry matter; CW control wet food; TW wet food with TM; HW wet food with HI; CD control dry food; TD dry food with TM; HD dry food with HI.

The diets were formulated and balanced to meet the nutrient requirements for dogs according to the nutrient guidelines of the European Pet Food Industry Federation (FEDIAF) [16]. Food intake was calculated based on the metabolic energy requirement (MER), which was determined after calculating body weight and applying the formula:

$$MER\ [kcal/day] = 1{,}8 \times 0{,}75 \times BW^{0,75},$$

where: BW – body weight [kg].

The dogs were fed a specific diet for 35 days, twice a day, according to the individual supplements as presented in the experimental scheme (Fig. 1). After a 5-day wash-out period, each dog was assigned a new diet, and the process was repeated for all treatments.

The food was weighed daily according to the energy requirements using the FEDIAF equation, divided into two equal portions, and provided to the animals at 9:00 AM and 5:00 PM in stainless steel bowls. Bowls were removed after consumption, and any leftover food was recorded.

In vivo digestibility

Apparent total tract digestibility coefficients (ATTDC) for individual dietary elements in the six diets were calculated using the Total Fecal Collection (TFC) method as follows:

ATTDC Xfood [%] = [(total Xfood − total Xfeces)/total Xfood] × 100,

where: X – the total content of dry matter (DM) or crude protein (CP) in the food or feces [g].

Fecal collection for digestibility analysis was performed twice daily on the 1st, 14th, 28th, and 35th days of the experiment. The feces were weighed and stored at − 20°C for subsequent analysis. The collected feces and food were analyzed for their DM, CP content to determine the digestibility of the respective dietary components. Triplicate samples were taken for each nutrient analysis.

In vitro digestibility

In vitro protein digestibility was assessed according to the methodology described by Hervera et al. [18] with modifications, which involved incubating the material in the Daisy II ANKOM incubator (ANKOM Technology, Macedon, NY, USA). The method simulates gastric and post-gastric digestion in two phases: gastric digestion (pH 2, 39°C, with added pepsin) and post-gastric digestion (pH 6.8, 39°C, with added pancreatin). After incubation in the ANKOM Daisy II Incubator, the residue was filtered, dried, and weighed to determine the contents of dry matter (DM) and crude protein (CP).

In vitro dry matter (DM) digestibility was calculated based on the difference between the amount of dry material (feed) before and after incubation. Crude protein (CP) digestibility was determined according to the following equation:

CP *in vitro* [%] = [1 − (total Xsample after × CP after) / (total Xsample before × CP before)] × 100,

where: X – sample weights [g], CP – the total contents of dry matter (DM) or crude protein (CP) in the samples [g].

Data from the *in vitro* digestibility analyses were used to estimate *in vivo* DM and CP digestibility using the regression equations reported by Hervera et al. [17]:

estimated digestibility of DM [%] = − 9.15 + 1.06 × *in vitro* DM digestibility [%],
estimated digestibility of CP [%] = 37.91 + 0.52 × *in vitro* CP digestibility [%].

Chemical analysis

After the collection period, the feces were freeze-dried (without thawing) using a laboratory freeze dryer (Alpha 1–4 LSC Basic, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and ground into a fine powder. The dry matter (DM) content of both the foods and feces was determined. The samples were analyzed according to the AOAC standard procedures, using methods described in the *in vitro* analysis section.

Both wet and dry foods, as well as insect larvae, were freeze-dried. All samples were then ground to pass through a 1 mm sieve before analysis. The foods and insect larvae were analyzed according to AOAC standard procedures for DM, CP, crude fat (EE).

