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Growth performance and chemical composition of *tenebrio molitor* larvae grown on substrates with different starch to fibre ratios

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ABSTRACT

Production of insects as animal protein source has increased recently but the information on nutrient requirements for optimal growth is still scarce. The effects of carbohydrate composition of the substrate on growth performance and chemical composition of *Tenebrio molitor* larvae were evaluated. Three substrates based on different proportions of wheat grain, wheat bran and soybean meal were formulated to contain three starch to neutral detergent fibre (NDF) ratios: low (1.0; LSF), medium (1.2; MSF) and high (1.4; HSF), at a common N level. Each treatment was replicated five times in trays containing 23 g of substrate and 40 larvae (2.95 mg average weight) each. Weight of the larvae and the substrate remaining per tray were weekly monitored from 21 to 49 days of experiment and larvae within the same experimental treatment were pooled at the end of the trial for chemical analysis. No treatment differences were observed for larval growth or mortality ($p > 0.05$). The starch to NDF ratio in the substrate did not affect larval composition in terms of dry matter, nitrogen or ether extact contents or the amino acid composition of the protein fraction. Within the range of ingredient composition considered, the starch to NDF ratio in the substrate has a minor influence on growth performance and chemical composition of *T. molitor* larvae.

HIGHLIGHTS

- a starch to NDF ratio within a range of 1.0 to 1.4 does not modify growth of *Tenebrio molitor* larvae
- larval composition remains unaffected when the starch to NDF ratio is maintained within a range of 1.0 to 1.4
- up to 326 g NDF/kg of a substrate with 25 g total N/kg is not limiting for *T. molitor* larval performance

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Introduction

Tenebrio molitor is an insect species from the order Coleoptera, called mealworm because it generally grows on stored cereal grains and meals as preferred feed substrates. Because of its growth performance and nutrient composition, industrial production of *T. molitor* larvae has a major interest as a potential protein source for feeding domestic animals (Veldkamp et al. 2012; Khan 2018; Secci et al. 2021). However, rearing substrate characteristics and chemical composition for optimum insect growth are not yet defined. Larvae of *T. molitor* are generally raised on mixtures based on cereal grains and by-products, commonly using wheat bran as the reference substrate (Ramos-Elorduy et al. 2002; Ribeiro et al. 2018). However, market prices of cereal grains and by-products of the milling industry have increased notably since 2019 and as a result, the potential use of other available fibrous sources that may reduce production costs has gained interest (Morales-Ramos et al. 2010; van Broekhoven et al. 2015; Kim et al. 2017). In any case, the wide variability in both the chemical composition and the potential nutritive value observed in agroindustrial by-products suggests that their use as alternative feeds would rely on their nutrient composition, particularly in terms of its protein and energy content.

The dietary level of protein is crucial to ensure an adequate larval growth (Fondevila and Fondevila 2023). Besides, as for other animal productive species such as pigs and poultry, starch can be assumed as

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the most suitable energy source for *T. molitor* larvae (Ramos-Elorduy et al. 2002; Ribeiro et al. 2018). However, these insect species can successfully grow on fibrous substrates and in fact, compared to other ingredients rich in starch (He et al. 2021; Rumbos et al. 2021), increased larval growth rates have been reported for diets based exclusively on wheat bran, a cereal by-product containing 371 to 403 g neutral detergent fibre (NDF) and 150 to 200 g starch per kg (FEDNA 2021). The presence of different types of polysaccharidases (amylase, cellulase, licheninase) and disaccharidases (β-glucosidase, cellobiase, trehalase) has been detected in the digestive tract of *T. molitor* (Terra et al. 1985; Genta et al. 2006). However, according to these authors, cellulase activity is low and mostly attributed to exogenous microorganisms. In this respect, Martin (1991) did not consider Tenebrioniidae among the cellulase-harbouring Coleoptera, and estimated fibre digestion between 0.14 and 0.16 in wheat bran-based diets (Fasce et al. 2022). In contrast, Oppert et al. (2010) suggested that *T. molitor* larvae degrade soluble and insoluble cellulose by endogenous (insect produced) and exogenous (from digestive microbiota) fibrolytic enzymes, and Rodjaroen et al. (2020) stated that the specific activity of cellulases increases respect to that of amylase at late stages of larval growth. Therefore, variations in the balance of the carbohydrate fraction present in the feed might determine the potential success of a substrate mixture made up with any fibrous by-products, included in the diet to reduce feeding costs respect to high starch feeds.

