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Influence of oilseed (*Camelina sativa* and *Linum usitatissimus*) cakes and insect (*Tenebrio molitor*) meal on immune gene expression, microbiota composition and gut morphology in broiler chickens

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Introduction: The search for alternative feed ingredients that can improve poultry health while reducing reliance on conventional protein sources is gaining increasing attention in the context of sustainability, animal welfare and public health. Oilseed by-products and insect meals are particularly promising, as they combine interesting nutritional profiles with favorable environmental and circular-economy features.
Methods: In this study, we evaluated the effects of diets including camelina cakes or *Tenebrio molitor* (TM) meal on nutrient digestibility, intestinal morphology, immune gene expression and gut microbiota in broiler chickens. Birds were assigned to a conventional corn–soy control diet, an oilseed-cake diet, or a TM-based diet.
Results: As expected, the inclusion of oilseed cakes partly reduced the digestibility of some nutrients, likely due to residual antinutritional factors, whereas TM mainly affected crude protein digestibility. Nevertheless, both alternative diets were associated with intestinal traits generally compatible with a favorable gut status, such as increased villus height and villus/crypt ratio in the oilseed group and a tendency towards similar improvements in TM-fed birds. At the molecular level, modulation of genes involved in apoptosis and immune regulation suggested a shift towards a more controlled inflammatory tone, particularly in oilseed- and insect-fed chickens. Microbiota analysis revealed only modest diet-driven changes but pointed to an enrichment of butyrate-producing taxa and a reduction of potentially detrimental families. Therefore, a likely combination of increased availability of antioxidants, polyunsaturated fatty acids (PUFAs), and other bioactive compounds—together with a modest modulation of the microbiome and its short-chain fatty acid

(SCFA) metabolism—may have contributed to enhanced intestinal functionality and improved immune regulation.

Discussion: Overall, these results indicate that appropriately balanced inclusion of oilseed cakes and TM meal can support gut health and immune homeostasis in broilers, while contributing to more sustainable feeding strategies.

KEYWORDS

Camelina sativa, chicken, digestibility, gene expression, intestine, microbiome

Introduction

Poultry meat and eggs represent a fundamental source of high-quality protein for the global population, due to the high production efficiency (i.e., low feed conversion ratio) and the broad consumer acceptance (Castro et al., 2023). Nowadays, in addition to productivity and cost, other aspects are gaining increasing attention from both consumers and public authorities, including environmental sustainability, animal welfare, meat safety, and the so-called "nutraceutical" properties (Verbeke et al., 2010; Di Pasquale et al., 2014). Feed composition clearly plays a pivotal role in all these domains. Notably, feed costs account for 60-80% of the total production costs in poultry farming, and the procurement of raw materials has a considerable environmental, and therefore social, impact (Elahi et al., 2022). Moreover, feed ingredient availability is subject to marked price fluctuations and geopolitical uncertainties. European policies are increasingly aimed at achieving raw material independence for feed production by reducing imports from foreign countries. In this context, the development and evaluation of alternative feed sources have become a major research focus. Oilseeds and oilseed meals, such as those derived from rapeseed, linseed, canola, and camelina, are important sources of energy, crude protein, and fats, including essential polyunsaturated fatty acids (PUFA) that can accumulate in animal tissues and improve the nutritional quality of the meat (Zając et al., 2020; Hajiazizi et al., 2024). These sources offer remarkable environmental and economic benefits, as they typically involve undemanding cultivars in terms of fertilization and irrigation. They are also resistant to diseases and parasites, requiring minimal treatments, and are used in various industrial applications. Their byproducts, such as oilseed cakes, can be further valorized as animal feed, thus contributing to the principles of circular economy by reintroducing agricultural and industrial residues into the production cycle (Singh et al., 2023). Among these crops, camelina (*Camelina sativa* L. Crantz) and linseed (*Linum usitatissimum* L.) have attracted particular interest. Camelina, a member of the Brassicaceae family, is highly adaptable to diverse environmental conditions, requires low agronomic inputs, and has a relatively short growing cycle. Its seeds contain 30-49% oil, rich in omega-3 (ω3) and omega-6 (ω6) fatty acids (FAs), 24-31% protein, and noteworthy levels of tocopherols, phytosterols, and phenolic compounds (Berti et al., 2016; Boyle et al., 2018; Mondor and Hernández-Álvarez, 2022). The cake obtained after oil extraction provides about 35% protein, a balanced amino acid profile, and 10-22% residual oil with a high proportion of ω3 FAs (Aziza et al., 2014). Linseed cake, a co-product of linseed oil production, is likewise rich in nutrients, with around 36% protein, 7-10% residual oil, and significant levels of lignans, antioxidants, and minerals (Duliński et al., 2017). Nevertheless, these crops are often rich in antinutritional factors (ANFs), and their excessive inclusion in poultry diets may compromise animal performance (Bischoff, 2021).

