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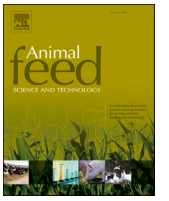

In vitro and *in vivo* investigations on the use of yellow mealworm (*Tenebrio molitor*) as a novel protein feed ingredient for fattening lambs

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A B S T R A C T

The quest for novel, alternative, and sustainable protein feed sources, including insects, has gained attraction by the feed industry. Here, two experiments (Exp.) explored the impacts of *Tenebrio molitor* meal (TMM) as a protein feed ingredient for fattening lambs, as compared to conventional plant-based and animal-based protein sources, namely soybean meal (SBM) and fish meal (FM), respectively. In Exp. 1, *in vitro* rumen gas kinetics and fermentation profile of three experimental diets [1- SBM at 150 g/kg dry matter (DM); 2- FM at 50 g/kg DM; and 3- TMM at 60 g/kg DM] were assessed using three fistulated lambs. In Exp. 2, twenty-four male Suffolk lambs [3 months of age; 24 ± 1.3 kg body weight (BW)] were randomly assigned for 30 days to one of the three experimental diets. Feed intake, digestibility, and nitrogen balance were assessed. At the end of the trial, the lambs were slaughtered, and samples of rumen (dorsal and ventral sacs), proximal intestine, and liver were collected and subjected to histomorphometric and

Abbreviations: ADF, acid detergent fiber; ADG, average daily gain; BW, body weight; CP, crude protein; Cd, crypt depth; DMI, dry matter intake; DM, dry matter; DMd, dry matter disappearance; EE, ether extract; FBW, final body weight; FM, fish meal; GP, gas production; GY, gas yield; IVGP, *in vitro* gas production; MCP, microbial crude protein; Muc, mucosa; Musc, muscular; NDF, neutral detergent fiber; OM, organic matter; RGY, relative gas yield; SCFA, short chain fatty acids; SBM, soybean meal; SD, standard deviation; Subm, submucosa; TMM, *Tenebrio molitor* meal; TMR, total mixed ration; Vh, villus height.

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histopathological evaluations. Data were analyzed using a general linear model and mixed models for the *in vitro* and *in vivo* trials, respectively; histopathological data were analyzed by a Kruskal-Wallis test. Results of Exp. 1 showed that total gas production was lower ($P = 0.021$; $\eta^2 = 0.359$), while DM disappearance ($P = 0.021$; $\eta^2 = 0.233$) and microbial crude protein production ($P = 0.015$; $\eta^2 = 0.347$) were higher, for the TMM diet when compared to the FM diet, while the SBM diet showed intermediate values. Results of Exp. 2 revealed that the DM intake of TMM-fed lambs was comparable to that of FM-fed lambs, but lower than that of SBM-fed lambs ($P = 0.035$; $\eta^2 = 0.407$). The average daily gain was the lowest in the TMM-fed lambs ($P = 0.033$; $\eta^2 = 0.373$), while the final BW remained unaffected by diet. Higher total tract apparent digestibility of DM and organic matter was obtained with the TMM and FM diets than with the SBM diet ($P < 0.001$; $\eta^2 = 0.411$ and 0.408, respectively). Nitrogen balance ranked in the following order: FM > TMM > SBM ($P < 0.001$; $\eta^2 = 0.385$). Ruminal morphometric indexes (i.e., papillae length and width, absorption area, and thickness of mucosa, submucosa and muscular layers) were not affected by diet. When compared to SBM-fed lambs, the TMM- and FM-fed ones exhibited significantly lower severity of epithelial keratinization in the ruminal dorsal sac ($P = 0.034$; $\varepsilon^2 = 0.299$). Intestinal inflammation was predominantly characterized by mononuclear cells and eosinophils in the TMM and SBM groups, respectively. No differences among the experimental groups were observed for other histopathological features at rumen, intestine, and liver levels. Overall, our results suggest that TMM can be used as a protein source for fattening lambs, but further studies should explore its use in combination with conventional protein sources to optimize the performance of lambs.

1. Introduction

Globally, meeting the increasing demand for meat, while considering socio-economic and environmental aspects, presents a challenging scenario for ruminant production systems (Gasco et al., 2020; Renna et al., 2023a). The sustainability of conventional protein feed ingredients, such as soybean meal (SBM) and fish meal (FM), in ruminant diets poses concerns due to climate change, depletion of natural resources, and food-feed-fuel competition (Ahmed and Nishida, 2023; Phesatcha et al., 2023). Therefore, over the last decades great efforts have been made to identify alternative feed ingredients that are able to sustain ruminant production and mitigate its undesirable footprints (Fukuda et al., 2022; Toral et al., 2022).

Insects reared using agro-industrial by-products and organic waste produce biomass rich in valuable proteins (30–68 % on a dry matter (DM) basis) with well-balanced amino acid profiles, fat (about 10–30 % DM), vitamins (particularly B_{12}), and bioavailable minerals (especially iron and zinc) (Ahmed and Nishida, 2023; Gasco et al., 2020). A great deal of research has focused on live or dried insects and insect meals as alternative protein sources for monogastrics, confirming their potential to sustain growth performance and improve the gut health status of farmed animals (Gasco et al., 2023). However, dietary insect application in ruminants has been limited so far, due to the potential risk of mad cow disease (Bovine Spongiform Encephalopathy) (Renna et al., 2023a). Ahmed et al. (2021) reported that insects (*Acheta domesticus*, *Gryllus bimaculatus*, *Bombyx mori*, and *Brachytrupes portentosus*) improved *in vitro* fermentation of a SBM-based concentrate. Cricket (*G. bimaculatus*) meal as a replacement for SBM (at levels of 100:0, 75:25, 50:50, 25:75, and 0:100) enhanced *in vitro* fermentation, while it decreased both ruminal protozoal population and methane production (Phesatcha et al., 2022). Astuti et al. (2019) showed, *in vivo*, that the dietary substitution of SBM with cricket (*G. bimaculatus*) meal had no negative effects on rumen fermentation of post-weaning goat kids. In a recently published study, Phesatcha et al. (2023) also reported that the substitution of SBM with cricket meal in the diet of Thai beef fed a rice straw-based diet resulted in improved nutrient digestibility and reduced methane production.

To verify the feasibility of using insects and insect-derived products in monogastric nutrition, different intestinal parameters have also been evaluated, including histomorphological features (Biasato et al., 2018, 2019; Colombino et al., 2023). Despite recent research has shown that insect-derived products could be considered suitable feed ingredients also for ruminant rations (Phesatcha et al., 2023; Renna et al., 2023a; Fukuda et al., 2022; Toral et al., 2022), the effects of their dietary utilization on rumen morphometry and histology (which are good indicators of rumen health; Wang et al., 2017), intestine and liver have not been investigated so far.

Using farmed insects as feed ingredients may contribute to sustainable livestock production, improving circular economy models. Nonetheless, more scientific data, both *in vitro* and *in vivo*, is needed to license the use of insects in ruminant diets. With this purpose, the current study investigated how the dietary inclusion of yellow mealworm (*Tenebrio molitor*) meal (TMM) (as an insect-based protein source) affected the rumen fermentation kinetics, the animal performance, as well as the histomorphometry and histopathology of rumen, proximal intestine, and liver in fattening lambs, when compared to conventional plant-based (SBM) and animal-based (FM) protein sources.