The analyses were conducted in the laboratory of the Department of Animal Nutrition and Feed Science, Faculty of Biology and Animal Breeding, University of Life Sciences in Wrocław. The dry matter (DM) was determined by drying the samples to a constant weight (AOAC Official Method 934.01). Crude protein (CP) was determined by the Kjeldahl method (AOAC Official Method 2001.11), while crude fat (ether extract, EE) was measured using petroleum ether extraction (AOAC Official Method 991.36). The total protein content was calculated based on the nitrogen content, using a factor of 6.25, as determined by the Kjeldahl method with a Büchi Distillation Unit K-355 (Athens, Greece). Janssen et al. [18] presented the following nitrogen-to-protein conversion factors: 4.76 for *Tenebrio molitor* and 5.60 for *Hermetia illucens*. The researchers highlight that these conversion factors are considerably below the conventional 6.25 factor, attributing this difference to non-protein nitrogen found in chitin and additional exoskeletal components of insects. Using the standard factor overestimates the protein content of insects. However, in this study, insects only constituted a small percentage of the feed composition. Therefore, the authors of the presented manuscript decided to use the standard conversion factor of 6.25, which is the most commonly used factor in pet food analysis and is the value recognized by most regulatory bodies and industry organizations.

Amino acids (AA) were analyzed in the laboratory of the Department of Food Chemistry and Biocatalysis, Faculty of Biotechnology and Food Science, University of Life Sciences in Wrocław, using ion-exchange chromatography. The amino acid composition of the samples was determined after hydrolysis for 23 hours with 6 N HCl at 110°C. The hydrolyzed amino acids were quantified using an AAA-400 analyzer (INGOS, Prague, Czech Republic), equipped with a photometric detector working at two wavelengths: 440 nm and 570 nm. A 350×3.7 mm column, packed with the ion exchanger Ostion LG ANB (INGOS), was used. The column temperature was maintained at 60–74°C, and the detector temperature was set at 121°C. Amino acid content was calculated relative to an external standard.

Quality of protein assessment

Quality of protein was assessed using the Oser method [19], which calculates the integrated index of exogenous amino acids (EAA). This method involves determining the amino acid composition of the feed and comparing it to the composition of hen egg white, which serves as the reference protein.

According to Oser [19], this mathematical model is described as the geometric mean of "the egg ratio," which represents the proportions of essential amino acids required by dogs (including arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine, but excluding tryptophan) in a protein compared to their corresponding quantities found in whole egg protein:

$$EAAI\ [\%] = \sqrt[n]{\left(\frac{EAA1p}{EAA1s} + \frac{EAA2p}{EAA2s} \ldots + \frac{EAAnp}{EAAns}\right)} \times 100\%$$

,

where: p – food protein [%]; s – standard protein (whole egg) [%], n – number of amino acids (n = 9).

From the EAA indexes, the biological value (BV) was calculated using the equation below [19]:

BV [%] = 1.09 (EAAI) – 11.7.

The nutritional index (NI) of the food samples was calculated using the formula below:

NI [%] = EAAI × protein [%] × 100.

Clinical observations and fecal assessment

To evaluate the health status of the dogs, clinical observations were conducted daily to monitor the occurrence of diarrhea. Body weight measurements and body condition assessments were performed on a 9-point BCS scale on the 1st and 35th days of the experiment. Fecal quality was assessed using the Walham scale on the 1st, 14th, 28th, and 35th days of the experiment.

Additionally, fecal consistency was evaluated using both the Purina and Walham scales. Throughout the experiment, no significant changes in fecal characteristics were observed, indicating good tolerance to the new feed and dietary additives.

The primary outcomes were apparent total tract digestibility of dry matter and crude protein. Secondary outcomes included in vitro digestibility and amino acid profile.

Statistical analyses

Statistical analyses were performed using Statistica 13 software (TIBCO Software Inc., Palo Alto, CA, USA). Due to the repeated-measures design and non-normal distribution of the data, differences among diets were evaluated using the non-parametric Friedman test, followed by Dunn’s post hoc test for multiple comparisons. The level of statistical significance was set at $p < 0.05$.

Blinding was not applied during the experiment due to the nature of the study.