Various insect species are currently being explored as promising alternative feed sources. Among them, *Tenebrio molitor* (TM) larvae have been primarily investigated in the form of full-fat or defatted meals as substitutes for conventional protein sources (Moula and Detilleux, 2019; Volek et al., 2021; Andrade et al., 2023). They can also be offered as live larvae, providing environmental enrichment and contributing to improved animal welfare (Bellezza Oddon et al., 2021; Colombino et al., 2021; Dalle Zotte et al., 2024). Nutritionally, TM larvae are rich in protein (approximately 50% of dry matter, DM) and contain high levels of essential amino acids. They also have a substantial fat content (around 30% of DM), which includes beneficial FAs, notably ω-3 and ω-6 PUFA (Kröncke et al., 2019; Trukhanova et al., 2022). In addition, TM larvae represent a valuable source of minerals and vitamins (Nowak et al., 2016). Their chitin-rich exoskeleton contains a bioactive polysaccharide with well-documented immunostimulatory and prebiotic properties (Swiatkiewicz et al., 2015; Elahi et al., 2022). Insect larvae also naturally produce antimicrobial peptides (AMP) as a defense mechanism against microbial infections (Elahi et al., 2022). These compounds have been shown to improve resistance to various pathogens, including zoonotic agents, potentially enhancing food safety and reducing the need for antimicrobial treatments (Hwang et al., 2022). The bioactive compounds present in insects have been reported to modulate the gastrointestinal microbiota when insects are used as full or partial replacements for conventional protein sources in chicken diets (Biasato et al., 2019; Moula and Detilleux, 2019). Similarly, oilseed cakes have been investigated as potential probiotic feed ingredients for livestock, with camelina cake shown to enhance the acid-forming activity of lactic acid bacteria and promote the growth of other beneficial bacterial populations (Mazanko et al., 2023). In addition, linseed cake, when combined with probiotics such as *Lactobacillus acidophilus*, has been reported to improve gut health in broiler chickens by supporting a more favorable intestinal microbiota and reducing pathogenic bacteria (Gheorghe et al., 2020). However, current knowledge on the effects of incorporating TM larvae and oilseed cakes into poultry diets, particularly regarding nutrient digestibility, gut morphology, immune function, and the microbiome, remains limited. Furthermore, direct comparisons between these two dietary

supplements remain scarce, making it difficult to determine which provides the greater benefits for chicken's health and performance.

The present study addresses these topics by comparing feed digestibility, morphometric parameters, inflammatory cell infiltration, gene expression, and microbiota composition across different intestinal segments of chickens fed conventional, oilseed cakes-enriched, or insect-enriched diets.

Material and methods

Animals and experimental design

The trial was conducted in a farm operating under a scientific agreement with the Department of Animal Medicine, Production, and Health (MAPS) at the University of Padova. An *in vivo* digestibility trial, was conducted on 30 male broiler chickens (Ross 308; Aviagen Group, UK), 33 days old, individually housed in digestibility cages (one bird per cage) and randomly assigned to three dietary treatments: (i) a corn-soybean-based control diet (CD); (ii) a diet containing 5% camelina cake (Pearl line, Smart Earth Camelina Corp.) and 5% linseed cake (Librazoo S.R.L., San Giorgio delle Pertiche, Padova, Italy) (oilseed cake diet, OD); or (iii) a diet containing 10% full-fat dried TM larvae (TM diet, TD), according to the procedure described by Dalle Zotte et al (Dalle Zotte et al., 2021). The diets, in mash form, were prepared at the University of Padova. Dried TM larvae were obtained from the INEF-Insect Novel Ecologic Food insect farm located in Piombino Dese, Padova, Italy. The ingredients and chemical composition of the experimental diets are reported in Table 1. Camelina and linseed cakes contained 319 and 208 g/kg crude protein (CP) (as fed basis) and 216 and 345 g/kg ether extract (EE) (as fed basis), respectively.

