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Digestibility of defatted insect meals for rainbow trout aquafeeds

L. Gasco[1], C. Caimi[1], A. Trocino[2], C. Lussiana[1], S. Bellezza Oddon[1], V. Malfatto[1], R. Anedda[3], G. Serra[4], I. Biasato[1], A. Schiavone[5], F. Gai[6*] and M. Renna[5]

[1]Department of Agricultural, Forest and Food Sciences, University of Turin, Largo P. Braccini 2, 10095 Grugliasco (TO), Italy; [2]Department of Comparative Biomedicine and Food Science, University of Padua, Viale dell'Università 16, 35020 Legnaro (PD), Italy; [3]Porto Conte Ricerche S.r.l., S.P. 55 Porto Conte, Capo Caccia km 8,400 Loc. Tramariglio, 07041 Alghero (SS), Italy; [4]Institute of BioEconomy, National Research Council, Traversa La Crucca, 3, 07100 Li Punti, Sassari, Italy; [5]Department of Veterinary Sciences, University of Turin, Largo Paolo Braccini 2, 10095 Grugliasco (TO), Italy; [6]Institute of Sciences of Food Production, National Research Council, Largo P. Braccini 2, 10095, Grugliasco (TO), Italy; francesco.gai@ispa.cnr.it

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RESEARCH ARTICLE

Abstract

The apparent digestibility coefficients (ADCs) of the dry matter (DM), crude protein (CP), ether extract (EE), gross energy (GE), amino acids (AA), and the main fatty acids (FA) of four defatted insect meals in rainbow trout (*Oncorhynchus mykiss*) have been assessed. The tested meals were obtained from two yellow mealworms (*Tenebrio molitor* – TM1 and TM2), one black soldier fly (*Hermetia illucens* – HI) and one lesser mealworm (*Alphitobius diaperinus* – AD). The experimental diets were prepared by means of the substitution method, with each test ingredient included in the diet at 30% on an as fed basis and using Celite® as an inert digestibility marker. Eighty rainbow trout (140±5.6 g) were stocked in tanks connected to an open water system. Faeces were collected over four consecutive weeks using an automatic collection device after feeding the fish. The ADCs of the DM, CP and GE of the insect meals differed significantly, with the AD meal displaying the lowest values. The ADC of the EE in the different meals did not vary. As far as AA digestibility is concerned, the ADC of methionine changed according to the following pattern TM2=HI>TM1>AD, whereas the ADCs of cysteine and tyrosine were significantly lower in the AD meal than in the other meals. The ADCs of the main FAs (C12:0, C14:0, C16:0, C18:1 *c*9, C18:2 n-6 and C18:3 n-3) were higher than 85% and did not differ significantly in the insect meals. Overall, the tested insect meals resulted to be highly digestible and the differences among them depended on both the insect species and the specific production techniques of the meals. These results provide useful data that may be considered to properly formulate compound diets for rainbow trout using innovative protein sources.

Keywords: apparent digestibility coefficient, *Oncorhynchus mykiss*, *Tenebrio molitor*, *Hermetia illucens*, *Alphitobius diaperinus*

1. Introduction

About 59 million tonnes of aquafeed were used in 2020, and this number is expected to rise to about 73 million tonnes by 2025 (Tacon, 2020). Because of its high nutritional value, digestibility and easy availability, huge quantities of fishmeal (FM) were used in aquafeeds in the past (Turchini *et al.*, 2019). However, to comply with public concerns about the unsustainable use of FM, and to face the increasing demand in aquaculture products, the dietary inclusion levels of FM have shown a decreasing trend in the last few years (FAO, 2020) and great efforts have been made to develop new formulae (Turchini *et al.*, 2019).

Nowadays, aquafeeds contain large quantities of plant proteins (PPs) and processed animal proteins (PAPs) (Hua *et al.*, 2019). The PPs that are used the most to replace FM are soybean meal (SBM), gluten meal, wheat meal and soybean protein concentrates (Cerqueira *et al.*, 2020; Ghosh and Ray, 2017), while poultry by-products, meat and bones, or meat and blood meals are considered the most promising PAPs (Davies *et al.*, 2019; Moutinho *et al.*, 2017).

However, PPs sometimes result in reduced performances and flawed intestinal integrity, especially in carnivorous fish, mainly due to an unbalanced nutrient profile or the presence of anti-nutritional factors (Colombo, 2020; Gai *et al.*, 2012). On the other hand, PAPs are very interesting from a nutritional point of view and do not contain any anti-nutritional factors. However, only non-ruminant PAPs from Category 3 animals are authorised in the European Union (EU), which limits the number of PAPs that can be used to a great extent (European Commission, 2001, 2017a).

Thus, there has been increasing interest in the use of insect meals (IMs) in aquaculture to substitute conventional protein sources (Gasco *et al.*, 2019, 2020; Lock *et al.*, 2019; Nogales-Mérida *et al.*, 2019). The EU has approved the use of insect derived PAPs from seven insect species in aquaculture, poultry and swine feeds (European Commission, 2017b, 2021) and a huge development has been forecast for the insect sector (IPIFF, 2019; Mancuso *et al.*, 2019).

Many studies have been conducted to assess the digestibility of diets with various levels of added IMs and their influence on the growth performances and product quality of both freshwater and marine species (Coutinho *et al.*, 2021; Gasco *et al.*, 2019; Guerreiro *et al.*, 2021; Hua, 2021; Nogales-Mérida *et al.*, 2019), but very few data are available about the digestibility of IMs as tested ingredients in aquafeeds (Basto *et al.*, 2020; Fontes *et al.*, 2019; Mo *et al.*, 2019). Such data are essential to properly formulate diets and avoid nutritional unbalances that could lead to a poor growth and welfare of fish, as well as environmental issues (Dam *et al.*, 2019). Moreover, considering that data about the bioavailability of macronutrients and essential amino acids (EAA) of commercially available insect meals for rainbow trout feeds is still scarce (Lee *et al.*, 2020), it is of utmost importance to provide such data for one of the most cultured fish species in Europe, and to extrapolate results that could be useful for other salmonid species.