Most of the studies in insect feeding are based on comparisons of a wide range of ingredients of very different nature and variable nutrient and chemical characteristics (Ramos-Elorduy et al. 2002; Jajić et al. 2022; Rumbos et al. 2022). Furthermore, information in literature about carbohydrate proportions in terms of starch and fibre is often missing, or limited to an estimation of the crude fibre and N-free extract contents, thus making difficult to draw firm conclusions on their effects. The nutrient composition and physical properties of agroindustrial by-products are affected by both the agronomical culture practices and the subsequent processing conditions, and thus the potential generalisation of in published research to broad nutritional principles is often limited, being primarily applicable to the specific ingredient used and the rearing conditions applied. Consequently, despite previous published approaches may be of interest in terms of productive and economic performance from a practical point of view, the productive results obtained cannot be directly extrapolable and they do not provide consistent information to define the nutritive requirements of insects accurately. It should be considered that, despite possible functional effects and the presence of secondary compounds (van Broekhoven et al. 2015), the potential interest of feeds including fibrous ingredients rely on their balance of nutrients, which in terms of energy availability is mostly defined by the fibrous to non-fibrous carbohydrates. Therefore, knowledge on the starch and fibre levels in the feeding substrate for adequate larval growth is essential to optimise protein and fat production as well as to reduce the production costs associated with the feed. This work aims to determine the effects of moderate variations in the starch to fibre ratio in the substrate on growth performance and chemical composition of *T. molitor* larvae to provide information on the carbohydrate requirements in this species in order to better establish their nutrient requirements.

Materials and methods

Experimental diets

Three substrates were formulated by combinations of wheat grain (starch, 658 mg/g dry matter, DM; NDF, 106 mg/DM; nitrogen, N, 21.1 mg/DM) and wheat bran (starch, 94 mg/g DM; NDF, 489 mg/g DM; N, 28.1 mg/g DM) to obtain different starch to NDF ratios. In addition, soybean meal (85.5 mg N/kg DM) was added when required to adjust total N proportion to a common level of 25 to 26 g/kg DM, equivalent to 156-163 g crude protein (N x 6.25) per kg. The final carbohydrate content of the experimental mixtures ranged from 330 to 390 g starch/kg DM and from 280 to 330 g NDF/kg DM, respectively. The resulting substrates were defined with three different starch to NDF ratios, namely low (1.0; LSF), medium (1.2; MSF) and high (1.4; HSF). Ingredient and chemical composition of substrates are shown in Table 1. All ingredients were ground to 2 mm particle size before being mixed in the substrate. In addition, fresh carrots (1.81 ± 0.46 g per tray, 909 g moisture per kg) were weekly provided as a source of water for larvae.

Experimental procedures

The trial was carried out in a room with homogeneous environmental conditions: average minimum and maximum daily temperatures ranged between 23 and 29° C (average 26.0 ± 2.6° C) and relative humidity values were between 38 and 58% (average 49.4 ± 9.8%).

Table 1. Ingredient (g/kg) and analysed chemical composition (g/kg dry matter, DM) of the experimental substrates.