Birds were weighed and assigned to the experimental groups (10 replicates per treatment) to ensure homogeneous live weight across treatments (1360 g ±186 g).

Each cage was equipped with a feeder and a poultry drinking cup. Feed and water were provided *ad libitum*. The photoperiod was set at 18 hours of light and 6 hours of darkness (18L:6D) throughout the experimental period. Birds were subjected to 12 days adaptation period to feed and five days to cage before the start of the 3-days digestibility trial.

Birds were fasted for 12 hours before the start of the *in vivo* digestibility assay, and the individual body weight was assessed along with the feeder tare and the weight of the feed provided. Aluminium foils were placed under each cage to collect the birds' droppings. The first droppings produced after the fasting period were discarded and not included in the chemical analyses. Droppings collection began on the second day of the trial. Every morning and evening (at 12-hour intervals), droppings were collected and weighed. Any extraneous material or feed residual was carefully removed. Feeders were also weighed to calculate feed consumption, and any feed waste was recorded. On the 3rd day of trial, at 6:00 p.m. feeders were removed, and birds were fasted for 24 hours. The last sample of droppings was collected on the 4th evening. The final weight of animals was recorder the same day.

TABLE 1 Ingredients and chemical composition of the experimental diets (CD, control diet; OD, oilseeds diet; TD, *Tenebrio molitor* diet).

Item	Experimental diets		
	CD	OD	TD
Ingredients (g/kg as fed)			
Corn meal	643.5	598.5	628.5
Soybean meal	280.0	230.0	205.0
Soybean oil	35.0	30.0	25.0
Camelina cake	0.00	50.00	0.00
Linseed cake	0.00	50.00	0.00
Full-fat *T. molitor* larvae	0.00	0.00	10.0
Calcium carbonate	17.0	17.0	17.0
Monocalcium phosphate	13.0	13.0	13.0
Sodium chloride	4.00	4.00	4.00
L-lysine HCl	2.00	2.00	2.00
DL-methionine	0.50	0.50	0.50
Mineral and vitamin premix[1]	5.00	5.00	5.00
Chemical composition (% as fed)			
Dry matter	89.25	89.8	89.61
Crude protein	19.08	19.60	18.96
Ether extract	6.14	8.01	7.92
Ash	6.06	7.60	6.26
Crude fiber	2.97	3.11	2.70
Lysine	1.04	1.03	1.10
Methionine	0.65	0.64	0.63
Calcium	0.95	0.97	0.94
Available phosphorous	0.38	0.38	0.37

[1]Premix ingredients (per kg premix): vit. A, 1600000 IU; vit. D3, 500000 IU; vit. E, 5000 mg; iron sulphate (Fe): 7000 mg; calcium iodate anhydrous (I): 100 mg; cupric sulphate (Cu): 1500 mg; manganese oxide (Mn): 21000 mg; zinc oxide (Zn): 8400 mg; sodium selenite (Se): 60 mg; 6-phytase (EC 3.1.3.26): 120000 OTU; Endo-1,4-beta-xilanasi EC 3.2.1.8 (4a7), 112000 TXU; Endo-1,4-beta-glucanasi EC 3.2.1.4 (4a7), 50000 TGU.

At the end of the digestibility trial, birds were slaughtered through cervical vertebrae relocation, as specified by Reg. (CE) n. 1099/2009 and immediately after, 5 birds from each group were collected and necropsies were performed. Sections of the duodenum, jejunum, ileum, and caecum, as well as one of the caecal tonsils, were aseptically collected and immediately fixed in 10% formalin. The other caecal tonsil was also collected, immediately refrigerated, and then stored at −80 °C within one hour of collection for subsequent molecular biology analyses. Droppings samples were collected from the jejunum, ileum, and caecum, immediately mixed with a DNA/RNA Shield stabilizing solution (Zimo Research), and then stored at −80 °C until processing.