Thus, the aim of this study has been to assess the *in vivo* apparent digestibility coefficient (ADC) of the dry matter (DM), crude protein (CP), ether extract (EE), gross energy (GE), amino acids (AA) and main fatty acids (FA) of four defatted IMs, chosen among the most promising insect species already authorised at a European level, for use in rainbow trout (*Oncorhynchus mykiss*) aquafeeds.

2. Materials and methods

The trial, which was designed according to the current European Directive guidelines (2010/63/EU) for animals used for scientific purposes, was conducted at the experimental facility of the Department of Agricultural, Forest and Food Sciences (DISAFA) of the University of Turin (UNITO, Italy). The UNITO Ethical Committee approved the experimental protocol (protocol No. 143811).

Insect meals and diet preparation

The defatted IMs used in this trial were obtained from different suppliers. One *Tenebrio molitor* meal (TM1) was obtained from larvae reared on wheat bran at the Institute of Ecosystem Studies in Sassari (Italy) and then dried and defatted at the Porto Conte Ricerche facilities (Alghero, SS, Italy). Another *T. molitor* meal (TM2), that is, a *Hermetia illucens* (HI) meal and an *Alphitobius diaperinus* (AD) meal were sourced from different European commercial producers. No specific information was provided about the rearing substrates or technological processes applied to obtain these meals, as such information is covered by intellectual property rights.

A high-quality reference diet (diet R) was formulated following the protocol recommended by Bureau *et al.* (1999) (Table 1). An aliquot of 1% Celite® (Fluka, St. Gallen, Switzerland) was added to the reference diet as an inert marker to assess the ADCs. Four experimental diets were then obtained by mixing the R diet with one of the above-mentioned IMs at a ratio of 70(R): 30(IM) (as fed basis). The ground ingredients were individually weighed and subsequently mixed with fish oil. The pellets were obtained using a 3.0 mm die meat grinder and dried at 50 °C for 48 h. The diets were stored in dark bags at -20 °C until utilisation.

Fish handling and faeces collection

Eighty rainbow trout (140±5.6 g) were obtained from a commercial farm (Troticoltura Bassignana, Cuneo, Italy). On arrival at the DISAFA facilities, the trout were put into five 250-l cylindroconical tanks (16 fish/tank; density: 8.96 kg/m^3) supplied with artesian well water (constant

Table 1. Ingredient composition of the reference diet (g/100 g as fed).

Fishmeal[1]	55.0
Wheat gluten meal[2]	14.5
Soybean meal[3]	7.0
Wheat meal[4]	8.0
Starch gelatinised, D500[5]	6.0
Fish oil	8.0
Premix vit/min	0.5
Celite	1.0

[1] Proximate composition (g/100 g, as fed): DM, 90.8; CP, 67.6; EE, 8.3; Ash, 16.4.
[2] Proximate composition (g/100 g, as fed): DM, 93.6; CP, 81.3; EE, 1.0; Ash, 0.5.
[3] Proximate composition (g/100 g, as fed): DM, 86.4; CP, 49.6; EE, 0.8; Ash, 6.4.
[4] Proximate composition (g/100 g, as fed): DM, 88.1; CP, 10.7; EE, 0.9; Ash, 0.9.
[5] Proximate composition (g/100 g, as fed): DM, 95.1; CP, 2.3; EE, 0.1; Ash, 0.6.

temperature of 13±1 °C) in a flow-through open system at a rate of about 8 l/min. The five diets were randomly assigned to the tanks (one tank per treatment). The trial was performed over three collection periods, randomly re-allocating the experimental diets to the tanks (Bureau *et al.*, 1999). After an initial housing of the fish, and between two consecutive collection periods, the fish were adapted to a new diet for 10 days (Glencross, 2007). The fish were fed by hand to visual satiety twice a day, seven days a week. The fish faeces were collected from each tank twice a day (8:00 and 15:00) during the collection periods for four consecutive weeks, using a continuous automatic device, as reported by Choubert *et al.* (1982); no faeces were collected during the adaptation period. The faeces were frozen (-20 °C) and then freeze-dried prior to the subsequent chemical analyses.

Analytical methods

Samples of the IMs, diets and faeces were ground using a cutting mill (MLI 204; Bühler AG, Uzwil, Switzerland) and analysed according to AOAC International (2000) for the DM (#934.01), CP (#984.13) and ash (#942.05) contents, and according to AOAC International (2003) for EE (#2003.05). The CP content of the IMs was calculated using a nitrogen-to-protein conversion factor (K_p) of 5.59 for the TM and AD meals, and of 5.62 for the HI meal (Janssen *et al.*, 2017). The chitin content of the meals was analysed according to Woods *et al.* (2020); the samples were totally defatted by means of solvent extraction and were subsequently subjected to demineralisation (with HCl) and deproteinisation (with NaOH). The GE content was determined using an adiabatic calorimetric bomb (C7000; IKA, Staufen, Germany).

The AA analysis was performed according to EC regulations (European Commission, 2009) and European Pharmacopoeia (2005; 2.2.56, method 1). Specific sample treatments were performed before protein hydrolysis to determine the cysteine/cystine (Cys) and methionine (Met) contents (European Pharmacopoeia, 2005; 2.2.56, methods 4, 5). After treatment of the samples, AA was determined using an HPLC Agilent 1260 Infinity device (Agilent Technologies, Santa Clara, CA, the USA) equipped with a diode array detector and a fluorescence detector (Agilent Technologies, 2008).

The FA composition of the IMs and of the experimental diets was assessed as reported by Dabbou *et al.* (2020). The total lipids of the faeces were extracted with dichloromethane/methanol (2:1, v:v), and fatty acid methyl esters (FAMEs) were prepared by acid-catalysed transesterification of the total lipids, as described in Caimi *et al.* (2020), using tridecanoic acid as the internal standard. The FAMEs of both the feeds and faeces were separated, identified, and quantified using the chromatographic conditions reported by Renna *et al.* (2019). The results were expressed as g FA/kg DM. The inert marker contents (%) in the diets and faeces were analysed by means of the Acid-Insoluble Ash (AIA) method (Atkinson *et al.*, 1984). All the analyses were performed in duplicate.