	LSF	MSF	HSF
Ingredients:			
Wheat grain	420	510	600
Wheat bran	580	475	370
Soybean meal	0	15	30
Chemical composition:			
Dry matter	898	898	894
Organic matter	961	961	962
Total N	25.3	26.4	26.4
Ether extract	19.0	18.3	17.2
Starch	330	391	392
Neutral detergent fibre	326	317	279
Starch to NDF ratio	1.01	1.23	1.41

NDF: neutral detergent fiber

The experiment was carried out in darkness, except for sampling procedures. Five open plastic trays (15 × 9 × 6 cm) with 23 g substrate were set up for each treatment, and 40 larvae with an initial individual weight of 2.95 mg (0.118 ± 0.012 g per tray) were placed on each tray. Prior to the start of the experiment, larvae were offered a substrate based on 1:1 wheat grain and wheat bran. Then, larvae were collected by sieving through a 0.6 mm mesh at 7 to 8 weeks after hatching. The experiment lasted for 49 days, until the proportion of pupae was over 5% (final proportion 6.5%).

From 21 to 49 days of experiment, the number and weight of the larvae, as well as the residual on each tray were weekly monitored. From these data, to estimate mass gain (total increase in larval weight), substrate intake (as the difference between initial substrate and final residue), larval mortality rate (by counting living larvae) and daily average individual larval weight gain (considered as larval growth) were calculated, either per week or along the whole experimental period. The estimation of daily intake was not corrected for the frass remaining in the residual substrate at the end of each period since these components of the residue could not be separated accurately from the finer particles of the substrate. Residual carrot remaining from the previous offer was weekly removed. The Feed to Gain ratio was calculated as the amount of substrate consumed (amount offered minus remaining residue after 49 days) per unit of weight gained, and expressed on fresh and DM basis, without considering the weight of the carrot provided. At the end of the experimental period, the number and weight of pupae appearing were recorded and removed from the tray. Pupae were not considered in the counts, but pupal weight was also considered in the calculation of the Feed to Gain ratio.

At the end of the experimental period, all the larvae from each tray were harvested by hand plucking, pooled by treatment, frozen at −80° C and lyophilised before being analysed for chemical composition. The total larval production of DM, N and ether extract (EE) per tray was calculated from larval mass production. Nitrogen efficiency was estimated as the amount retained per unit of ingested N. Feed residue (including larval excretion) from each tray was individually sampled for further laboratory analysis, as reference.

Laboratory analyses

Substrate feeds and residues were ground through a 1 mm size sieve and analysed following the AOAC (2005) procedures for DM (method 934.01), organic matter (OM, method 942.05), total N (method 976.05) and EE (method 2003.05, only substrates). Besides, the concentration of NDF was analysed as described by Mertens (2002) with an Ankom 200 Fibre Analyser (Ankom Technology, New York), using α–amylase and sodium sulphite, and the results were expressed exclusive of residual ashes. Total starch content was determined enzymatically from samples ground to 0.5 mm by using a commercial kit (Total Starch Assay Kit K-TSTA 07/11; Megazyme, Bray, Ireland).

Larvae were lipohylised (initial temperature −45 °C; 48 h at −25 °C, 55 μbar; 24 h at 25 °C) and analysed for DM, total N and EE, by the procedures reported above. In addition, amino acid composition was analysed by ion-exchange chromatography (Hewlett-Packard 1100, Waldbronn, Germany). Briefly, samples were hydrolysed with 6 N HCl for 22 h at 110° C under reflux conditions while bubbling with nitrogen (Jones et al. 1981). A 400 mL to 200 mg acid to sample ratio was used to reduce amino acid losses in the presence of carbohydrates. Protein hydrolysates and amino acid calibration mixture were derivatised with *o*-phthalaldehyde. For the determination of methionine and cysteine, separate samples were previously oxidised with performic acid and measured as Met sulphone and cysteic acid, respectively (Moore 1963). Tryptophan was determined after alkaline hydrolysis for 20 h at 110° C.