Histology

Formalin-fixed samples were routinely processed and paraffin-embedded. Four-µm sections were cut on microtome, stained with hematoxylin and eosin (H&E), digitally acquired using a slide scanner and systematically reviewed in a blinded manner by an

EBVS-certified veterinary pathologist, using the SlideCenter software (3D Histech Ltd., Budapest, Hungary). Morphometric measurements were performed by measuring the height of 10 villi and the depth of 10 crypts for each intestinal segment (duodenum, jejunum, ileum), and the mean value per subject was calculated (Hampson, 1986). The ratio between villus height and crypt depth was also calculated. The number of goblet cells was counted along 300 µm-long segments of the villus length, in 5 villi (Fonsatti et al., 2025). In the caecal tonsils, the average diameter of lymphoid follicles within the mucosa-associated lymphoid tissue (MALT) was determined, based on the mean value of 10 lymphoid follicles per subject, expressed in micrometers. Finally, in all intestinal segments, inflammatory cell infiltration was assessed and expressed as the range of cells in the inter glandular lamina propria per high-power field (HPF; 40x, equal to 0.237 mm^2), with reference to heterophils, lymphocytes and plasma cells, and macrophages. Variations in the mean leukocyte count were assessed as increases in mucosal leukocytes, according to the criteria adapted from Day et al (Day et al., 2008); the severity was graded on a 0–3 scale, corresponding to normal (0), mild (1), moderate (2), and marked (3) increases.

Chemical analyses

Once collected, droppings samples were pooled to obtain one homogeneous sample per cage/treatment and subsequently dried in oven at 60 °C. Chemical analyses of dried excreta were carried out in accordance with the AOAC (2019) methods to determine dry matter (DM; method no. 934.01), crude protein (CP; method no. 2001.11) and ash (method no. 967.05) (Association of Official Analytical Chemists (AOAC), 2019). The CP content of excreta was corrected for uric acid content, which was analyzed according to the procedure described by Cullere et al (Cullere et al., 2016). Ether extract (EE) was determined after acid hydrolysis. Gross energy (GE) was measured with an adiabatic bomb calorimeter (ISO 9831:1998) (ISO9831:1998. Animal feeding stuffs, animal products, and faeces or urine — Determination of gross calorific value — Bomb calorimeter method, 1998), and starch content of the experimental diets was analyzed using the amyloglucosidase–α-amylase method (method 996.11). The same analyses were performed on the experimental diets.

RNA extraction

Caecal tonsils were weighed and homogenized in phosphate-buffered saline (PBS) at a 10% (w/v) ratio using a T10 basic ULTRA-TURRAX® homogenizer (IKA®-Werke GmbH & Co. KG, Staufen, Germany) equipped with sterile, disposable plastic probes to avoid cross-contamination between samples. The homogenates were centrifuged at 2000 rpm for 15 minutes at 4 °C, and the resulting supernatants were collected. RNA extraction was carried out with the High Pure RNA Isolation Kit (Roche Diagnostics, Marnes-la-Coquette, France) following the manufacturer's protocol. Complementary DNA (cDNA) synthesis was performed from 5 µL of RNA using random primers and the Maxima™ H Minus cDNA Synthesis Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), including a DNase treatment step to remove residual genomic DNA. The synthesized cDNA was stored at 4 °C and used for relative quantification within one hour of preparation.

Relative quantification

Gene expression was evaluated by relative quantification using the ΔCt method, normalizing to the geometric mean of the Ct values of the housekeeping genes ACTIN and GAPDH. Primers and amplification protocols for BAX, BCL-2, CASP-3, IgA, IL-10, IL-12, IL-1β, IL-2, IL-4, IL-5, IL-6, IFN-γ, IFN-β, ACTIN, and GAPDH were previously described (Franzo et al., 2024). Reactions were carried out with the PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) on a LightCycler 95 instrument (Roche, Basel, Switzerland). For each sample, all target genes were analyzed within the same run. Each 10 µL reaction mixture contained 2 µL of cDNA, 1× SYBR™ Green Master Mix, 0.8 µL of each primer, and nuclease-free water. Thermal cycling consisted of an initial polymerase activation at 95 °C for 2 min, followed by 45 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 1 min. A melting curve analysis was subsequently performed by increasing the temperature from 40 °C to 90 °C at a ramp rate of 0.1 °C/s, with continuous fluorescence monitoring. Previously determined amplification efficiencies were applied to obtain efficiency-corrected Cq values, which were then used to calculate the relative expression ratio between each target gene and the reference genes.