Calculations of the ADCs

The ADCs of each nutrient and energy in the reference and experimental diets were calculated according to Bureau *et al.* (1999) as follows:

$$ADC = 1 - \left(\frac{F}{D} \times \frac{Di}{Fi}\right) \quad (1)$$

where: F = % nutrient (or kJ/g GE) in the faeces; D = % nutrient (or kJ/g GE) in the reference or experimental diet; Di = % marker indicator (AIA) in the diet; Fi = % marker indicator (AIA) in the faeces.

The ADC of DM was calculated as:

$$ADC_{DM} = 100 \times \left(1 - \frac{Di}{Fi}\right) \quad (2)$$

The ADCs of the nutrients and energy of each of the tested IMs were obtained as:

$$ADC_{ing} = ADC_{test} + \left((ADC_{test} - ADC_{ref}) \times \left(\frac{0.7 \times D_{ref}}{0.3 \times D_{ing}}\right)\right) \quad (3)$$

where: ADC_{test} = ADC (%) of the experimental diet; ADC_{ref} = ADC (%) of the reference diet; D_{ref} = g/100 g nutrient (or MJ/kg GE) of the reference diet (DM basis); D_{ing} = g/100 g nutrient (or MJ/kg GE) of the test ingredient (DM basis).

Only FAs that were higher than 3 g/kg DM in at least one IM were retained for the ADC calculations and subsequent statistical analysis. The digestible essential amino acids (EAA) were calculated by multiplying the content of each EAA by the corresponding ADC.

Statistical analysis

Data were analysed by means of one-way ANOVA using IBM SPSS Statistics, v. 27.0 (IBM, Armonk, NY, USA) for Windows with the following model:

$$Y_{ij} = \mu + D_i + \varepsilon_{ij} \quad (4)$$

where: Y_{ij} = observation; μ = overall mean; D_i = effect of the IM; ε_{ij} = residual error.

The assumption of normality and equal variances was assessed by means of the Kolmogorov-Smirnov test and Levene's homogeneity of variance test, respectively. If such an assumption did not hold, the Brown-Forsythe statistic was applied, instead of the F one, to test the equality of group means. Pairwise multiple comparisons were performed to test the difference between each pair of means (Tukey's test

and Tamhane's T2 in the cases of assumed or not assumed equal variances, respectively). The results were expressed as the mean and pooled standard error of the mean (SEM). Significance was declared at $P \leq 0.05$.

3. Results

Proximate composition and energy content of the insect meals

The CP content of the four IMs used in the trial varied from the lowest value of 56.4 g/100 g DM in the HI meal to the highest value of 76.3 g/100 g DM in the AD meal (Table 2). The lowest EE content was recorded in the AD meal (2.5 g/100 g DM), while the highest was found in the HI meal (12.0 g/100 g DM). The latter also showed the highest ash content (10.3 g/100 g DM). The GE content ranged from 19.2 MJ/kg DM in the HI meal to 23.5 MJ/kg DM in the TM1 meal. Finally, chitin ranged from 6.3/100 g DM in TM2 to 7.8/100 g DM in TM1.

As for the AAs (Table 3), the AD meal showed the highest total AA content, followed by TM1 and TM2, while the HI meal showed about a 30% lower total AA content than AD. Such a difference is only about 5.5% when the AA values are expressed as a percentage of the total CP. The same trends were observed for both the EAA and non-essential (NEAA) amino acids. In all the IMs, the amount of the total NEAAs was higher than that of the total EAAs. Glutamic acid was the most abundant individual AA in all the tested IMs. The second and third most abundant AAs were proline and alanine (Ala) in TM1 and HI, Ala, and aspartic acid (Asp) in TM2, and Asp and tyrosine (Tyr) in AD.

As for the FA composition (Table 4), the highest amount of total fatty acids (TFA) was recorded in HI (64.66 g/kg DM), followed by TM2 (43.54 g/kg DM), TM1 (33.14 g/kg DM), and finally by AD (21.19 g/kg DM). The most abundant individual FAs were oleic acid (C18:1 *c*9) in TM1, lauric acid (C12:0) in HI and linoleic acid (C18:2 n-6) in AD. TM2 showed almost equal amounts of oleic and linolenic acids (14.81 and 14.74 g/kg DM, respectively).

Apparent digestibility coefficients of the insect meals

The digestibility of the nutrients changed significantly among the IMs (Table 5). Overall, TM1 showed a higher digestibility than the other meals: the ADC_{DM} of TM1 was higher than that of HI and AD, while TM2 showed intermediate values ($P<0.05$); the ADC_{CP} of TM1 was higher than that of AD, while TM2 and HI showed intermediate values ($P<0.05$); ADC_{GE} was ranked in the following order: TM1>HI=TM2>AD. On the other hand, ADC_{EE} did not vary significantly among the IMs. As for the AAs (Table 6), the highest ADC of Met was in TM2 and HI, followed by TM1 and AD ($P<0.001$); the ADCs of Cys and Tyr were significantly lower in AD than in the other IMs ($P<0.05$ and $P<0.001$, respectively). Regarding the contents of digestible EAAs (Table 7), the AD meal showed higher values than the other IMs, except for Met, while the HI meal had the lowest values. The digestible lysine (Lys) and digestible Met contents ranged from 3.2 (HI) to 5.54 (AD) g/100 g DM and from 1.24 (HI) to 2.32 (TM1) g/100 g DM, respectively.

Finally, no significant differences were found for the ADC of the lauric, myristic (C14:0), palmitic (C16:0), oleic, linoleic, and α-linolenic (C18:3 n-3) acids for the four IMs (Table 8).