2. Materials and methods

2.1. Ingredients and experimental diets

Dehydrated full-fat TMM was obtained from the Institute of Biology of the Universidad Nacional Autónoma de México. The *T. molitor* larvae were fed with a diet containing 90 % bran and 10 % yeast, with a rearing duration of 95 d; the larvae were dehydrated

at 60ºC for 24 h and then ground in a mill to pass a 2-mm screen sieve (Arthur Hill Thomas Corporation, Philadelphia, Pennsylvania, USA). Both SBM and FM were purchased from a commercial feed manufacturer (Grupo La Moderna, Toluca de Lerdo, México).

Three experimental total mixed ration (TMR) diets were formulated to be isoenergetic (8.45 MJ/kg DM of metabolizable energy) and isonitrogenous [198 g/kg DM of crude protein (CP)] and to meet energy and protein requirements of growing sheep (NRC, 2007). The diets were formulated to contain different protein sources, as follows: 1- plant-based (SBM at 150 g/kg DM), 2- animal-based (FM at 50 g/kg DM), and 3- insect-based (TMM at 60 g/kg DM). The chemical composition of the ingredients used to formulate the experimental diets is shown in Table 1, while the ingredients and chemical composition of the experimental diets are shown in Table 2.

2.2. Exp. 1: In vitro trial

In vitro gas production (IVGP) was performed using a batch technique, incubating the substrate with rumen fluid and buffer as described by Theodorou et al. (1994). Rumen liquor was donated by three growing Suffolk lambs [35 ± 3.5 kg average body weight (BW); mean ± standard deviation (SD)] fed a TMR [40 % forage (corn silage) and 60 % concentrate (crushed rapeseed, corn grain, soybean meal, wheat grain, corn stover and mineral-vitamin premix)], with free access to clean drinking water. The ruminal inoculum (300 mL / animal) was collected before the morning feeding, mixed from the three animals, filtered through four layers of cheesecloth and immediately transported to the laboratory in pre-warmed thermo flasks under a continuous flux of CO_2 in a water bath kept at 39°C. A total of 0.8 g DM of each experimental diet was put into glass flasks bottles of 125 mL in triplicate in each tandem and repeated for four incubation runs (for a total of 12 bottles for each diet), with 90 mL of buffer solution and 10 mL of rumen fluid, and homogenized with CO_2 at 39 °C (Menke and Steingass, 1988). All the bottles were incubated in a water bath at 39 °C and the volume of gas (mL of gas/g DM) was recorded at 0, 3, 6, 9, 12, 24, 36, 48, 72, and 96 h of incubation. Blank bottles containing only buffered rumen fluid were incubated simultaneously. After the incubations, the dry matter disappearance (DMd) was determined. Moreover, the kinetic parameters of IVGP were estimated through an iterative procedure of non-linear regression analysis (PROC NLIN, SAS Institute, version 9.0; SAS Institute Inc., Cary, NC, USA) according to the model proposed by France et al. (1993), calculated as:

$$Y = A\{1- \exp [-B(t-T) - C(\sqrt{t} -A/T)]\}$$

where Y is the cumulative gas production (mL), t is the incubation time (h^{-1}), A is the asymptote curve (total gas produced, mL), B(h^{-1}) and C($h^{-1/2}$) are the gas production constants, T is the time of delay (hours) for microbial colonization to begin the fermentation.

Short chain fatty acids (SCFA) concentration was calculated according to Getachew et al. (2002), as follows:

$$SCFA\ (mmol/200mg\ DM) = 0.0222\ GP - 0.00425$$

where GP is the 24 h net gas production (mL/200 mg DM).

Microbial crude protein (MCP) production was calculated according to Blümmel et al. (1997), as follows:

$$MCP\ (mg/g\ DM) = mg\ \textit{in vitro}\ DMd - (mL\ gas \times 2.2mg/mL)$$

where 2.2 mg/mL is a stoichiometric factor, which expresses mg of C, H and O required for the SCFA gas associated with production of 1 mL of gas.

Metabolizable energy (MJ/kg DM) was calculated according to the equations proposed by Menke and Steingass (1988) for roughages (1) and (2) other feeds, as follows:

$$ME\ (MJ/kg\ DM) = 2.20 + 0.1357\ GP + 0.0057\ CP + 0.0002859 \times EE^2 \quad (1)$$

$$ME\ (MJ/kg\ DM) = 1.06 + 0.157\ GP + 0.0084\ CP + 0.022\ EE - 0.0081 \times ash, \quad (2)$$

where GP is 24 h net gas production (mL/200 mg DM), while CP and EE are crude protein and ether extract (g/kg DM), respectively.

2.3. Exp. 2: In vivo trial

2.3.1. Animals and experimental design

Twenty-four male Suffolk lambs (3 months of age; 24 ± 1.3 kg of BW; mean ± SD) were randomly assigned to the three dietary treatments ($n = 8$ per treatment). The animals were housed in individual cages (1.2 m × 0.8 m) for 30 days, including 23 days of feeding period and the last 7 days as sampling period. The diets were administered as TMRs twice daily (08:00 and 17:00 h) for *ad libitum* consumption and the lambs had free access to clean drinking water.

During the trial, the offered feed and refusals were recorded daily and data from the last 7 days were accounted for statistical analysis. Individual BW was measured at the beginning and end of the experimental period after a 16-h fasting period, before the first feeding in the morning, using a calibrated scale (WA01, AGRETO electronics Co. Pommersdorf, Austria).

During the sampling period, individual feces were collected daily; the 10 % by weight of the collected feces were frozen at −20 °C for further analysis. The total urine excreted by each lamb daily was collected into carboys containing 10 % sulfuric acid (to maintain pH < 3.0). Daily urine output was determined by weight. Urine subsamples from each lamb on each day were diluted by mixing 15 mL of sample with 60 mL of 0.072 N H_2SO_4 and stored at −20 °C until analyzed.

Table 1
Chemical composition (g/kg DM, unless otherwise stated) of the ingredients used in the experimental diets (values are reported as mean ± standard deviation)[a].

Item	SBM	FM	TMM	Bran wheat	Corn grain	Barley hay	Triticale grain	Soya oil	Fish oil	Urea
DM, g/kg	892 ± 13.4	895 ± 17.9	900 ± 13.5	880 ± 17.1	946 ± 14.2	905 ± 18.0	885 ± 13.3	995 ± 1.8	995 ± 2.0	990 ± 1.4
OM	925 ± 9.3	844 ± 25.3	967 ± 9.7	941 ± 28.2	986 ± 9.9	906 ± 27.2	930 ± 9.3	995 ± 1.9	995 ± 1.7	999 ± 0.9
CP	537 ± 13.4	596 ± 8.9	567 ± 14.2	160 ± 2.4	80 ± 2.0	120 ± 1.8	120 ± 3.0	n.a.	n.a.	2810 ± 70.3
EE	70 ± 1.0	153 ± 3.8	189 ± 2.8	106 ± 2.7	102 ± 1.5	96 ± 2.4	93 ± 1.4	990 ± 14.8	990 ± 15.3	n.a.
aNDFom	172 ± 1.7	424 ± 6.4	248 ± 2.5	407 ± 6.1	228 ± 2.3	594 ± 8.9	355 ± 3.6	n.a.	n.a.	n.a.
ADFom	64 ± 1.6	153 ± 3.1	162 ± 4.0	117 ± 2.3	47 ± 1.2	377 ± 7.5	61 ± 1.5	n.a.	n.a.	n.a.
ADL	15 ± 0.2	47 ± 0.7	39 ± 0.6	43 ± 0.6	16 ± 0.2	89 ± 1.3	24 ± 0.4	n.a.	n.a.	n.a.