Microbiome analysis

Fecal samples were processed using the Genomic Mini AX Stool Spin 100 kit (A&A Biotechnology) following the manufacturer's protocol. DNA concentration was measured with a Qubit fluorometer using the Qubit dsDNA HS Assay Kit (Invitrogen). Prokaryotic metagenome sequencing was performed with the Rapid Sequencing DNA – 16S Barcoding Kit 24 V14 (Oxford Nanopore Technologies, Oxford, UK), targeting the full 16S rRNA hypervariable region (V1–V9) using the 27F forward and 1492R reverse primers. Sequencing libraries were prepared according to the kit instructions and loaded onto MinION Flow Cells (R10.4.01, Oxford Nanopore Technologies). MinKNOW software (v21.06.13, Oxford Nanopore Technologies) was used for sequencing control, while raw FAST5 files were basecalled in real-time to FASTQ format using the GPU-accelerated Guppy basecaller (v5.0.16, Oxford Nanopore Technologies) in super-accurate mode with barcode and adapter trimming. During sequencing, the EPI2ME platform was used for periodic analysis using the Metagenome workflow. Runs were terminated at least 4 hours after the lowest-read sample showed no further gain in sequencing depth or taxonomic diversity, as assessed by rarefaction curve analysis. Given the high capacity of the MinION Flow Cells compared with the 24-sample multiplexing limit of the SQK-16S024 barcoding kit, flow cells were washed after each run using the Flow Cell Wash Kit (EXP-WSH004, Oxford Nanopore Technologies), stored, and reused until pore depletion.

Statistical analysis

Data of apparent digestibility coefficient were analyzed using SAS software (Version 9.4, SAS Institute Inc., Cary, NC, USA). Prior to conducting the analysis of variance (ANOVA), the distribution of the data for each dependent variable was examined to verify the assumptions required for parametric analysis. The normality of residuals was assessed using the Shapiro–Wilk test (PROC UNIVARIATE), while the homogeneity of variances among dietary treatments was verified with Levene's test (PROC GLM). When the assumption of normality was not met, data were further evaluated using the non-parametric Kruskal–Wallis test. A one-way ANOVA was then performed using the general linear model procedure (PROC GLM). The experimental group (diet) was included as a fixed factor, and individual cages were considered as the experimental units. When a significant effect of diet was detected ($P < 0.05$), mean differences among treatments were evaluated using Tukey's multiple comparison test. The statistical significance was set at $P < 0.05$.

The effect of diet on the histomorphometric parameters were evaluated for each gut tract using a hierarchical model implemented in the *nlme* package in R. The dietary group CD, OD, TD were considered as the fixed effect of interest. To account for repeated measures (multiple measurement for each slides) each measure was nested within the sample. Model selection was carried out using maximum likelihood (ML) estimation by comparing the following nested models: Intercept-only generalized least squares, random-intercept, random-intercept model with diet as fixed effect and Random-intercept and random-slope model. Nested models were compared using likelihood ratio tests (LRTs). Random-intercept model with diet as fixed effect was constantly reported as the best fitting method.

Differences in gene expression ratios among groups were assessed using the non-parametric Kruskal–Wallis test. When significant, pairwise comparisons were performed with the Mann–Whitney U test, applying Bonferroni correction for multiple testing. All statistical analyses were conducted in R, with a significance threshold set at $p < 0.05$. Graphs were produced using the ggplot2 and ggpubr packages (Ginestet, 2011; Kassambara, 2018).

For the microbiome analysis the taxonomic assignment on curated reads was performed with the emu (Curry et al., 2022) tool, using the Greengeen/silvs dataset. The following analysis were performed in R (R Core Team, 2022) benefitting of the packages vegan, microbiome and phyloseq, and related dependencies (Dixon, 2003; McMurdie and Holmes, 2013; Lahti and Shetty, 2019). Alpha diversity indices, including Chao1 richness, Shannon diversity, Simpson evenness, and dominance, were calculated and compared across groups and tissues w using the Kruskal–Wallis test, followed by pairwise Mann–Whitney U tests with Bonferroni correction for multiple comparisons. Beta diversity was assessed using Bray–Curtis dissimilarity indices. Principal Coordinates Analysis (PCoA) was performed to visualize the dissimilarity patterns among samples. Permutational Multivariate Analysis of Variance (PERMANOVA) was applied using the adonis2 function in the vegan package to evaluate the association between beta diversity and explanatory variables (intestinal segment and dietary group), with 999 permutations. Differences in specific taxa level abundance among groups were assessed with DESeq2 (Love et al., 2014; Nearing et al., 2022).