4. Discussion

Insects are part of the natural diet of carnivorous fish species. Insect and insect-derived meals show a good nutritional value, as they are rich in proteins and EAAs, lipids, vitamins, and minerals. Their main chemical composition (Meneguz *et al.*, 2018a; Oonincx and Finke, 2020), their AA content (Basto *et al.*, 2020; Fuso *et al.*, 2021) and their FA content (Melis *et al.*, 2018, 2019) depend on the insect species, life stage, rearing substrate and conditions,

Table 2. Proximate composition (g/100 g DM, unless otherwise stated) and energy content of the insect meals and of the reference diet.[1]

	TM1	TM2	HI	AD	REF
DM (g/100 g)	91.8	95.2	94.8	89.5	90.2
CP	71.3	63.0	56.4	76.3	58.7
EE	5.7	8.1	12.0	2.5	13.3
Chitin	7.8	6.3	6.8	7.0	–
Ash	5.0	4.9	10.3	4.9	10.6
NFE[2]	18.0	24.0	21.3	16.3	17.4
GE (MJ/kg DM)	23.5	23.0	19.2	23.1	22.2

[1] AD = *Alphitobius diaperinus* larvae meal; CP = crude protein; DM = dry matter; EE = ether extract; GE = gross energy; HI = *Hermetia illucens* larvae meal; NFE = nitrogen free extracts; REF = reference diet; TM1 and TM2 = *Tenebrio molitor* larvae meal.
[2] Calculated as: 100–(crude protein + ether extract + ash).

Table 3. Amino acid composition of the insect meals and of the reference diet, expressed as g/100 g DM or as g/100 g CP (in brackets).[1]

	TM1	TM2	HI	AD	REF
Essential amino acids (EAA)					
Arginine	3.80 (5.33)	3.62 (5.75)	2.88 (5.11)	4.49 (5.88)	3.03 (5.16)
Histidine	2.39 (3.35)	2.28 (3.62)	1.53 (2.71)	3.23 (4.23)	2.44 (4.16)
Isoleucine	2.65 (3.72)	2.44 (3.87)	2.01 (3.56)	3.12 (4.09)	2.25 (3.83)
Leucine	4.90 (6.87)	4.66 (7.40)	3.72 (6.60)	5.49 (7.20)	4.75 (8.09)
Lysine	3.97 (5.57)	3.97 (6.30)	3.27 (5.80)	5.72 (7.50)	4.00 (6.81)
Methionine	2.65 (3.72)	1.41 (2.24)	1.31 (2.32)	2.67 (3.50)	1.54 (2.62)
Phenylalanine	2.30 (3.23)	2.21 (3.51)	1.87 (3.32)	3.29 (4.31)	2.54 (4.33)
Threonine	3.00 (4.21)	2.82 (4.48)	2.38 (4.22)	3.63 (4.76)	2.68 (4.57)
Valine	3.79 (5.32)	3.51 (5.57)	2.64 (4.68)	3.98 (5.22)	2.73 (4.65)
Non-essential amino acids (NEAA)					
Alanine	5.91 (8.29)	5.50 (8.73)	4.87 (8.63)	6.07 (7.96)	3.67 (6.25)
Aspartic acid	5.45 (7.64)	5.29 (8.40)	4.60 (8.16)	6.89 (9.03)	4.65 (7.92)
Cysteine	0.44 (0.62)	0.40 (0.63)	0.37 (0.66)	0.57 (0.75)	0.74 (1.26)
Glutamic acid	8.68 (12.17)	8.40 (13.33)	7.58 (13.44)	7.50 (9.83)	8.44 (14.38)
Glycine	3.73 (5.23)	3.39 (5.38)	3.08 (5.46)	3.86 (5.06)	3.26 (5.55)
Proline	5.97 (8.37)	4.54 (7.21)	5.13 (9.10)	4.78 (6.26)	3.08 (5.25)
Serine	3.66 (5.13)	3.37 (5.35)	2.88 (5.11)	4.02 (5.27)	2.44 (4.39)
Tyrosine	5.10 (7.15)	4.51 (7.16)	2.65 (4.70)	6.18 (8.10)	2.09 (3.56)
Σ EAA	29.45 (41.30)	26.91 (42.73)	21.61 (38.32)	35.64 (46.68)	25.96 (44.22)
Σ NEAA	38.93 (54.61)	35.40 (56.19)	31.17 (55.25)	43.78 (52.25)	28.37 (48.56)
Σ Amino acids (AA)	68.38 (95.92)	62.32 (98.92)	52.78 (93.56)	75.51 (98.94)	54.33 (92.78)
Σ EAA / Σ AA (%)	43.07 (43.05)	43.18 (43.20)	40.94 (40.96)	47.20 (47.18)	47.78 (47.66)

[1] AD = *Alphitobius diaperinus* larvae meal; CP = crude protein; DM = dry matter; HI = *Hermetia illucens* larvae meal; REF = reference diet; TM1 and TM2 = *Tenebrio molitor* larvae meal.

Table 4. Fatty acid (FA) composition of the insect meals and of the reference diet (g/kg DM).[1]

	TM1	TM2	HI	AD	REF
C12:0	0.16	0.15	17.25	0.03	0.39
C14:0	1.05	0.87	4.09	0.10	4.68
C16:0	5.32	8.57	14.84	5.32	16.61
C18:1 *c*9	14.24	14.81	12.09	5.77	25.63
C18:2 n-6	9.12	14.74	10.24	6.91	11.71
C18:3 n-3	0.59	0.62	0.94	0.19	2.70
Other[2]	2.67	3.79	5.30	2.88	35.70
TFA	33.14	43.54	64.66	21.19	97.42

[1] AD = *Alphitobius diaperinus* larvae meal; DM = dry matter; HI = *Hermetia illucens* larvae meal; REF = reference diet; TM1 and TM2 = *Tenebrio molitor* larvae meal. Only FA with a content higher than 3 g/kg DM in at least one insect meal are reported.
[2] C10:0 + C14:1 *c*9 + C15:0 + C15 iso + C16 iso + C16:1 *c*9 + C17:0 + C17 iso + C17 aiso + C18:0 + C18 aiso + ∑C18:1 t + C18:1 *c*11 + C18:3 n-6 + C20 + C20:1 *c*9 + C20:1 *c*11 + C20:2 n-6.