Abbreviations: SBM, soybean meal; FM, fish meal; TMM, *Tenebrio molitor* meal; DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; aNDFom, neutral detergent fiber; ADFom, acid detergent fiber; ADL, acid detergent lignin; n.a., not analyzed.
[a] Samples were analyzed in triplicate.

2.3.2. Laboratory analyses

All ingredients, TMRs, orts, and fecal samples were dried in a forced air oven (60 °C, 48 h), then they were ground using a hammer mill (Willey, 2 mm Ø Arthur H. Thomas Philadelphia, PA, USA). The samples were analyzed in triplicate, according to AOAC methods AOAC (1990), for DM (method no. 934.01), organic matter (OM, method no. 942.05), ash (method no. 942.05), CP (Kjel-Foss Automatic 16210 Analyzer, Foss Electric, Hillerød, Denmark; method no. 976.05), and ether extract (EE; Soxhlet System HT Analyzer; Foss Electric, Hillerød, Denmark; method no. 973.18). Ash-free neutral detergent fibre (aNDFom) and acid detergent fiber (ADFom) (Fibertec 1020 Analyzer; Foss Analytical AB, Höganäs, Sweden) were analyzed according to Van Soest et al. (1991). Feces and urine samples were used to determine nitrogen (N; method no. 991.20). Total tract apparent digestibility of nutrients was determined as follows (Wiseman, 2018):

Digestibility (g/kg) = (nutrient intake – nutrient excreted) / (nutrient intake) × 1000.

2.3.3. Histomorphometry and histopathology

At the end of the sampling period, the animals were slaughtered in a commercial slaughterhouse, and samples of rumen (dorsal and ventral sacs), proximal intestine and liver were collected and fixed in 10 % neutral buffered formalin for subsequent morphometric and histopathological assessments. Following fixation for at least 48 h, a minimum of 10 papillae from the ruminal ventral sac per lamb (Dieho et al., 2016) were isolated by cutting at the base (Van Niekerk et al., 2021) and photographed in the presence of a measuring ruler (Dieho et al., 2016). Morphometric macroscopic measurements (height from the apex to the base and width in three different points – base, apex, and widest part) of each ruminal papilla were conducted utilizing the ImageJ software with Fiji distribution (Schindelin et al., 2012). For microscopic measurements/evaluations and histopathological analyses, all the organs were trimmed, embedded in paraffin wax blocks, cut at 5 µm thickness, submitted to standard Haematoxylin and Eosin (H&E) staining (Van Niekerk et al., 2021) and mounted on glass slides. Slides for histomorphometry were then examined using a Zeiss Axiophot light microscope (Carl Zeiss, Oberkochen, Germany) at 2.5 × magnification, and images were captured using a Zeiss camera (Zeiss AxioCam 208, Oberkochen, Germany) coupled with a computer for further analysis. ImageJ software was used for measurements (height and width - base, apex and widest part - of at least 10 well-oriented primary papillae per lamb). Histopathological evaluations were conducted using a semi-quantitative scoring system (0 = none, 1 = mild, 2 = moderate, 3 = severe) (Colombino et al., 2023), wherein the severity of inflammation in the rumen (dorsal and ventral sac) and proximal intestine [mucosa (Muc), submucosa (Subm) and muscular layer (Musc)] and vacuolar degeneration and keratinization of the ruminal epithelium (dorsal and ventral sacs) were assessed (Martel et al., 2021). Additionally, the pattern of inflammation, vacuolar degeneration and keratinisation (focal = 1, multifocal = 2, disseminated = 3, diffused = 4), and the type of inflammatory cells (lymphoplasmacellular = 1, mixed or with neutrophils = 2, eosinophilic = 3) were evaluated (Pacorig et al., 2022). The scores were considered as continuous numerical variables, resulting from the sum of the partial inflammation scores of the three portions (Muc, Subm and Musc). A similar semiquantitative scoring system was assigned to the degree of inflammatory infiltration and the vacuolar degeneration in the liver (Colombino et al., 2023).

2.4. Statistical analysis

Data were statistically analyzed using the SAS software (SAS Institute, version 9.0; SAS Institute Inc., Cary, NC, USA). The Shapiro-Wilk and Levene's tests were used to check the assumptions of normality and homoscedasticity, respectively.

Data from Exp. 1 were analyzed using the general linear model procedure (PROC GLM), using the following model:

$$y_{ij} = \mu + D_i + R_j + \varepsilon_{ij},$$

where y_{ij} is the dependent variable, μ is the overall mean, D_i is the diet effect, R_j is the incubation run effect, and ε_{ij} is the residual error.

Data from Exp. 2 (except for histopathological ones) were analyzed using a mixed procedure (PROC MIXED), using the following model:

Table 2
Ingredients and chemical composition (g/kg DM, unless otherwise stated) of the experimental diets[a].

	Diet		
	SBM	FM	TMM
Ingredients			
Soybean meal	150	0	0
Fish meal	0	50	0
Tenebrio molitor meal	0	0	60
Bran wheat	78	68	69
Corn grain	250	300	300
Barley hay	250	300	300
Triticale whole grain	250	250	250
Oil[b]	12	12	0
Urea	0	10	11
Vit-Min mix[c]	10	10	10
Chemical composition			
DM, g/kg	903.6	892.0	886.2
OM	949.7	943.8	957.8
CP	197.2	200.4	197.2
EE	88.6	104.2	86.8
NFC	319.8	344.8	252.0
aNDFom	360.1	394.4	326.8
ADFom	170.3	164.9	146.4
ADL	29.8	26.9	23.7

Abbreviations: DM; dry matter; SBM, soybean meal; FM, fish meal; TMM, *Tenebrio molitor* meal; OM, organic matter; CP, crude protein; EE, ether extract; NFC, non-fibrous carbohydrates; aNDFom, neutral detergent fiber; ADFom, acid detergent fiber; ADL, acid detergent lignin.

[a] Samples were analyzed in triplicate.

[b] Soybean oil and fish oil used in SBM and FM diets, respectively.

[c] Multitec de Malta (1.0 kg DM) containing antioxidant 25 mg, calcium carbonate 4.5 g, salt 6 g, ionophore 30 g, zinc oxide 50 g, sodium bicarbonate 6 g, copper sulphate 6 g, ferrous sulphate 20 g, sodium sulphate 125 g, vitamins E18 000 IU, A 3 000 000 IU, D 3 750 000 IU, potassium chloride 140 g, E.D.D. I ethylene-dynamine 0.500 g, cobalt carbonate 0.090 g, magnesium oxide 500 mg, manganese oxide 36 g, and selenium 0.090 g.

$$y_{ijkl} = \mu + D_i + A_{j(i)} + T_k + D_i \times T_k + \varepsilon_{ijkl},$$

where y_{ijkl} is the dependent variable, μ is the overall mean, D_i is the fixed effect of diet, $A_{j(i)}$ is the random effect of j^{th} lamb within i^{th} diet, T_k is the effect of time, considered as a repeated measure (for intake, total tract apparent digestibility, and nitrogen evaluations), $D_i \times T_k$ is the fixed effect of the interaction between diet and time (for intake, total tract apparent digestibility, and nitrogen evaluations), and ε_{ijkl} is the residual error.

Least-square means were computed and tested for differences using the Tukey's test.

Histopathological data from Exp. 2 were not normally distributed; therefore, they were subjected to a non-parametric Kruskal-Wallis test, and subsequent Dunn's post hoc tests for assessment of pairwise comparisons.