Results

Digestibility trial

Significant differences were observed across all evaluated apparent digestibility coefficients, with the sole exception of starch digestibility, which did not differ among the experimental groups. The digestibility of DM, OM, and EE were higher in CD and TD groups compared to OD ($0.001<P<0.01$). Differently, the digestibility coefficient of CP resulted lower in both OD and TD groups compared to CD ($P = 0.038$). The digestibility of GE was similar between the CD and TD groups and significantly higher than that of the OD group ($P < 0.001$). Consequently, the CD and TD diets showed a higher metabolizable energy (ME) concentration compared to the OD diet ($P = 0.001$) (Table 2).

Morphometric and histologic evaluation

The length of the villi was significantly higher in the duodenum and jejunum of the OD group compared to the CD group, while no statistically significant differences were observed in the ileum for either group. A decrease in crypt depth was observed in the ileum of both treatment groups (OD and TD) compared to CD, and in the duodenum of the OD group only. A statistically significant increase in the villus-to-crypt ratio was recorded in all three intestinal segments in the OD group (Table 3). No significant differences were detected in the number of goblet cells or in the diameter of lymphoid follicles in the tonsils (Table 3). No differences were detected in the two treatment groups, with the exception of a reduction of villi length in the jejunum of TD compared to OD.

TABLE 2 Apparent digestibility coefficients of the experimental diets (CD, control diet; OD, oilseeds diet; TD, *Tenebrio molitor* diet).

Item	Experimental diets			RMSE	P-value
	CD	OD	TD		
Feed intake (g)[1]	359	382	373	57.6	0.721
Dry matter	0.737[a]	0.702[b]	0.745[a]	0.023	0.001
Organic matter	0.773[a]	0.738[b]	0.775[a]	0.023	0.002
Crude protein	0.827[a]	0.777[b]	0.793[b]	0.038	0.035
Ether extract	0.912[a]	0.843[b]	0.907[a]	0.034	<0.001
Starch	0.986	0.973	0.985	0.017	0.205
Gross energy	0.788[a]	0.754[b]	0.792[a]	0.019	<0.001
Metabolizable energy ME (kcal/kg)	3255[a]	3155[b]	3302[a]	80.18	0.001

RMSE: root mean square error.
[1] Total feed intake per bird during the 4-day *in vivo* digestibility trial period.
[a,b] Different superscript letters indicate significant differences ($P < 0.05$).

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TABLE 3 Summary of the differences among groups in the considered parameters.

Tissue	Measure	CD	OD	TD	RMSE	P-value OD vs CD	P-value TD vs CD	P-value TD vs OD
Duodenum	Villi Height (μm)	1648.05[a]	1983.70[b]	1757.68[ab]	247.01	0.042	0.433	0.169
	Crypt Depth (μm)	237.42[a]	209.94[b]	225.56[ab]	18.51	0.038	0.344	0.206
	Goblet Cells	11.72	10.96	9.52	2.17	0.975	0.169	0.161
	Villi/Crypt Ratio	6.967[a]	9.40[b]	7.934 [ab]	1.232	0.005	0.119	0.123
Jejunum	Villi Height (μm)	955.86[a]	1165.52[b]	1021.04[a]	84.39	0.0037	0.272	0.030
	Crypt Depth (μm)	181.14	177.14	170.94	15.03	0.6851	0.319	0.544
	Goblet Cells	12.32	14.36	11.80	2.55	0.186	0.7675	0.293
	Villi/Crypt Ratio	5.309[a]	6.63[b]	5.73[ab]	0.60	0.0095	0.0914	0.236
Ileum	Villi Height (μm)	784.4	784	728.66	54.62	0.9952	0.137	0.138
	Crypt Depth (μm)	198.3[a]	172.84[b]	162.42[b]	14.01	0.0149	0.001	0.271
	Goblet Cells	17.44	16.92	14.31	3.62	0.7688	0.4735	0.668
	Villi/Crypt Ratio	4.01[a]	4.547[b]	4.341[ab]	0.39	0.0367	0.0539	0.8344

P-values are reported within brackets, as estimated using the multilevel approach, is reported among brackets. Different superscript letters indicate significant differences ($P < 0.05$).