Statistical analysis

Results were analysed by one-way ANOVA with the Statistix 10 package (Analytical Software, Tallahasee, USA), considering the tray with 40 initial larvae each, as the experimental unit for all measurements. All parameters were explored for normality using the Shapiro-Wilk test. Differences were considered significant at $p < 0.05$ and a trend to significance was considered at $p < 0.10$. Tukey's t test was used for comparison among means.

Results

No treatment differences on the initial larval weight were detected ($p = 0.950$, Table 2). The overall mortality averaged 0.176 and did not differ among treatments ($p = 0.252$). No differences among treatments were detected ($p > 0.050$) for larval weight at any of the phases considered (Figure 1).

Larval production from 0 to 49 days of experiment, either considered as total mass gained per tray or growth per living larvae, were not affected by the starch to fibre ratio. Similarly, no differences among experimental treatments were detected for the total DM, total N or EE production or the daily substrate intake per tray. However, the Feed to Gain ratio tended to be lower in MSF than in HSF when expressed either in fresh ($p = 0.087$) or DM ($p = 0.090$) basis. The Efficiency of N utilisation averaged 0.875 g of larval N produced per unit of dietary N used, and did not differ among treatments ($p = 0.383$). The chemical composition of the feed residue is shown in Table 3.

Although the results on larval composition were not statistically contrasted, no apparent treatment differences were detected in DM, total N and EE contents (on DM basis), that averaged 388, 77 and 358 mg/g, respectively (Table 4). However, the N content of the larvae increased numerically as the starch to NDF ratio

Table 2. Effect of different starch to neutral detergent fibre (NDF) ratios in the substrate on mortality (as proportion of initial larvae), growth performance, feed to Gain ratio, efficiency of N deposition (g retained/g ingested) and total nutrient production of *T. molitor* larvae from 0 to 49 days.

	LSF	MSF	HSF	SEM	*p*-value[a]
Mortality	0.145	0.169	0.215	0.0284	0.252
Initial weight (mg/larvae)	2.95	2.94	3.00	0.141	0.950
Final weight (mg/ larvae)	87.1	95.7	89.4	4.46	0.447
Mass gained (g)	2.880	3.078	2.696	0.2124	0.515
Larval growth (mg/d)	1.72	1.89	1.76	0.088	0.437
Intake (g, fresh basis)	3.780	3.623	3.722	0.2491	0.915
Intake (g, DM basis)	3.444	3.303	3.384	0.2231	0.916
Feed to Gain (g/g, fresh basis)	1.326	1.186	1.375	0.0521	0.087
Feed to Gain (g/g, DM basis)	3.122	2.792	3.228	0.1219	0.090
Nitrogen efficiency	0.881	0.827	0.917	0.0413	0.383
Total production (g)					
Dry matter	1.115	1.192	1.044	0.0830	0.512
Total N	0.085	0.092	0.081	0.0064	0.534
Ether extract	0.405	0.422	0.371	0.0297	0.516

SEM: standard error of means; DM: dry matter.
[a]*p*-values from the analysis of variance.