Effect size (Iacobucci et al., 2023) was calculated using the SPSS software (version 29.0 for Windows; IBM SPSS Statistics, Armonk, NY, USA). The partial eta squared statistic (η^2) was used for parametric data (small, medium and large effects were considered for η^2 values equal to 0.01, 0.06 and 0.14, respectively). The epsilon squared statistic (ε^2) was instead used for histopathological data (values varying from 0 to 1, indicating no relationship and perfect relationship, respectively).

Significance was declared at $P \leq 0.05$.

3. Results

3.1. Exp. 1: In vitro trial

Results of Exp. 1 showed that the TMM diet resulted in lower total gas production (parameter A, $P = 0.021$; $\eta^2 = 0.359$) when compared to the FM diet, while the SBM diet showed intermediate values (Table 3 and Fig. 1). The fermentation rate (parameter B; $P < 0.001$; $\eta^2 = 0.054$) was the highest for the SBM diet, followed by the TMM diet and finally by the FM diet. Lag time was comparable among the diets ($P > 0.05$; $\eta^2 = 0.302$). The TMM diet yielded higher DMd 96 h and MCP when compared to the FM diet ($P = 0.021$; $\eta^2 = 0.233$), while the SBM diet showed intermediate values. The highest relative gas yield at 96 h (RGY) was obtained with the FM diet, followed by SBM and TMM ($P = 0.022$; $\eta^2 = 0.209$). However, gas yield at 24 h (GY 24 h) remained unaffected by treatment ($P > 0.05$; $\eta^2 = 0.306$). The amount of SCFA was higher for the FM diet when compared to both the TMM and SBM diets ($P < 0.001$; $\eta^2 = 0.245$).

Table 3
Effect of the experimental diets containing soybean meal (SBM), fish meal (FM) and *Tenebrio molitor* meal (TMM) as protein sources on *in vitro* rumen fermentation profile[a,b,c].

Item	Diet			SEM	*P*-value	Effect size[d]
	SBM	FM	TMM			
A, mL gas / g DM incubated	250.82^{ab}	276.89^{a}	232.81^{b}	7.920	0.021	0.359
B, h^{-1}	0.046^{a}	0.042^{c}	0.044^{b}	< 0.001	< 0.001	0.054
C, $h^{-1/2}$	−0.002	−0.016	−0.014	0.006	0.124	0.018
Lag time, h	0.489	0.672	0.642	0.090	0.376	0.302
DMd, mg/g DM	83.00^{ab}	80.67^{b}	84.00^{a}	0.608	0.021	0.233
RGY, mL gas 96 h / g DMd	304.33^{ab}	341.67^{a}	278.00^{b}	11.55	0.022	0.209
GY 24 h, mL gas / g DMd	193.67	204.33	171.33	7.824	0.061	0.306
SCFA, mmol/g DM	18.00^{b}	21.00^{a}	18.00^{b}	< 0.001	< 0.001	0.245
MCP, mg/g DM	719.67^{ab}	685.33^{b}	736.33^{a}	8.569	0.015	0.347
ME, MJ/kg DM	8.45	8.68	8.23	0.178	0.116	0.201

Abbreviations: DM, dry matter; A, total gas production; B, fermentation rate; C, fermentation rate; Lag time, initial delay before gas production begins; DMd, DM degraded substrate; RGY, relative gas yield after 96 h of incubation; GY 24 h, gas yield at 24 h; SCFA, short chain fatty acids production; MCP, microbial crude protein production; ME, metabolizable energy; SEM, standard error of the mean.

[a] Rumen liquor was donated by three growing Suffolk lambs. Three flasks per sample were used, in four incubation runs, for a total of 12 bottles for each experimental diet.

[b] Data were statistically analyzed using the General Linear Model procedure (PROC GLM), considering diet as a fixed effect and run as a fixed blocking factor.

[c] Values within rows followed by different superscript letters (a-c) are significantly different ($P < 0.05$).

[d] Effect size was calculated using the partial eta squared statistic (η^2). Small, medium and large effects are reflected in η^2 values equal to 0.01, 0.06 and 0.14, respectively (Iacobucci et al., 2023).

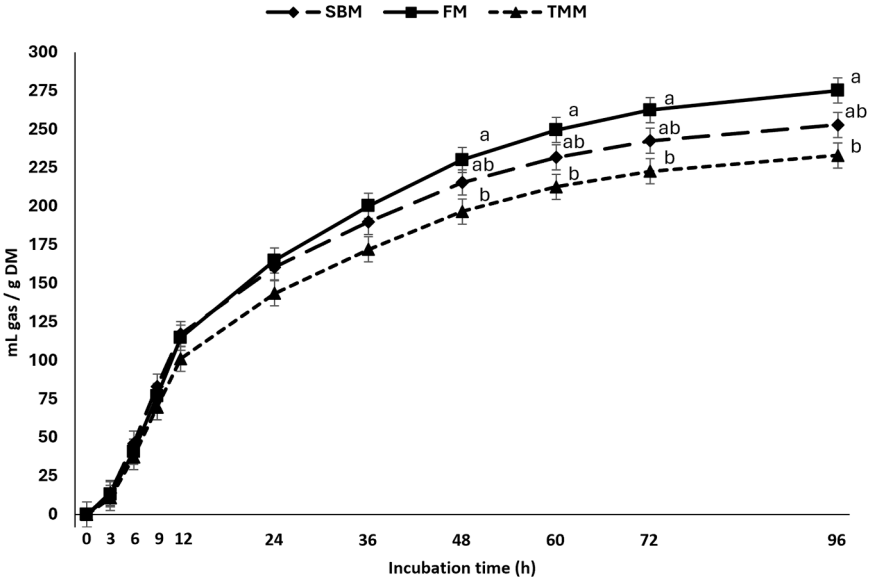

Fig. 1. Effects of the experimental diets containing soybean meal (SBM, ◆), fish meal (FM, ■) and *Tenebrio molitor* meal (TMM, ▲) as protein sources on *in vitro* rumen gas kinetics (mL gas/g DM)[1,2,3]. Abbreviations: DM, dry matter; h, hours. [1] Rumen liquor was donated by three growing Suffolk lambs. Three flasks per sample were used, in four incubation runs, for a total of 12 bottles for each experimental diet. [2] Data were statistically analyzed using the General Linear Model procedure (PROC GLM), considering diet as a fixed effect and run as a fixed blocking factor. [3] For each incubation time, values with different letters (a, b) are significantly different ($P < 0.05$).

3.2. Exp. 2: In vivo trial

3.2.1. Growth performance, total tract apparent digestibility, and nitrogen balance

The average daily gain (ADG, kg/d) of the lambs fed with the TMM diet was lower than that of both SBM-fed and FM-fed lambs ($P = 0.033$; $\eta^2 = 0.373$; Table 4). However, the final BW (FBW) of the lambs remained unaffected by diet ($P > 0.05$; $\eta^2 = 0.348$). The absolute lowest DM intake (DMI) was recorded for the TMM-fed lambs, both when the DMI was expressed as g/d ($P = 0.035$; $\eta^2 = 0.407$) and g/kg $BW^{0.75}$/d ($P = 0.041$; $\eta^2 = 0.408$). The intakes of NDF, ADF, and EE were lower in the TMM-fed compared to both the SBM-fed and the FM-fed lambs ($P < 0.001$; $\eta^2 = 0.371$, $\eta^2 = 0.457$ and $\eta^2 = 0.414$, respectively; Table 4). The total tract apparent

Table 4
Effects of the experimental diets containing soybean meal (SBM), fish meal (FM) and *Tenebrio molitor* meal (TMM) as protein sources on feed intake and total tract apparent digestibility in lambs[a,b,c].