Table 5. Apparent digestibility coefficients (ADC, %) of main nutrients and gross energy (n=3).[1,2]

	TM1	TM2	HI	AD	SEM	*P*-value
ADC_{DM}	89.05[a]	76.45[ab]	68.86[b]	66.19[b]	3.535	0.018
ADC_{CP}	91.01[a]	79.48[ab]	89.86[ab]	75.26[b]	2.512	0.023
ADC_{EE}	100.00	97.36	96.42	96.55	0.738	0.336
ADC_{GE}	86.21[a]	81.42[b]	81.86[b]	75.26[c]	1.202	0.000

[1] AD = *Alphitobius diaperinus* larvae meal; CP = crude protein; DM = dry matter; EE = ether extract; GE = gross energy; HI = *Hermetia illucens* larvae meal; TM1 and TM2 = *Tenebrio molitor* larvae meal.
[2] Different letters in the same row stand for statistical differences between groups ($P<0.05$).

Table 6. Apparent digestibility coefficients (ADC, %) of amino acids (n=3).[1,2]

	TM1	TM2	HI	AD	SEM	*P*-value
Essential amino acids						
Arginine	99.99	100.00	99.98	100.00	0.003	0.164
Histidine	97.23	97.92	100.00	94.39	0.983	0.231
Isoleucine	93.78	93.38	93.78	91.99	0.590	0.733
Leucine	94.92	94.49	95.24	94.81	0.671	0.989
Lysine	98.47	98.45	97.72	96.87	0.441	0.595
Methionine	87.48[b]	93.68[a]	94.84[a]	82.78[c]	1.479	0.000
Phenylalanine	93.37	93.43	92.99	91.82	0.866	0.932
Threonine	93.76	93.25	94.21	92.00	0.610	0.671
Valine	94.47	94.21	95.22	91.82	0.910	0.646
Non-essential amino acids						
Alanine	92.96	92.86	94.01	91.33	0.548	0.433
Aspartic acid	92.44	92.32	92.66	90.98	0.637	0.833
Cysteine	80.71[a]	79.01[a]	80.59[a]	68.93[b]	1.772	0.030
Glutamic acid	94.87	94.85	97.06	94.47	0.685	0.592
Glycine	90.97[ab]	90.70[ab]	94.63[a]	60.33[b]	4.472	0.000
Proline	82.82	79.61	82.78	83.50	0.580	0.059
Serine	92.33	92.20	93.09	90.97	0.657	0.778
Tyrosine	91.76[a]	91.29[a]	90.87[a]	63.12[b]	3.709	0.000

[1] AD = *Alphitobius diaperinus* larvae meal; HI = *Hermetia illucens* larvae meal; TM1 and TM2 = *Tenebrio molitor* larvae meal.
[2] Different letters in the same row stand for statistical differences between groups ($P<0.05$).

and on the processing treatment. The few studies that are available show that the digestibility of insect-derived ingredients also depends on the species of farmed fish, as it does for the insect species and all the conditions related to insect rearing and processing (Basto *et al.*, 2020; Fontes *et al.*, 2019).

Nowadays, insect producers are oriented towards the production of defatted IMs to increase the protein content of the meals and to modulate feed extrudability. The defatting process also avoid having insect meals with a high lipid content which may hinder the inclusion of these innovative ingredients in feed formulae for monogastric animals (Cadinu *et al.*, 2020; Chemello *et al.*, 2020; Choi *et al.*, 2017). Thus, this study has investigated the nutritional value and digestibility of four defatted IMs that could be used to formulate compound diets, on a digestible value basis, for rainbow trout, one of the most frequently cultured fish species in Europe.

Proximate composition, and the amino acid and fatty acid contents of insect meals

Mass rearing conditions and feed substrates influence the metabolism and, consequently, the chemical composition of insects to a great extent (Fuso *et al.*, 2021; Melis *et al.*, 2019; Rumbos *et al.*, 2021). Technological processes can have a further impact on them, and can lead to defatted IMs

Table 7. Contents of digestible essential amino acids (EAA) of the tested insect meals, digestible EAA of conventional protein sources (fishmeal and soybean meal), and EAA requirements of rainbow trout of 0.2-20 g and 100-500 g of body weight (g/100 g DM).[1]

	Insect meals				Conventional protein sources		Fish requirements[2]	
	TM1	TM2	HI	AD	FM[3]	SBM[3]	0.2-20 g	100-500 g
Arginine	3.80	3.62	2.88	4.49	3.58	3.38	1.68	1.54
Histidine	2.32	2.23	1.53	3.05	1.48	1.14	1.04	0.95
Isoleucine	2.49	2.28	1.88	2.87	2.66	1.99	1.37	1.25
Leucine	4.65	4.40	3.54	5.21	4.48	3.16	2.47	2.26
Lysine	3.91	3.91	3.20	5.54	4.23	2.96	2.52	2.30
Methionine	2.32	1.32	1.24	2.21	1.61	0.58	1.01	0.92
Phenylalanine	2.15	2.06	1.74	3.02	2.31	2.29	1.57	1.43
Threonine	2.81	2.63	2.24	3.34	2.64	1.55	1.37	1.25
Valine	3.58	3.31	2.51	3.65	3.14	1.96	1.75	1.60

[1] AD = *Alphitobius diaperinus* larvae meal; FM = fishmeal; HI = *Hermetia illucens* larvae meal; SBM = soybean meal; TM1 and TM2 = *Tenebrio molitor* larvae meal.
[2] Reported by Hua and Bureau (2019) for diets having 44% digestible protein and 22 MJ/kg digestible energy.
[3] Elaborated from Lee *et al.* (2020). The digestible EAA of FM are calculated as the average values of sardine and menhaden meals.

Table 8. Apparent digestibility coefficients (ADC, %) of main fatty acids (n=3).[1]

	TM1	TM2	HI	AD	SEM	*P*-value
C12:0	97.65	98.74	96.75	100.00	0.747	0.571
C14:0	100.00	93.39	96.69	100.00	1.780	0.561
C16:0	100.00	93.87	96.17	90.27	1.994	0.416
C18:1 *c*9	100.00	98.89	89.19	91.81	2.498	0.343
C18:2 n-6	100.00	93.04	91.53	96.19	1.604	0.225
C18:3 n-3	98.72	99.96	90.73	94.29	1.625	0.101

[1] AD = *Alphitobius diaperinus* larvae meal; *c* = *cis*.; HI = *Hermetia illucens* larvae meal; TM1 and TM2 = *Tenebrio molitor* larvae meal.

that change dramatically from one to the other in terms of their nutrient contents (Melis *et al.*, 2018; Ravi *et al.*, 2020). Thus, the differences in the compositions of the meals tested in the present trial and published data depend on the different origins, production processes and treatments.