Similarly, no statistically significant differences were observed in the parameters related to inflammation (i.e., lymphocytes, heterophils, and macrophages), with the different treatment groups showing comparably low scores and cell counts per field. A few cases of mild heterophilic enteritis were observed across all groups in the jejunum, ileum, and caecum.

Gene expression

Differences in gene expression were identified for BCL-2 ($p = 0.012$), IL-10 ($p = 0.030$), IL-1β ($p = 0.004$), and IL-5 ($p = 0.049$). Specifically, BCL-2 expression was significantly higher in both the OD ($p = 0.016$) and TD ($p = 0.011$) groups. IL-10 expression was increased in the OD group compared to CD ($p = 0.032$). IL-1β expression was higher in OD compared to both CD ($p = 0.018$) and TD ($p = 0.016$), and in TD compared to CD ($p = 0.034$). IL-5 expression was significantly lower in the TD group compared to CD ($p = 0.048$). Although not statistically significant, increased expression of IL-12, IL-2, and IL-4 was observed in both treatment groups, with generally higher values in the OD group, except for INF-γ, which did not follow this trend. Conversely, IgA, IL-6, and INF-β showed higher average expression levels in the CD group (Figure 1).

Microbiome composition

The evaluation of alpha diversity revealed a significant effect ($p < 0.001$) of the intestinal tract on all the indices considered, with significantly higher Chao1 richness, Shannon diversity, and Simpson evenness, as well as lower Simpson dominance in the caecum compared to other intestinal segments. Within diet, a significant difference among tissues pairs was constantly detected ($p<0.001$) with the exception of Simpson evenness and Shannon diversity did not differ significantly between the caecum and ileum in the CD group ($p=0.19$), but this was likely due to the high variability observed in the ileal values. Overall, much lower heterogeneity in alpha diversity indices was observed in the caecum compared to other intestinal tracts, regardless of diet (Figure 2).

The evaluation of beta diversity using PCoA and Bray–Curtis distances revealed strong clustering based on intestinal location, clearly differentiating the caecum from the jejunum and ileum, although a certain degree of separation was also evident among the latter segments. No clear clustering based on diet was observed (Figure 3).

The PERMANOVA test, assessing the strength of the association between beta diversity and explanatory variables, was performed on Bray–Curtis dissimilarities using the adonis2 function (999 permutations, marginal effects). Tissue had a strong and highly significant effect on community composition ($df = 2$, $F = 29.49$, $R^2 = 0.568$, $p = 0.001$), explaining approximately 56.8% of the observed variance. Group also showed a statistically significant but comparatively modest effect ($df = 2$, $F = 2.39$, $R^2 = 0.046$, $p = 0.032$), accounting for about 4.6% of the variance.

Such variation in microbiome composition across different sections of the intestinal tract could also be observed qualitatively at multiple taxonomic levels. On the other hand, no relevant discrepancies were observed among groups. At the phylum level, all intestinal tracts and experimental groups were dominated by Firmicutes, with Proteobacteria representing the second most abundant phylum in the jejunum, and particularly in the ileum. In contrast, in the caecum, Bacteroidota and, to a lesser extent, Proteobacteria constituted the most relevant minor phyla (Supplementary Figure 1).

At the family level, Lactobacillaceae were the dominant group in the jejunum, followed by Lachnospiraceae, Enterobacteriaceae, and Oscillospiraceae. In the ileum, alongside Lactobacillaceae, a notable contribution was provided by Peptostreptococcaceae, and to a lesser extent by Lachnospiraceae, Clostridiaceae, and Enterobacteriaceae. The caecum exhibited a more heterogeneous and balanced microbial composition, with Lachnospiraceae, Oscillospiraceae, and Ruminococcaceae present in approximately equal proportions, followed by Lactobacillaceae and Enterobacteriaceae (Figure 4).