Item	Diet			SEM	*P*-value	Effect size[d]
	SBM	FM	TMM			
Initial body weight, kg	25.75	21.65	24.09	1.323	0.113	0.279
Final body weight, kg	32.69	28.50	29.10	1.498	0.127	0.348
Growth rate, kg/period	6.93[a]	6.85[a]	5.01[b]	0.541	0.033	0.392
Average daily gain, kg/d	0.231[a]	0.228[a]	0.167[b]	0.018	0.033	0.373
Initial metabolic body weight, $BW^{0.75}$	11.40	10.01	10.86	0.448	0.112	0.073
Final metabolic body weight, $BW^{0.75}$	13.64	12.31	12.52	0.408	0.129	0.078
Average daily gain, g/kg $BW^{0.75}$	74.59[ab]	76.66[a]	55.26[b]	5.737	0.029	0.395
Intake, g d^{-1}						
Dry matter	1343.11[a]	1309.85[ab]	1163.52[b]	50.303	0.035	0.407
Organic matter	1275.55	1236.23	1114.43	47.782	0.065	0.394
Neutral detergent fiber	483.62[a]	516.56[a]	380.24[b]	18.355	< 0.001	0.371
Acid detergent fiber	228.73[a]	215.95[a]	170.37[b]	8.079	< 0.001	0.457
Acid detergent lignin	40.03[a]	35.31[b]	27.61[c]	1.347	< 0.001	0.423
Ether extract	118.99[b]	136.51[a]	101.00[c]	4.776	< 0.001	0.414
Intake, g/kg $BW^{0.75}$ d^{-1}						
Dry matter	99.07[ab]	105.94[a]	93.26[b]	3.421	0.041	0.408
Organic matter	94.08	99.99	89.31	3.255	0.078	0.314
Neutral detergent fiber	35.67[b]	41.79[a]	30.47[c]	1.214	< 0.001	0.420
Acid detergent fiber	16.88[a]	17.47[a]	13.64[b]	0.544	< 0.001	0.313
Acid detergent lignin	2.96[a]	2.85[a]	2.22[b]	0.090	< 0.001	0.432
Ether extract	8.77[b]	11.04[a]	8.11[b]	0.316	< 0.001	0.448
Total tract apparent digestibility, g/kg						
Dry matter	639.62[b]	719.14[a]	716.07[a]	8.785	< 0.001	0.411
Organic matter	639.76[b]	736.65[a]	729.91[a]	8.844	< 0.001	0.408
Crude protein	668.91[c]	791.56[a]	743.05[b]	8.545	< 0.001	0.518
Neutral detergent fiber	409.31[c]	578.66[a]	510.27[b]	15.371	< 0.001	0.385
Acid detergent fiber	348.96[b]	407.60[ab]	410.49[a]	17.698	0.029	0.320
Acid detergent lignin	0.10[c]	1.80[a]	1.30[b]	0.002	< 0.001	0.534

Abbreviations: SEM, standard error of the mean; BW, body weight; d, day.

[a] Samples were collected from 24 lambs (8 lambs per dietary treatment).

[b] Data were statistically analyzed using a mixed procedure (PROC MIXED), considering diet as a fixed effect and the random effect of lamb within diet. The model for intake and total tract apparent digestibility additionally included the effect of time (as a repeated measure) and the effect of the interaction between diet and time.

[c] Values within rows followed by different superscript letters (a-c) are significantly different ($P < 0.05$).

[d] Effect size was calculated using the partial eta squared statistic (η^2). Small, medium and large effects are reflected in η^2 values equal to 0.01, 0.06 and 0.14, respectively (Iacobucci et al., 2023).

digestibility of DM and OM was higher for the TMM and FM diets when compared to the SBM diet ($P < 0.001$; $\eta^2 = 0.411$ and 0.408, respectively; Table 4).

The TMM-fed lambs showed lower N intake (g/d) when compared to the lambs fed with the FM diet, while the lambs fed with the SBM diet showed intermediate values ($P = 0.019$; $\eta^2 = 0.357$; Table 5). Urinary N excretion was lower in the SBM-fed and TMM-fed lambs than in the FM-fed ones ($P = 0.002$; $\eta^2 = 0.340$), while fecal N excretion showed higher values in the SBM-fed lambs when compared to both the FM-fed and TMM-fed lambs ($P < 0.001$; $\eta^2 = 0.425$). The highest N balance value was obtained with the FM diet, followed by the TMM diet, and finally by the SBM diet ($P < 0.001$; $\eta^2 = 0.385$).

3.2.2. Histomorphometry and histopathology

The dietary treatment did not significantly affect either the macroscopic morphometric measurements (papilla length and papilla width) alongside the area of absorption, or the thickness of Muc, Subm and Musc layers of the ruminal wall ($P > 0.05$; Table 6).

Table 7 shows the histopathological conditions (inflammation, vacuolation, and keratinization) across the rumen (dorsal and ventral sacs), proximal intestine and liver. Significant differences were observed among the three groups of lambs for the severity of keratinization ($P = 0.034$; $\varepsilon^2 = 0.299$) in the dorsal sac of the rumen (Fig. 2A and B) and for the type of inflammation ($P = 0.027$; $\varepsilon^2 = 0.313$) in the proximal intestine, only. Regarding the severity of keratinization in the dorsal sac of the rumen, a higher rank was observed in the SBM group when compared to both the FM and TMM groups. Regarding the type of inflammation in the proximal intestine, a higher rank was observed for the SBM-fed lambs when compared to the TMM-fed ones, while no significant differences were observed when comparing the TMM and FM groups or the SBM and FM groups. In TMM-fed lambs, the intestinal inflammation was predominantly characterized by the presence of lymphocytes, plasma cells, macrophages and rare neutrophils (Table 8 and Fig. 2C), while the SBM-fed lambs predominantly exhibited eosinophils (Table 8 and Fig. 2D).

Table 5
Effects of the experimental diets containing soybean meal (SBM), fish meal (FM) and *Tenebrio molitor* meal (TMM) as protein sources on nitrogen (N) balance and N retention of lambs[a,b,c].

Item	Diet			SEM	*P*-value	Effect size[d]
	SBM	FM	TMM			
	N intake					
N intake, g/d	38.94[ab]	42.02[a]	35.58[b]	1.552	0.019	0.357
N intake, g/d $BW^{0.75}$	2.87[b]	3.39[a]	2.87[b]	0.106	0.001	0.212
	N excreted					
Urinary, g/d	24.21[b]	28.61[a]	23.29[b]	1.069	0.002	0.340
Feces, g/d	12.90[a]	8.74[b]	9.14[b]	0.271	< 0.001	0.425
	N balance					
N balance, g d^{-1}	1.82[c]	4.66[a]	3.17[b]	0.271	< 0.001	0.385
	N utilization					
Fecal N / N Intake, %	33.09[a]	20.85[c]	25.70[b]	0.853	< 0.001	0.334
Urinary N/ N Intake, %	62.12[c]	68.10[a]	65.37[b]	0.705	< 0.001	0.306
Excreted N / N Intake, %	4.75[c]	11.09[a]	8.92[b]	0.598	< 0.001	0.324

Abbreviations: SEM, standard error of the mean; d, day; BW, body weight.
[a] Samples were collected from 24 lambs (8 lambs per dietary treatment).
[b] Data were statistically analyzed using a mixed procedure (PROC MIXED), considering the fixed effect of diet, the random effect of lamb within diet, time as a repeated measure, and the effect of the interaction between diet and time.
[c] Values within rows followed by different superscript letters (a-c) are significantly different ($P < 0.05$).
[d] Effect size was calculated using the partial eta squared statistic (η^2). Small, medium and large effects are reflected in η^2 values equal to 0.01, 0.06 and 0.14, respectively (Iacobucci et al., 2023).