The main chemical composition of TM1 and TM2 was consistent with literature for other defatted TM meals (CP 58-68% DM; EE 4-13.6% DM; ash 3-8% DM) (Basto *et al.*, 2021; Botella-Martínez *et al.*, 2021; Cho *et al.*, 2020; Rema *et al.*, 2019). However, some differences were found (higher CP and lower EE contents) compared to the defatted TM meal used by Basto *et al.* (2020) as a protein source for European sea bass. The EAA contents found for TM1 and TM2 were comparable with those reported by Basto *et al.* (2020) for defatted TM meals, except for arginine (Arg) and Met, which were lower and higher, respectively, in our trial than in the above-mentioned study (5.13 and 0.96 g/100 g DM for Arg and Met, respectively). Additionally, the Σ EAA / Σ AA ratio in TM1 and TM2 (as in all the IMs tested in the current study), was higher than 40%, thus confirming previous results pertaining to HI meal (Huang *et al.*, 2019). As far as the FA profile is concerned, only scant information is available for defatted TM meals. Under our conditions, the TM meals showed a preponderance of oleic acid that is consistent with the available data (Lawal *et al.*, 2021). The differences in the C16:0 and C18:2 n-6 contents between TM1 and TM2 likely depended on the different feed formulations (protected by intellectual properties rights in the case of TM2) that were used to rear the insects (Dreassi *et al.*, 2017; Lawal *et al.*, 2021; Melis *et al.*, 2019), even though the processing technique can also impact the FA profile of yellow mealworm larvae (Melis *et al.*, 2018). According to the literature, the n-6/n-3 FA ratio in yellow mealworms is highly variable (from 6.76 to 71.07) (Dreassi *et al.*, 2017; Mattioli *et al.*, 2021), and our data (15.52 and 23.95 for TM1 and TM2, respectively) fall within this range.

The chemical composition of the HI meal tested in the present trial was consistent with data referring to partially

defatted HI meals used for animal feeding (Biasato *et al.*, 2019; Caimi *et al.*, 2021). As expected, when compared with highly defatted HI meals, our HI meal showed lower CP and higher EE contents (Caimi *et al.*, 2020; Schiavone *et al.*, 2017). Nevertheless, some of the differences in the CP content of IMs among studies can be ascribed to the use of different K_p for nitrogen conversion into CP. In fact, Schiavone *et al.* (2017) used 6.25 as K_p to calculate the CP content of HI meal, which resulted to be equal to 65.5% DM. The use of the correct K_p value (Janssen *et al.*, 2017) would have resulted in a CP content equal to 59% DM and would therefore have been consistent with the CP content of the HI meal used in our trial. Our results also confirm that the K_p proposed by Janssen *et al.* (2017) avoids overestimation of the protein content, which may result in the formulation of unbalanced diets for farm or companion animals (Boulos *et al.*, 2020). In fact, the total AA content (obtained as the sum of the contents of the individual AAs) was only slightly lower for the tested IMs than the CP content calculated using the K_p suggested by Janssen *et al.* (2017). The sum of the individual AAs measured in a matrix represents its true protein content (Hayes, 2020) and this approach would therefore appear to be the optimal one to assess the protein in an ingredient.

As for the amino acids in the HI meal, the EAA contents found in our study were very similar to those reported by Basto *et al.* (2020), but overall higher than those reported by Crosbie *et al.* (2020) for other defatted HI larva meals used in animal feeding and digestibility trials. The HI meal also showed a prevalence of saturated (SFAs) and monounsaturated FAs, compared to polyunsaturated FAs, thus confirming previously published data (Hender *et al.*, 2021; Huang *et al.*, 2019). The sum of the main SFAs (C12:0 + C14:0 + C16:0) (36.17 g/kg DM) accounted for 56% of the TFA. Large quantities of SFAs in black soldier fly larvae have been linked to its subtropical origin, as the high melting point of SFA enables the HI to prevent lipid oxidation and to survive at the typical high temperatures of subtropical areas (Meneguz *et al.*, 2018b).

The composition of the AD meal used in the current trial is not comparable with previous data, as a strong defatting process was applied and this resulted in a very low lipid content and a high protein content (2.5 and 76.3 g/100 g DM, respectively). The DM and CP data for AD are consistent with those reported by Janssen *et al.* (2019) for freeze-dried AD defatted with diethyl ether as the solvent. According to a recent review (Rumbos *et al.*, 2019), the CP content in full-fat AD meals ranges from 58 to 65% DM while the crude lipid content ranges from 13.4 to 29.0% DM. The EAA contents obtained in the current study were comparable with those reported for a defatted AD meal with a crude lipid content of 8.9% DM (Jensen *et al.*, 2019), and higher than the contents measured in whole AD larvae (Soetemans *et al.*, 2020) or full-fat AD meal (Jensen *et al.*, 2019). This latter result was expected because of the defatting process (Basto *et al.*, 2020). Finally, the FA profile of full-fat AD larvae has been reported to be rich in C16:0, C18:1 n-9 and C18:2 n-6, with a low level of C18:3 n-3, thus leading to a high n-6/n-3 FA ratio (Jensen *et al.*, 2019; Oonincx *et al.*, 2020). This latter ratio reached a value of 37.0 in the AD meal tested in the present trial, consistently with what Oonincx *et al.* (2020) measured for full-fat AD larvae.

Digestibility of the macronutrients, amino acids and fatty acids

In the present trial, all the tested IMs showed a higher DM digestibility than 65%, where ADC_{DM} gives a measure of the overall digestibility of a feedstuff or a diet (Basto *et al.*, 2020; Che *et al.*, 2017; Lee *et al.*, 2020), and low values are related to the presence of indigestible materials, such as complex carbohydrates and minerals, or antinutritional factors. The DM digestibility was always higher than the one reported for PPs (SBM, barley, canola meal, cotton seed meals, distiller dried grains with solubles, sunflower meal, and wheat meal) and higher or equal to values reported for other PAPs, such as feathers, poultry by-products, meat and bone meals in rainbow trout (Lee *et al.*, 2020). When compared to the ADC_{DM} of fishmeal (86.6% for both sardine and menhaden meals, as reported by Lee *et al.* (2020)), our values were slightly higher (TM1) or substantially lower (TM2, HI and AD).