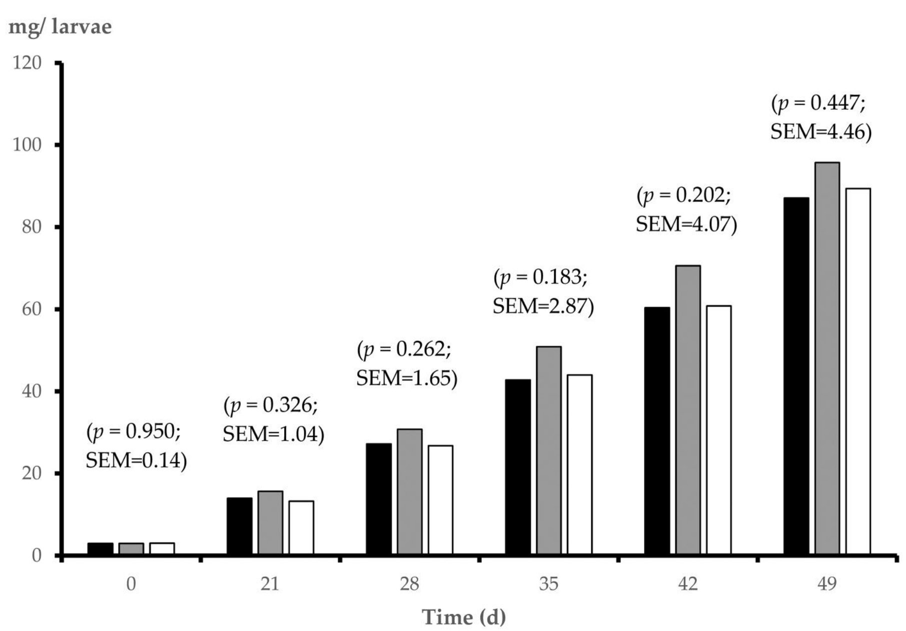

Figure 1. Average weight (mg fresh matter) along the experimental period of *T. molitor* larvae given substrates with low (LSF, black columns), medium (MSF, grey columns) or high (HSF, white columns) starch to neutral detergent fibre (NDF) ratios. The values in brackets indicate *p*-values and standard error of means (SEM) for each time interval. Interaction substrate x time, $p = 0.331$.

Table 3. Chemical composition (mg/g dry matter, DM) of the residual substrate of *T. molitor* larvae after 49 days receiving diets with different starch to neutral detergent fibre (NDF) ratios. The results have not been analysed statistically.

	LSF	MSF	HSF
Feed residue composition			
Dry matter	898	897	899
Organic matter	950	956	956
Total N	24.6	25.0	26.1
Starch	302	350	351
Neutral detergent fibre	385	325	320
Starch to NDF ratio	0.77	1.01	1.08

LSF: low starch to fibre ratio; MSF: medium starch to fibre ratio; HSF: high starch to fibre ratio

Table 4. Effect of different starch to neutral detergent fibre (NDF) ratios in the substrate on the chemical (mg/g DM) and amino acid (g/16 g N) composition of *T. molitor* larvae after 49 days of experiment receiving diets with different starch to NDF ratios. These results have not been analysed statistically.

	LSF	MSF	HSF
Larval composition			
Dry matter	387	389	387
Total N	75.9	77.0	77.5
Ether extract	364	354	356
Amino acid composition			
Lys	5.16	5.18	4.97
Met	1.25	1.25	1.21
Cys	0.98	1.00	0.92
Thr	3.73	3.74	3.62
Trp	1.21	1.21	1.18
Ile	5.05	5.09	4.96
Leu	7.70	7.80	7.58
Val	5.69	5.64	5.56
Phe	3.32	3.33	3.27
Arg	5.17	5.16	5.05
His	2.73	2.76	2.63

increased, whereas the EE content was numerically higher in larvae fed LSF. Similarly, no apparent substrate effect was observed on the amino acid composition of the larvae, as it is shown by a coefficient of variation lower than 2.3% for all parameters.

Discussion

A previous work from our group showed that a level of 100-120 g crude protein (CP, total N x 6.25) per kg DM in the substrate is enough for ensuring an optimum growth of *T. molitor* larvae (Fondevila and Fondevila 2023), and no differences were detected in total larval mass production within a range of 100 to 160 g CP/kg DM (equivalent to 16 to 19 g N/kg DM). However, because of the natural CP proportion of wheat bran as the main substrate ingredient, the N concentration of the substrates used in the current work was adjusted to 26 g N/kg DM to avoid any protein deficiency effect on the results.