Table 6
Effects of the experimental diets containing soybean meal (SBM), fish meal (FM) and *Tenebrio molitor* meal (TMM) as protein sources on macroscopic (Mac) and microscopic (Mic) morphometric measurements of rumen papillae and width of mucosa, submucosa and muscular layers of rumen papillae in lambs[a,b].

Item	Diet			SEM	*P*-value	Effect size[c]
	SBM	FM	TMM			
Mac_PL (cm)	0.19	0.19	0.18	0.012	0.954	0.009
Mac_PW (cm)	0.10	0.13	0.12	0.010	0.534	0.094
Area of absorption (cm^2)	10.17	12.38	12.24	1.504	0.853	0.026
Mic_PL (mm)	0.75	0.82	0.62	0.162	0.135	0.234
Mic_PW (mm)	0.21	0.22	0.22	0.010	0.892	0.016
Muc (mm)	0.72	0.66	0.57	0.060	0.639	0.046
Submuc (mm)	0.09	0.09	0.06	0.005	0.117	0.202
Musc (mm)	0.76	0.82	0.68	0.054	0.640	0.046

Abbreviations: SEM, standard error of the mean; PL, papilla length; PW, papilla width; Muc, mucosa; Submuc, submucosa; Musc, muscular.
[a] Samples were collected from 24 lambs (8 lambs per dietary treatment).
[b] Data were statistically analyzed using the Mixed procedure (PROC MIXED), considering diet as a fixed effect and the random effect of lamb within diet.
[c] Effect size was calculated using the partial eta squared statistic (η^2). Small, medium and large effects are reflected in η^2 values equal to 0.01, 0.06 and 0.14, respectively (Iacobucci et al., 2023).

4. Discussion

4.1. Exp. 1: In-vitro trial

Our results show that the TMM diet yielded the lowest total gas production, and the highest DMd and MCP production, among the tested diets. Phesatcha et al. (2022) demonstrated that incorporating cricket (*G. bimaculatus*) meal into ruminant diets lowered IVGP and enhanced *in vitro* DMd. Jayanegara et al. (2017) reported that the IVGP of diets containing 50 % black soldier fly (*Hermetia illucens*) larvae meal instead of SBM was decreased and these authors attributed such result to the fat content of insect larvae. Also, Ahmed and Nishida (2023) showed that, in a 60:40 forage to concentrate ruminant diet, the substitution of the concentrate with insects (powdered adults of *G. bimaculatus* and *B. mori*) at levels beyond 20 % (on a DM basis) led to lower IVGP, which was mainly attributed to the high chitin and fat contents of the insect meals. Conversely, Kahraman et al. (2023) observed that the IVGP and *in vitro* DMd of TMRs containing 20 % and 40 % of defatted *H. illucens* meal as a substitute for SBM were enhanced compared to a control TMR containing no insect meal, a result which seems to confirm the key role of insect fat. In a review paper, Renna et al. (2023a) stated that the high EE and chitin contents of full-fat insect meals explain their detrimental impact on nutrient digestibility. In fact, the presence of high amounts of unsaturated lipids can be toxic for the ruminal cellulolytic microbiota. In addition, chitin, a long-chain polymer of N-acetylglucosamine present in the exoskeleton of insects, has been shown to be a hard-to-degrade compound that can reduce nutrient digestibility. Available published literature on the effects of insect-derived products on *in vitro* rumen fermentation parameters is still

Table 7
Effects of the experimental diets containing soybean meal (SBM), fish meal (FM) and *Tenebrio molitor* meal (TMM) as protein sources on various pathological conditions (inflammation, vacuolation, and keratinization) in the rumen (dorsal and ventral sacs), proximal intestine, and liver in lambs[a,b,c,d].

Organ	Pathological condition		Diet			SEM	*P*-value	Effect size[e]
			SBM	FM	TMM			
Rumen (dorsal sac)	Inflammation	*Pattern*	0.50	0.25	0.38	0.375	0.506	0.016
		Severity	0.38	0.25	0.13	0.250	0.451	0.038
		Type	0.38	0.13	0.13	0.183	0.380	0.084
	Vacuolation	*Pattern*	3.75	3.13	2.25	0.675	0.189	0.186
		Severity	1.50	1.13	0.75	0.250	0.094	0.212
	Keratinization	*Pattern*	2.38	1.25	1.75	0.701	0.292	0.098
		Severity	1.13[a]	0.50[b]	0.50[b]	0.320	0.034	0.299
Rumen (ventral sac)	Inflammation	*Pattern*	0.75	0.50	0.75	0.526	0.987	0.009
		Severity	0.38	0.38	0.38	0.263	1.000	0.000
		Type	0.38	0.38	0.38	0.263	1.000	0.000
	Vacuolation	*Pattern*	2.00	2.00	1.63	0.756	0.872	0.010
		Severity	1.00	0.88	0.88	0.423	0.989	0.004
	Keratinization	*Pattern*	1.38	1.38	0.63	0.565	0.504	0.062
		Severity	0.63	0.50	0.25	0.263	0.476	0.073
Proximal intestine	Inflammation	*Pattern*	4.00	3.88	4.00	0.125	0.368	0.087
		Severity	3.00	3.00	2.88	0.125	0.368	0.087
		Type	2.75[a]	1.88[ab]	1.63[b]	0.350	0.027	0.313
Liver	Inflammation	*Pattern*	3.25	3.13	3.13	0.515	0.656	0.004
		Severity	1.13	0.88	1.13	0.125	0.278	0.113
		Type	1.00	0.88	1.00	0.125	0.368	0.087
	Vacuolation	*Pattern*	0.00	0.88	0.00	0.581	0.124	0.178
		Severity	0.00	0.25	0.00	0.164	0.124	0.182

Abbreviations: SEM, standard error of the mean.
[a] Pattern (focal = 1, multifocal = 2, disseminated = 3, diffused = 4); Severity (0 = none, 1 = mild, 2 = moderate, 3 = severe); Type (lymphoplasmacellular = 1, mixed or with neutrophils = 2, eosinophilic = 3).
[b] Samples were collected from 24 lambs (8 lambs per dietary treatment).
[c] Data were statistically analyzed by a Kruskal Wallis test, followed by Dunn's post hoc tests for assessment of pairwise comparisons.
[d] Values within rows followed by different superscript letters (a, b) are significantly different ($P < 0.05$).
[e] Effect size was calculated using the epsilon squared statistic (ε^2); values vary from 0 (no relationship) to 1 (perfect relationship) (Iacobucci et al., 2023).

very limited and inconsistent results may be the consequence of various factors, including insect species, insect-derived product used (e.g., insect oil, full-fat insect meal, defatted insect meal), and basal diet composition (Renna et al., 2023a; Phesatcha et al., 2022).