The ADC_{DM} of TM1 was higher than that reported by Basto *et al.* (2020) for a defatted TM meal fed to European sea bass (72.4%), but lower than what Fontes *et al.* (2019) obtained when assessing the digestibility of a full-fat TM meal in tilapia fingerlings (95.8%). Indeed, the discrepancies in these levels may also be due to the methodology used to collect the fish faeces, as well as to the variability in the composition of the tested raw materials. The automatic Choubert collection device used in our trial enables a rapid removal of the faeces from the water, thus minimising nutrient leaching. However, other faeces collection methods can overestimate digestibility values (i.e. sedimental column) due to nutrient leaching, or underestimate it (i.e. stripping) because of the contamination of faeces with undigested material (Bureau *et al.*, 1999; da Mota *et al.*, 2015; Lee *et al.*, 2020). In our trial, the lowest ADC_{DM} values were obtained for the HI and AD meals. The low ADC_{DM} value of HI may be due to its high ash content, as ash can negatively affect the dry matter digestibility of PAPs (Bureau *et al.*, 1999; Mo *et al.*, 2019).

Overall, the ADC_{CP} values obtained in our trial for all the IMs were higher than those reported by Lee *et al.* (2020) for PAPs in rainbow trout. As for TM1, the ADC_{CP} was higher than those of sardine and menhaden meals (Lee *et al.*, 2020) or comparable with other menhaden meals (NRC,

2011). Additionally, the ADC_{CP} of TM1 was consistent with values reported by Basto *et al.* (2020) and higher than the value reported for a full-fat TM meal by Fontes *et al.* (2019). As far as HI is concerned, the comparisons with literature results are not consistent. We found values that were higher than those of sardine and menhaden meals (Lee *et al.*, 2020); comparable with the HI studied by Dumas *et al.* (2018) in rainbow trout (85%) and by Basto *et al.* (2020) in European sea bass (87.2%), but higher than those found in rainbow trout (68.8%) by Lee *et al.* (2020), and by Kroeckel *et al.* (2012) in turbot (63.1%). In most studies, low ADC_{CP} values are attributed to the presence of high chitin levels. In fact, chitin may interfere with the dietary utilisation of protein, thus decreasing protein digestibility (Longvah *et al.*, 2011). Fontes *et al.* (2019) showed a low ADC_{CP} in tilapia fed meals based on *Nauphoeta cinerea* (67.7%), *Zoophobas morio* (74.3%), *Gromphadorhina portentosa* (58.3%) and *Grillus assimilis* (38.9%), and observed a chitin content of 22.3 to 28.9% DM. In contrast, the ADC_{CP} found by the same authors for a full-fat TM meal containing 12.1% DM of chitin was much higher (92.4%) and was similar to that obtained in our trial for TM1. Recently, Eggink *et al.* (2022) demonstrated that different HI meal size fractions, corresponding to different chitin contents of the meals, significantly affected the digestibility of nutrients in rainbow trout. These authors showed that the highest size fraction (>400 mm), which corresponded to the HI meal with the highest chitin content, reduced the apparent digestibility of DM, CP, nitrogen-free extract and chitin, despite an upregulated exochitinase activity along the intestinal tract of the fish. Such results clearly suggest that, even if rainbow trout is somewhat able to digest chitin, the latter also acts as an anti-nutrient.

A positive correlation has also been reported between the protein content and its digestibility in carnivorous species, such as European sea bass or rainbow trout (Basto *et al.*, 2020; Kaiser *et al.*, 2021), as also observed for the TM1 tested in our trial. In our study, the lowest ADC_{CP} was reported for the AD meal. Protein digestibility is related to the ability of fish to hydrolyse proteins into small peptides and free AAs (NRC, 2011). Processing treatments, such as heating or pressure, are used to improve protein digestibility, particularly for PPs (Almeida Sá *et al.*, 2020; Joye, 2019). Nevertheless, these treatments, when excessive, could decrease CP digestibility by creating new protein linkages, thus increasing resistance towards the digestion process (NRC, 2011). As far as the AD meal used in the current trial is concerned, no details about the manufacturing process (temperature or defatting methods) were provided by the manufacturer. However, by considering at its very low fat content (2.5% DM), it is possible to assume that a strong defatting process was applied. A correlation between protein proprieties and defatting methods has been reported for insect meals (Gravel *et al.*, 2021; Ravi *et al.*, 2020). Therefore, we can assume that the lowest CP digestibility found for AD meal may be due to the technological process applied for its production. Furthermore, considering the similar amounts of chitin detected in the tested meals, the species and the processing technology likely had major impacts on the overall and CP digestibility (Mancini *et al.*, 2021; Mo *et al.*, 2019).

Regarding ADC_{EE}, the length of the aliphatic chain, the saturation level, and the melting point of lipids can all affect EE digestibility. Lipid digestibility is usually higher than 80% in fish (Hua and Bureau, 2009) and the higher the SFA content is, the lower the digestibility (Bélanger *et al.*, 2021). However, HI is naturally very rich in C12:0 (Table 4), which is known to be much more digestible than other SFAs (Belghit *et al.*, 2019). Thus, we did not observe any significant differences in the tested meals (ADC_{EE} always >96%). We could argue that the unsaturated FA introduced by fish oil and, to a lesser extent, by fish meal in the reference diet generally improved ADC_{EE}, even for the HI meal that contained the greatest SFA content (Bélanger *et al.*, 2021; Hua and Bureau, 2009; NRC, 2011). Moreover, the differences in the contents of the FA classes at the used substitution rate (30%) were not too high to affect the digestive utilisation of fat.