To our knowledge, no previous publications in the literature have specifically studied the effects of the starch to NDF ratio in the substrate on the growth performance of *T. molitor* larvae, and in fact previous research was frequently based on a wide range of ingredients and rearing conditions that resulted in variable and inconsistent data. In this respect, the larval performance was better with an estimated starch to NDF ratio of 1.96 compared to 0.68 and 5.70 in diets based on different proportions of wheat grain and wheat bran (Li et al. 2016), whereas the larval growth increased as the estimated starch to NDF ratio decreased from 5.92 to 1.01 in diets based on various agro-industrial by-products such as bread remains, brewer's yeast, or courgette remains (Montalbán et al. 2022). Discrepancies among research might be explained by the wide range of starch to NDF ratios tested as well as potential variations in the nature of the ingredients under test. The results obtained in the present study showed that, within the range considered, the starch to NDF ratio did not affect larval growth, suggesting that the larvae can adapt their feed intake and growth performance according to variations in the starch or fibrous sources included in the diet. Probably, under *ad libitum* conditions, the larvae could select the proportions of particles and ingredients (either wheat grain or wheat bran) required to maximise their growth (Morales-Ramos et al. 2020; Kröncke and Benning 2022). Thus, the starch content of wheat grain and the fibre and protein concentrations present in wheat bran should be conveniently used by the larvae as appropriate sources of energy, an effect that is apparently independent of the larval age. In fact, comparing the chemical composition in the substrate residue with to the initially offered substrate, the starch to NDF ratio decreased in the residue for all treatments (from 1.01 to 0.77 for LSF; from 1.23 to 1.03 for MSF; and from 1.41 to 1.08 for HSF), suggesting that there is some extent of preference for starch over fibre in the larval selection for energy intake. In this sense, Morales-Ramos et al. (2011) indicated that *T. molitor* larvae show a selective feeding behaviour by giving preference to certain ingredients to maintain a constant nutrient intake, and Kröncke and Benning (2022) showed the larval preference for high grain substrates in feed mixtures. Li et al. (2016) and Morales-Ramos et al. (2020) reported that fibre intake in *T. molitor* larvae negatively affects nutrient assimilation from feed, and suggested proportions of NDF in selected feeds between 22.5 and 34.9% to maximise intake, that should agree with the NDF range recorded in our experiment.

It could be considered that the results presented in this work might be in part biased by the presence of larval excreta mixed with the substrate residue at the end of the growth period, and thus affecting its composition and the calculations of feed intake. Some authors (Fasce et al. 2022) have proposed to separate the excreta by sieving, assuming a different particle size compared to that of the substrate. In this respect, previous screening in our laboratory when using ground cereal grains as substrate, showed that 68% of the particles in the frass are between 0.3 and 0.6 mm, whereas 32% are smaller, whereas 42 and 25% of particles of the original wheat grain ingredient were smaller than 0.6 and 0.3 mm, respectively. Consequently, assuming the sieving method as a tool to separate the excreta from the particles of the residual substrate might result in important mistakes when using cereal meals as feed.

Despite the larvae require some level of fibrous sources for maintaining a bulky substrate that ensures their environmental comfort, they do not have a relevant capacity to degrade fibre (Genta et al. 2006; Fasce et al. 2022), which should not contribute at a major extent to their nutrition. However, Yang et al. (2019) reported digestibility rates of cellulose and hemicelluloses from wheat bran and other cereal by-products by *T. molitor* larvae that reached 0.59, highlighting the ability of this insect to digest fibre polysaccharides. Probably, larvae could adapt their growth to variations in the starch to NDF ratios considered in the current research, although excessive NDF contents respect to those observed in fibrous ingredients such as cereal and legumes harvested by-products (Ruschioni et al. 2020; He et al. 2021; Kröncke and Benning 2022) might compromise the nutrient availability. Furthermore, wheat bran, a low starch by-product with a starch to NDF ratio that generally ranges from 0.35 to 0.55 (FEDNA 2021), has been used as the reference substrate in previous research (Kim et al. 2017; Ribeiro et al. 2018) as it usually results in an adequate larval growth rate. This information suggests that *T. molitor* larvae can adapt their feed intake to the nutrient composition of the substrate to meet their energy requirements from other components different to starch such as protein.