4.2. Exp. 2: In-vivo trial

4.2.1. Growth performance, digestibility and nitrogen balance

The available published literature on the *in vivo* use of insects as feed ingredients for ruminants is scant and inconclusive. In line with our results, Fukuda et al. (2022) showed that the dietary inclusion of full-fat black soldier fly larvae meal (36 % diet DM) in substitution of SBM led to lower DMI in beef steers but, conversely to our results, these authors observed no detrimental effect of the insect meal on nutrients digestibility. Analyzing the effects of the dietary incorporation of defatted silkworm (*B. mori*) pupae meal as a replacement for SBM at levels of 0, 10, 20, and 30 % in crossbred cattle, Rashmi et al. (2022) observed no negative effects on intake or digestibility of nutrients. However, the dietary inclusion (up to 50 % of the concentrate feed) of mealworm frass, the main by-product obtainable from insect rearing which contains high chitin levels, resulted in lower DMI of sheep, most probably because of reduced palatability (Ayaz et al., 2023). In the current study, the lower DMI of fattening lambs following TMM feeding may also be attributable to a palatability issue stemming from the disparity in fat level and fatty acid composition among animal-, vegetable-, and insect-based protein sources (Finke, 2002). The results obtained in our study show that TMM, as a protein source, acted more similarly to an animal protein source (i.e., FM) than to a plant protein source (SBM), which confirms previous findings obtained *in vitro* by Renna et al., (2022b). Our results are also in line with the findings by Toral et al. (2022), who reported lower ruminal degradation of TMM when compared to SBM.

In the present study, the ADG of TMM-fed lambs was lower than that of SBM-fed and FM-fed lambs, while the FBW of the lambs remained unchanged. Similarly, Astuti et al. (2019) reported that feeding milk replacers containing 15 % and 30 % (on a DM basis) of cricket (*G. bimaculatus*) meal did not affect the DMI and ADG of goat kids. Also, the replacement of 0, 33, 67, and 100 % (on a fresh matter basis) of SBM with *G. bimaculatus* meal did not affect DMI and digestibility in Thai native beef cattle fed a rice straw-based diet, while it enhanced FBW and ADG (Phesatcha et al., 2023).

In summary, we can mention two key factors that may have contributed to the reduced DMI and ADG in the TMM-fed lambs in our study: (1) the chitin present in the exoskeleton of the TMM larvae and (2) their fat content (as we used a full-fat meal). Chitin's interaction with the organism varies depending on its type and origin, and its structure and solubility can affect its bioaccessibility and

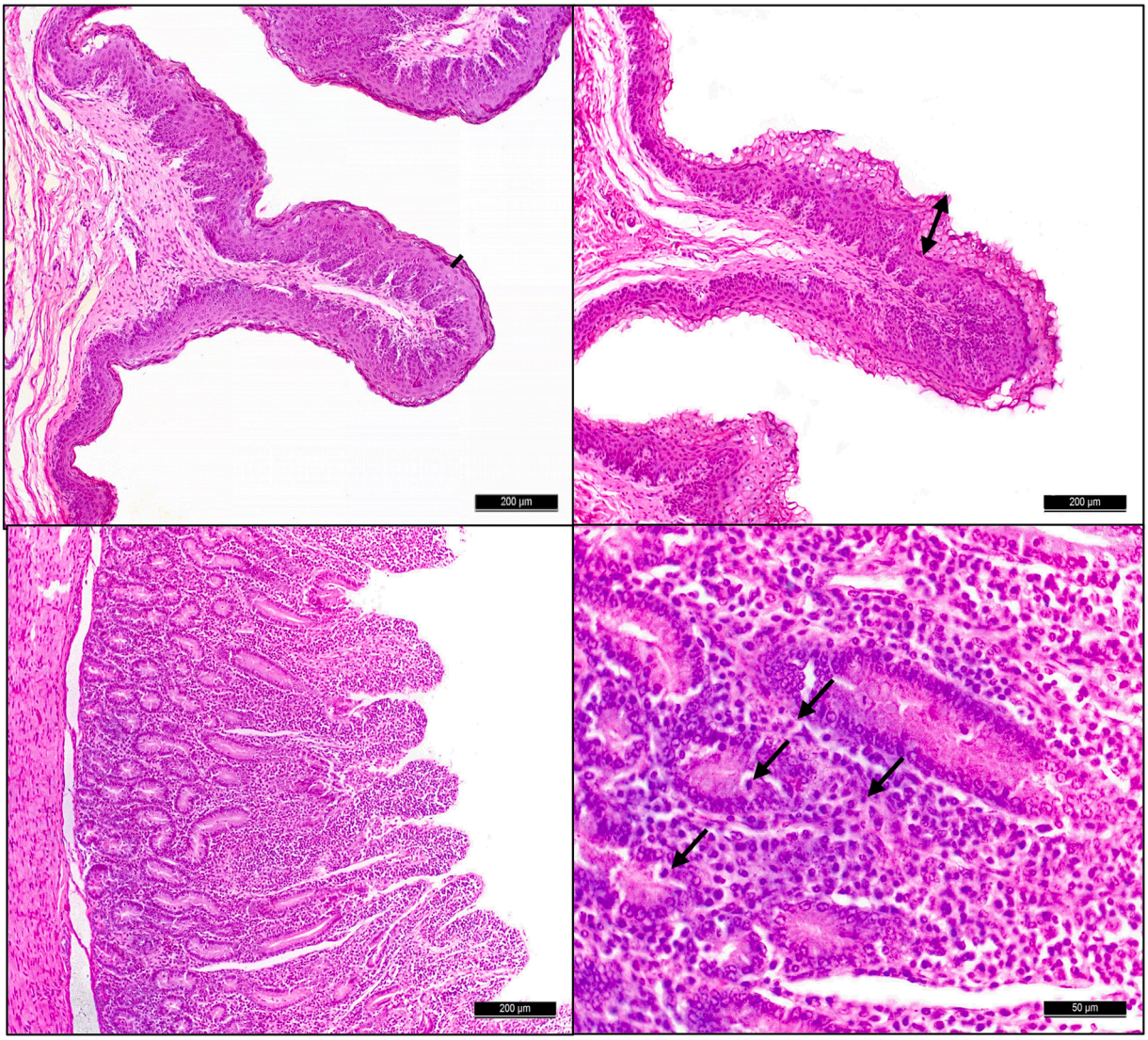

Fig. 2. Effects of the experimental diets containing soybean meal (SBM) and *Tenebrio molitor* meal (TMM) as protein sources on histopathology of ruminal papillae and proximal intestine of lambs. Legend. A: TMM-fed lamb, rumen, dorsal sac: normal papilla; B: SBM-fed lamb, rumen, dorsal sac: hyperkeratosis characterized by increased thickness of stratum corneum; C: TMM-fed lamb, proximal intestine: non suppurative inflammation; D: SBM-fed lamb, proximal intestine: eosinophilic inflammation characterized by the presence of mononuclear cells and eosinophils (arrows).

Table 8
Effects of the experimental diets containing soybean meal (SBM), fish meal (FM) and *Tenebrio molitor* meal (TMM) as protein sources on the number (percentage within parentheses) of lambs exhibiting various types of intestinal inflammation [1 = lymphocytes only; 2 = mixed (lymphocytes and neutrophils); 3 = eosinophils][a].