As expected, the digestibility of the gross energy differed among the tested IMs, consistently with ADC_{DM} and ADC_{CP}. The ADC_{GE} of the TM meals was similar to those reported in the literature (82.1% in Fontes *et al.*, 2019; 86.1% in Basto *et al.*, 2020). The ADC_{GE} of the HI meal was higher than the value reported for turbot (75.1%; Kroeckel *et al.*, 2012) and similar to that reported for European sea bass (81.7%; Basto *et al.*, 2020). Lee *et al.* (2020) found that the ADC_{GE} of a defatted HI larva meal averaged at 61.8% in rainbow trout with no significant improvement when protease was added (63.6%). Additionally, our IMs showed higher ADC_{GE} values than those of PAPs (from 58.1% for meat and bone meal to 74.4% for poultry by-product meal) and PPs (from 32.9% for sunflower meal to 70.2% for algae meal) in rainbow trout (Lee *et al.*, 2020), except for the ADC_{GE} of sardine and menhaden meals (87.0 and 90.2%, respectively). Moreover, raw grain legume seeds, such as chickpea and faba beans, when tested as alternative protein sources for rainbow trout, showed lower ADC_{GE} values (Magalhães *et al.*, 2018) than our IMs.

Only a limited number of papers are currently available about the AA digestibility of IMs as tested ingredients. The ADCs of the EAAs were high for all the tested IMs and higher than the ADCs of the AAs of several animal and plant protein sources commonly used in rainbow trout feeds, except for the ADC of Met in TM1 and AD (Lee *et al.*, 2020). Lys is generally the most limiting EAA in plant protein sources used to formulate aquafeeds (Yun *et al.*, 2016) and its requirements are higher in rainbow trout than in various other aquaculture fish species (NRC, 2011). The

average ADC of Lys of the TM meals tested in our trial was about 3% higher than the values reported by Basto *et al.* (2020) for full-fat (95.8%) and partially defatted (95.5%) TM meals. The available data for HI report a digestibility of 90% for a partially defatted HI meal in rainbow trout (Dumas *et al.*, 2018). This low value can be explained by both the lower CP content (42.3% DM if calculated with the appropriate K_p) and Lys content (2.79% DM) in the HI meal used by these authors. Basto *et al.* (2020) found ADC Lys values equal to 92.6% and 96.1% in full-fat and defatted HI meals, respectively, for European seabass.

The TM1, TM2 and AD meals used in the current trial showed larger amounts of digestible EAAs than the average values reported by Lee *et al.* (2020) for sardine and menhaden meals in rainbow trout; except for isoleucine (Iso), Lys, and phenylalanine (Phe) in TM1 and TM2, and showed lower values than the above-mentioned fish meals. On the other hand, the HI meal had lower than or very similar (histidine) digestible EAA contents to FM. In comparison with the digestible EAA profile of SBM (Lee *et al.*, 2020), both of the TM meals, HI and the AD meal showed higher contents of individual digestible EAAs, except for Arg, Iso, and Phe in the HI meal. Unlike SBM, which is deficient in digestible Met, all the IMs can fulfil the digestible EAA requirements of different sized rainbow trout by using diets with different combinations of digestible protein and digestible energy contents (Hua and Bureau, 2019). Nogales Merida *et al.* (2019) also reported that different IMs, such as *Musca domestica*, *H. illucens*, *T. molitor*, *Acheta domesticus* and *Z. morio*, were able to cover the requirements of omnivorous fish species, while a dietary supplementation with Met is necessary for carnivorous species, such as *Psetta maxima*, to cover their requirements (1.49-1.59%) (Ma *et al.*, 2013).

Finally, as far as the FAs are concerned, to the best of our knowledge, this is the first study to show the ADC of FA of IMs in fish. High ADC values were found, ranging from the lowest value of 90.27% for palmitic acid in AD to 100% for several FAs in both TM1 and AD. Such high values are comparable with or lower than those found in a previous study on rainbow trout conducted by Sevgili *et al.* (2019) who tested the digestibility of microbial raw materials and two different batches of anchovy meal. As mentioned above, lipid digestibility is impacted by its FA profile and especially by SFAs (Bélanger *et al.*, 2021; Hua and Bureau, 2009). Care should be taken when using IMs characterised by a high SFA content in cold water fish to include sufficient quantities of unsaturated FAs to guarantee high digestibility values. Under the conditions of the present trial, the reference diet contained 8% fish oil that decreased to 5.6% in the tested diets. Bureau *et al.* (2008) showed that an inclusion of 8% of fish oil is able to guarantee high lipid digestibility, even when 8% of tallow was present. The common lipid sources in commercial diets for rainbow trout are plant oils, such as soybean, rapeseed, or linseed oils (Bruni *et al.*, 2021; Caimi *et al.*, 2021; Chemello *et al.*, 2020), which are characterised by high levels of unsaturated FAs, and fish oil, when included at low rates, guarantees a supply of the long chain PUFAs. When using alternative ingredients in diets for rainbow trout, an SFA threshold of 23% TFA has been proposed to avoid negative effects on lipid digestibility (Hua and Bureau, 2009). Considering the HI meal used in the present trial, which was the diet that contained the highest level of SFAs (about 60%), even inclusions levels of 30% did not represent an issue for lipid or FA digestibility.

5. Conclusions

Over the past decades, a great deal of attention has been paid to searching for cost-effective alternative protein sources to FM in aquafeeds. Nowadays, PPs or PAPs are used extensively. However, these alternatives sometimes show sub-optimal ADC and digestible nutrient contents. The use of defatted IMs in fish feeding is still at its infant stage, compared to other protein sources, and the proper determination of their ADC is a key element for their correct inclusion in diets for carnivorous and omnivorous fish species. The overall high ADC values found in this research for macronutrients, AAs and FAs show that these innovative feed ingredients could be a valuable protein source for the formulation of sustainable rainbow trout feeds. According to our results, strong defatting processes, such as the one AD likely underwent, seem to negatively impact digestibility. Therefore, particular attention should be paid to the kind of process that is applied (i.e. in terms of temperature, lipid extraction methods and solvents used) in order to avoid compromising the potential use of very valuable high protein sources in aquafeeds. Finally, considering the great variability found in literature, and which has been confirmed in our trial, about the composition of IMs and, consequently, about their digestibility, further efforts need to be made by the industry to deliver more standardised IMs to the market.

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Conflict of interest

The authors declare no conflict of interest.

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