At the level of dietary CP of the present study, the efficiency of N deposition averaged 0.88 and was not affected by the treatment, indicating that protein utilisation might not depend on the source of energy. In any case, the results observed were higher to those previously recorded with the same N level in the substrate (0.67 for a 26.6 g N/kg DM substrate, equivalent to 166 g CP/kg; Fondevila and Fondevila 2023). Probably, differences in the ingredient composition of the substrates or in the environmental conditions among experiments, as well as potential variations related to the larvae used in the study, might explain these differences.

The chemical composition of the larvae was within the range reviewed from previous research (Veldkamp et al. 2012; Sánchez-Muros et al. 2014) in terms of N and EE contents, that ranged from 75.9 to 77.5 and from 354 to 364 mg/g DM, respectively. In this respect, although differences among treatments were not evident, the N content of the larvae increased numerically, and the EE decreased as the starch to NDF ratio increased. These results agree with previous research, in which larvae reared on wheat bran (with a low starch to NDF ratio) showed greater N and lower fat contents than those reared on wheat grain (with a high starch to NDF ratio) flour (Ruschioni et al. 2020). A decrease in certain amino acids such as histidine, isoleucine and tyrosine was also detected in that research, whereas an opposite trend was observed for leucine in larvae fed wheat bran compared to wheat grain. In contrast, Stull et al. (2019) observed an increase in the fibre content of the diet reduced the methionine and tyrosine contents of the larvae, whereas no major effects of the estimated starch to NDF ratio on the essential amino acid composition of the larvae has been reported by others (Montalbán et al. 2022). It should be considered that the research from these authors analysed the chemical composition of larvae reared on a vast range of substrates that differed substantially in nature as well as in nutrient content. In the current research, the amino acid profile of the protein fraction of the larvae did not differ substantially among substrates with different starch to NDF ratios, resulting in a standard deviation (SD) that was lower than 1.2% in all cases. In general, the information available on the amino acid composition of *T. molitor* larvae is variable and inconsistent (Adámková et al. 2020; Fasce et al. 2022; Montalbán et al. 2022). For example, amino acid contents reported in the literature vary from 2.50 to 6.52 g/16 g N for lysine (Heidari-Parsa et al. 2018; Nascimento-Filho et al. 2021), and from 0.52 to 2.72 g/16 g N for methionine (Heidari-Parsa et al. 2018; Montalbán et al. 2022). These results suggest that several factors might affect protein deposition in *T. molitor* larvae, and in fact variations in literature do not allow for an accurate estimation of the protein profile of these insects. In any case, no differences on the amino acid composition of the larvae could be expected when comparing substrates with similar ingredient composition such as those used in the current research, despite of the starch to NDF ratio considered.

Conclusions

From the results obtained in the current research, there are no apparent differences in the growth and chemical composition of *T. molitor* larvae within the range of starch to NDF ratios assayed. These results suggest that variations in the proportions of dietary ingredients with different starch and fibre contents such as wheat grain and wheat bran might not necessarily affect the larval performance. However, the results obtained by the inclusion in diet of other by-products different to those tested here may depend on the nature of the fibre source and consequently they cannot be directly extrapolated to any other fibrous feed, without considering potential variations. From an economic perspective, the proportion of starch and fibre contents in diets for *T. molitor* production larvae could be conveniently established according to market costs and ingredient availability, without major effects on their productive performance.

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Ethical approval

The authors confirm that the study was conducted in an ethical and responsible manner. Using insects as laboratory animal does not require approval from an Ethical Commission.

Authors' contributions

Conceptualisation, M.F.; methodology, A.R.; formal analysis, A.R. and G.F.; data curation, G.F.; writing-original draft preparation, G.F.; writing-review and editing, M.F.; supervision, M.F.; funding acquisition, M.F. All authors have read and agreed to the published version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The authors confirm that the data generated in the current study are available within the article.

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