	Diet		
Inflammation type	SBM	FM	TMM
1	0/8 (0 %)	4/8 (50 %)	4/8 (50 %)
2	2/8 (25 %)	1/8 (12.5 %)	3/8 (37.5 %)
3	6/8 (75 %)	3/8 (37.5 %)	1/8 (12.5 %)

[a] Samples were collected from 24 lambs (8 lambs per dietary treatment).

digestibility by animal species (Pascon et al., 2024). In insects, chitin is cross-linked to a protein matrix, making it difficult to generate intestinal protein and lipid absorption (Hahn et al., 2018). Using the *in situ* technique, Fadel El-Seed et al. (2003) observed a very low rate of chitin degradation in the rumen of sheep. Chitinase activity has been described in some rumen bacteria and protozoa (Williams

et al., 2020), which may explain a possible role in chitin digestion. An interesting strategy to be tested in the near future could be to add chitinases to ruminant diets containing insect meals, as already tested in diets destined to monogastric animals (Hasan et al., 2023). Instead, to overcome the high-fat issue, defatted rather than full-fat insect meals could be used, as already seen for monogastrics. In fact, the defatting process offers meals with a higher protein content and higher resistance to degradation (Chemello et al., 2020). Insect manufacturers are now producing defatted insect meals, with the extracted oil being used for other purposes (including ruminant nutrition as well; Nekrasov et al., 2022). However, only a few studies are currently available on the evaluation of defatted insect meals in ruminant nutrition (Renna et al., 2023b).

Regarding N balance and N retention, Robles-Jimenez et al. (2022) showed that the dietary inclusion of full-fat TMM for growing lambs did not change DMI and N intake, while it decreased N retention when compared to a diet containing SBM as protein source. It has been reported that the presence of chitin in insects could hinder the absorption and metabolism of amino acids, resulting in reduced N retention (Phesatcha et al., 2022). However, it is important to highlight that, in the current investigation, the N balance in TMM-fed lambs was better than that of SBM-fed lambs. In line with such results, Volek et al. (2021) reported a higher N retention in rabbits fed TMM containing diets compared to rabbits fed diets containing SBM. In fish, it was also observed that substituting FM with TMM (up to 100 %) increased N retention (Basto et al., 2021).

4.2.2. Histomorphometry and histopathology

Currently, no study has investigated the rumen and intestinal histomorphometry in insect-fed ruminants, making our research the pioneering assessment of this subject in scientific literature. In the current trial, the rumen morphometric features were not influenced by TMM administration. Similarly, in monogastric species, gut morphology remained unaffected by the administration of low dietary inclusion levels of insect meals. In particular, no significant alterations of villus height (Vh), crypt depth (Cd), as well as Muc, Subm or tunica Musc thickness have been recorded in the gut of broiler chickens (Colombino et al., 2023) and free range chickens (Biasato et al., 2018) fed TMM (0.75, 2.5, 5, 7.5, and 10 % of inclusion) when compared to control groups fed SBM-based diets. Recently, Malla et al. (2024) showed that feeding black soldier fly meal, lesser mealworm (*Alphitobius diaperinus*) meal, and TMM at 8.35, 9, and 7.66 % diet DM, respectively, did not affect gut health parameters of piglets when compared to a CTRL diet containing SBM. Similarly, the inclusion of 5 and 10 % of black soldier fly meal did not alter gut morphology of weaned pigs in terms of Vh, Cd and Vh/Cd ratio (Biasato et al., 2019). Comparable results were also observed in rabbits, where the dietary inclusion of TM or black soldier fly oil did not change gut morphology (Gasco et al., 2019).

Interestingly, in the present trial, we observed a significant decrease of epithelial keratinization of the ruminal dorsal sac and different degrees and types of inflammation in the proximal intestine in the lambs fed with TMM when compared to the SBM-fed and FM-fed lambs. Epithelial keratinization in the rumen, particularly with concentrate feeds, may indicate mucosal damage due to accelerated fermentation. The rumen epithelium, a protective barrier between rumen and portal circulation, may respond to rapid fermentation by thickening keratinized layers (Na and Guan, 2022). In the present study, the SBM-fed lambs showed greater keratinization in the ruminal dorsal sac when compared to the FM-fed and TMM-fed lambs; such a result may be due to higher DMI and modifications of the SCFA production. The obtained results suggest that the use of TMM as a protein source for fattening lambs does not negatively compromise the ruminal absorption processes when compared to conventional protein sources such as SBM and FM.

All the lambs involved in the current trial showed inflammation of various severities in the proximal tract of the intestine. However, the TMM-fed lambs mainly showed chronic inflammation composed of mononuclear cells. On the contrary, in the SBM-fed and FM-fed lambs the phlogosis was often mixed or with the presence of eosinophils. Intestinal inflammation can depend on many factors such as infections, allergies or unbalanced diets that may directly damage the mucosa or contribute to microbiota alterations (Beam et al., 2021). Unbalanced diets can be responsible for low-grade chronic inflammation (Soares et al., 2022), whereas the presence of neutrophils and/or eosinophils is more often attributable to bacterial/parasitic infections or allergic reactions (Ondari et al., 2021). The lambs involved in this study were not infected by intestinal parasites, but the presence of neutrophils in the mucosa could be the result of intestinal dysbiosis with bacterial proliferation of microbiota. Previous studies showed that, once the integrity of the intestinal epithelium is impaired, toxic compounds or pathogenic bacteria in the lumen may translocate across it, further causing local or systemic inflammation (Di Vincenzo et al., 2023). It has been reported that, in dairy cattle fed high-grain diets, an excessive flow of undigested starch or SCFA from the rumen into the small intestine may induce excessive fermentation, imbalance the bacterial communities in the small intestine, and accumulate lipopolysaccharides in digesta responsible for local inflammation and barrier injury in the jejunal and ileal epithelia (Lai et al., 2022). Soybean meal-based diets seem also to increase the density of B and T lymphocytes in the small intestine of calves (Lallès et al., 1996). A possible explanation of the presence of eosinophils in the intestine of SBM-fed lambs in the current trial could be the presence of antinutritional factors in SBM (Pan et al., 2018). Especially soybean agglutinin can bind to various carbohydrate compounds, reaching the proximal intestinal tract (Pan et al., 2018), and modulating the local immunological response resulting in the eosinophils recruitment, as reported by Furusawa et al. (2013).

5. Conclusion

In the framework of sustainable-based and circular-based ruminant production systems, novel protein feed ingredients are advocated. This study aimed to examine different protein sources, including plant-based (SBM), animal-based (FM) and insect-based (TMM) ones, for fattening lambs by means of *in vitro* and *in vivo* approaches. The dietary inclusion of TMM (60 g/kg DM) was accompanied by lower IVGP, but higher DMd and MCP, when compared to the FM-containing diet. When compared to the SBM-based diet, the dietary inclusion of TMM negatively impaired the DMI of the lambs, but the total tract apparent digestibility of DM and OM, as well as the N balance, were improved.

The ruminal morphometric indexes were not significantly influenced by the dietary inclusion of TMM, although a decrease of epithelial keratinization of the ruminal dorsal sac and a mononuclear inflammation in the proximal intestine were observed in the TMM-fed lambs when compared to the SBM-fed ones. These results allow us to hypothesize that TMM utilization in lambs does not negatively alter the gut microbiota, allowing the maintenance of a regular function of the intestinal barrier.

Further studies should explore the effects of dietary TMM inclusion on the ruminal and intestinal microbiota. In addition, future research should also focus on the use of TMM in combination with conventional protein sources with the aim of optimizing the performance of lambs.

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Ethical statement

This experiment was carried out in compliance with the guidelines established by the Professional Committee on Standardization of Experimental Animals of the Universidad Autónoma del Estado de México, under the approved ID project CAT2021–0070COMECyT.

CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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