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Abbreviations

AA
Amino Acids

AOAC
Association of Official Analytical Chemists
ATTDC
Apparent Total Tract Digestibility Coefficient
BCS
Body Condition Score
BV
Biological Value
CD
Control Dry Food
CP
Crude Protein
CW
Control Wet Food
DM
Dry Matter
EE
Ether Extract (crude fat)
EAA
Essential Amino Acids
EAAI
Essential Amino Acid Index
EU
European Union
FEDIAF
European Pet Food Industry Federation (Fédération Européenne de l'Industrie des Aliments pour Animaux Familiers)
GLU
Glutamic Acid
HCl
Hydrochloric Acid
HD
Dry diet supplemented with *Hermetia illucens* larvae
HI
Hermetia illucens (black soldier fly)
HW
Wet diet supplemented with *Hermetia illucens* larvae
ILE
Isoleucine
LCA

Life Cycle Assessment
LEU
Leucine
LYS
Lysine
MET
Methionine
MER
Metabolic Energy Requirement
NI
Nutritional Index
p
Probability value (level of statistical significance)
PRO
Proline
SER
Serine
TD
Dry diet supplemented with *Tenebrio molitor* larvae
THR
Threonine
TM
Tenebrio molitor (yellow mealworm)
TFC
Total Fecal Collection
TW
Wet diet supplemented with *Tenebrio molitor* larvae
TYR
Tyrosine
VAL
Valine

Declarations

- **Ethics approval and consent to participate**
  All procedures were carried out in accordance with relevant national regulations and the principles outlined in Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. The study protocol was reviewed by the Animal Welfare Committee of the Wrocław University of Environmental and Life Sciences (opinion no. 4.2026), which confirmed that formal ethical approval was not required under the Polish Act on the

Protection of Animals Used for Scientific or Educational Purposes (Journal of Laws 2015, item 266), as the study involved only non-invasive feeding procedures conducted under standard husbandry conditions. No animals were euthanized during the study. The animals were owned by the institution, and no external owner consent was required.

- **Consent for publication**
  Not applicable.
- **Availability of data and materials**
  Data supporting the findings of this study are available within the article.
- **Competing interests**
  The authors declare no conflicts of interest.
- **Funding**

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- **Authors' contributions**
  Conceptualization, A.B.-W.; methodology, A.B.-W., M.W.; investigation, A.B.-W., M.W., K.L.; writing—original draft preparation, A.B.-W.; writing—review and editing, all authors; supervision, R.K.
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- **Authors' information (optional)**

References

1. Hammer L, Moretti D, Abbühl-Eng L, Kandiah P, Hilaj N, Portmann R, et al. Mealworm larvae (Tenebrio molitor) and crickets (Acheta domesticus) show high total protein in vitro digestibility and can provide good-to-excellent protein quality as determined by in vitro DIAAS. Front Nutr. 2023;10:1150581.
2. Penazzi L, Schiavone A, Russo N, Nery J, Valle E, Madrid J, et al. In vivo and in vitro digestibility of an extruded complete dog food containing black soldier fly (Hermetia illucens) larvae meal as protein source. Front Vet Sci. 2021;8:653411.
3. Abd El-Wahab A, Meyer L, Kölln M, Chuppava B, Wilke V, Visscher C, et al. Insect larvae meal (Hermetia illucens) as a sustainable protein source of canine food and its impacts on nutrient digestibility and fecal quality. Anim (Basel). 2021;11:2525.
4. Veldkamp T, van Duinkerken G, van Huis A, Lakemond CMM, Ottevanger E, Bosch G et al. Insects as a sustainable feed ingredient in pig and poultry diets – a feasibility study. Wageningen Livest Res Rep. 2012;638.

5. Makkar HP, Tran G, Heuzé V, Ankers P. State-of-the-art on the use of insects as animal feed. Anim Feed Sci Technol. 2014;197:1–33.
6. Baiano A. Edible insects: an overview on nutritional characteristics, safety, farming, production technologies, regulatory framework, and socio-economic and ethical implications. Trends Food Sci Technol. 2020;100:35–50.
7. Costa S, Pedro S, Lourenço H, Batista I, Teixeira B, Bandarra NM, et al. Evaluation of Tenebrio molitor larvae as an alternative food source. NFS J. 2020;21:57–64.
8. Kohlmeier M, Phenylalanine. Food Sci Technol Nutr Metab. 2003:314–21.
9. Barroso FG, Sanchez-Muros MJ, Segura M, Morote E, Torres A, Ramos R, et al. Insects as food: enrichment of larvae of Hermetia illucens with omega-3 fatty acids by means of dietary modifications. J Food Compos Anal. 2017;62:8–13.
10. Bosch G, Vervoort JJM, Hendriks WH. In vitro digestibility and fermentability of selected insects for dog foods. Anim Feed Sci Technol. 2016;221:174–84.
11. Jacuńska W, Biel W, Zych K. Evaluation of the nutritional value of insect-based complete pet foods. Appl Sci. 2024;14:10258.
12. Lemos IA, Ribeiro AG, Madureira ER, Rabello CBV, Dias GAO, dos Santos Nascimento JC, et al. Hematological changes in dogs fed with Tenebrio molitor larvae meal. Acta Vet Bras. 2023;17:85–90.
13. Kröger S, Heide C, Zentek J. Evaluation of an extruded diet for adult dogs containing larvae meal from the black soldier fly (Hermetia illucens). Anim Feed Sci Technol. 2020;270.
14. Freel TA, McComb A, Koutsos EA. Digestibility and safety of dry black soldier fly larvae meal and black soldier fly larvae oil in dogs. J Anim Sci. 2021;99.
15. Lei XJ, Kim TH, Park JH, Kim IH. Evaluation of supplementation of defatted black soldier fly (Hermetia illucens) larvae meal in beagle dogs. Ann Anim Sci. 2019;19:767–77.
16. FEDIAF. Nutritional guidelines for complete and complementary pet food for cats and dogs. Eur Pet Food Ind Fed. 2023.
17. Hervera M, Baucells MD, Blanch F, Castrillo C. Prediction of digestible energy content of extruded dog food by in vitro analyses. J Anim Physiol Anim Nutr. 2007;91:205–9.
18. Janssen RH, Vincken JP, van den Broek LAM, Fogliano V, Lakemond CMM. Nitrogen-to-protein conversion factors for three edible insects: Tenebrio molitor, Alphitobius diaperinus, and Hermetia illucens. J Agric Food Chem. 2017;65:2275–8.
19. Oser BL. An integrated essential amino acid index for predicting the biological value of proteins. Protein and Amino Acid Nutrition. New York: Academic; 1959. pp. 281–96.
20. Biagi G, Cipollini I, Grandi M, Pinna C, Vecchiato CG, Zaghini G. A new in vitro method to evaluate digestibility of commercial diets for dogs. Ital J Anim Sci. 2016;15:617–25.
21. Seo K, Cho HW, Lee MY, Kim CH, Kim KH, Chun JL. Prediction of apparent total tract digestion of crude protein in adult dogs. J Anim Sci Technol. 2024;66:374–86.

22. Rumpold BA, Schlüter OK. Nutritional composition and safety aspects of edible insects. Mol Nutr Food Res. 2013;57:802–23.
23. Barragan-Fonseca KB, Dicke M, van Loon JJA. Nutritional value of the black soldier fly (Hermetia illucens L.) and its suitability as animal feed – a review. J Insects Food Feed. 2017;3:105–20.
24. Bosch G, Zhang S, Oonincx DG, Hendriks WH. Protein quality of insects as potential ingredients for dog and cat foods. J Nutr Sci. 2014;3:e29.
25. Liland NS, Biancarosa I, Araujo P, Biemans D, Bruckner CG, Waagbø R, et al. Modulation of nutrient composition of black soldier fly larvae by feeding seaweed-enriched media. PLoS ONE. 2017;12:e0183188.
26. Yu DW, Yang T, Sonoda T, Gong Y, Cao Q, Gaffney K, et al. Osteopontin gene is expressed in the dermal papilla of pelage follicles in a hair-cycle-dependent manner. J Invest Dermatol. 2001;117:1554–60.
27. Spranghers T, Ottoboni M, Klootwijk C, Ovyn A, Deboosere S, De Meulenaer B, et al. Nutritional composition of black soldier fly prepupae reared on different organic waste substrates. J Sci Food Agric. 2017;97:2594–600.
28. Caligiani A, Marseglia A, Leni G, Baldassarre S, Maistrello L, Dossena A, et al. Composition of black soldier fly prepupae and systematic approaches for extraction and fractionation of proteins, lipids, and chitin. Food Res Int. 2018;105:812–20.
29. Deschamps C, Denis S, Humbert D, Zentek J, Priymenko N, Apper E, et al. In vitro models of the canine digestive tract as an alternative to in vivo assays: Advances and current challenges. Altex. 2022;39:235–57.

Figures

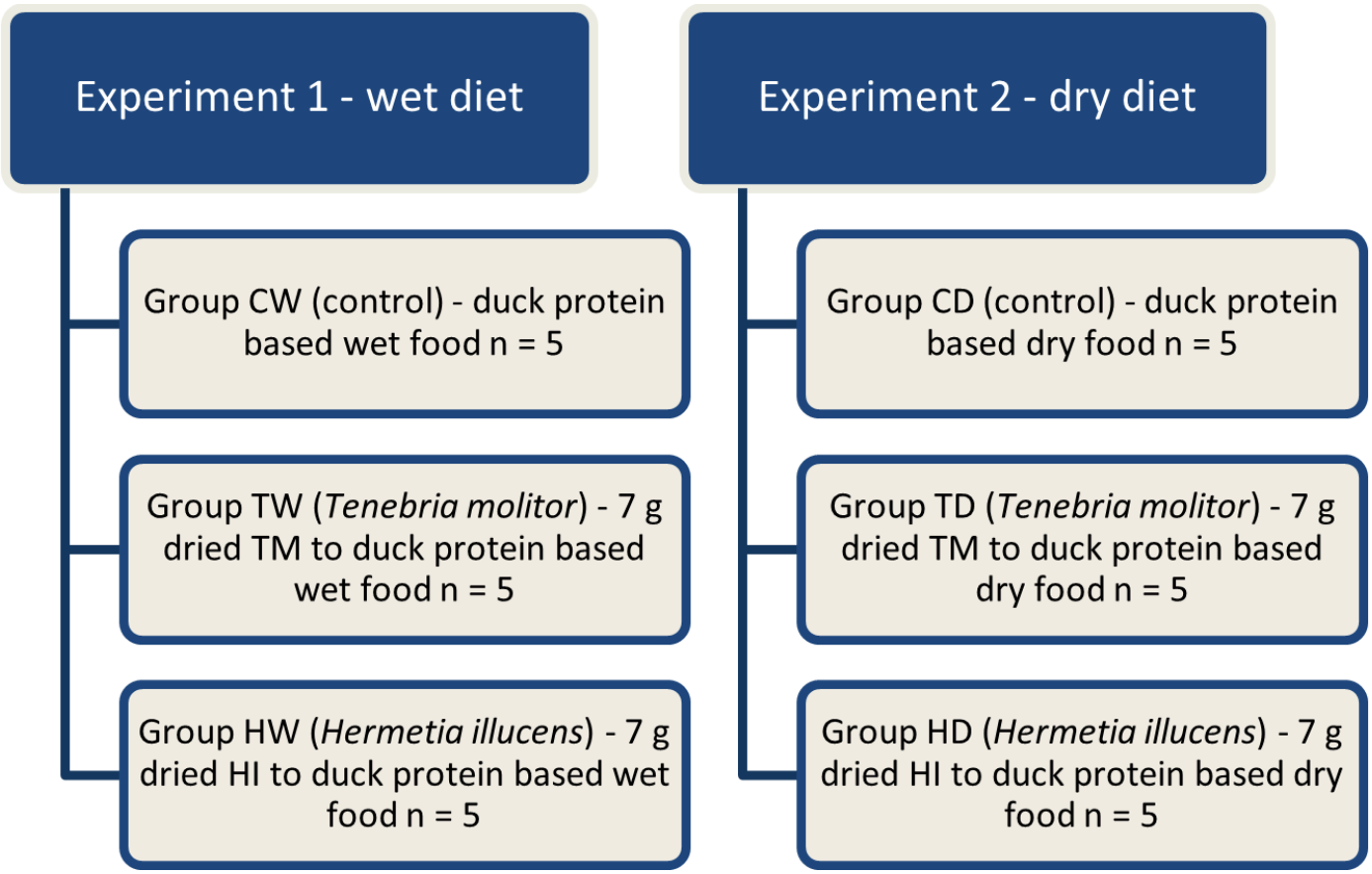


Figure 1

Experimental design.