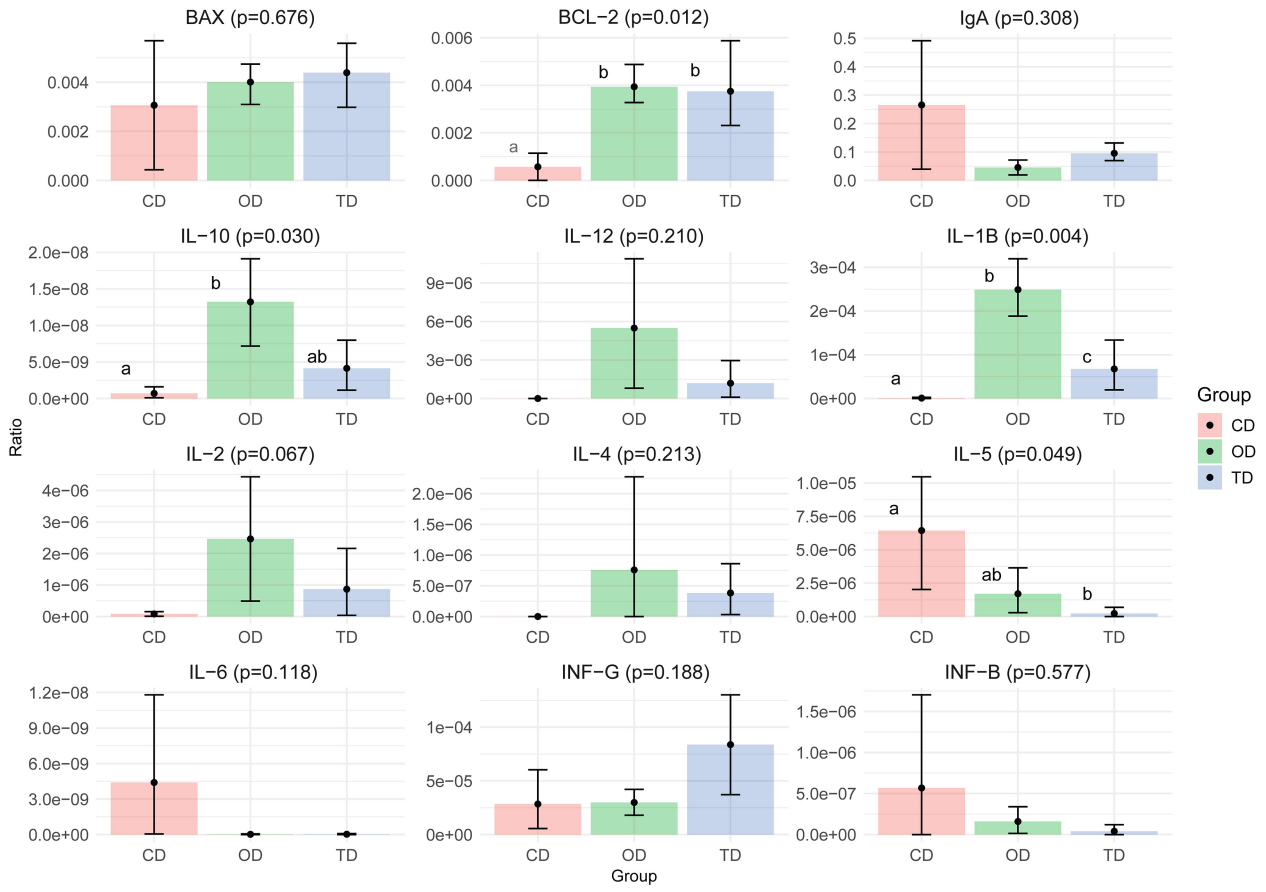


FIGURE 1
Expression ratios of genes and cytokines related to apoptosis and immune response in the intestinal tissue of broilers fed different diets. Bars represent mean ± SD for each experimental group. CD, Control Diet; OD, Oilseed Diet and TD, Tenebrio Diet. Five birds for each experimental group were considered. Differences between groups were assessed using the Kruskal–Wallis test, followed by pairwise Mann–Whitney U tests with Bonferroni correction.

No differences in specific taxa were observed in the Jejunum and Ileum, while in the caecum Rikenellaceae, Peptostreptococcaceae, Desulfotomaculaceae and Enterobacteriaceae were differentially ($p<0.001$) represented in the CD vs OD. Peptostreptococcaceae in CD vs TD and Rikenellaceae and Desulfotomaculaceae in the OD vs TD (Figure 5).

Discussion

The present study investigated the effects of diets incorporating alternative protein sources and FA supplements, specifically camelina and linseed cakes as well as TM meal, on diet digestibility, intestinal morphology, immune responses, and microbiome composition in broiler chickens.

The apparent digestibility coefficients indicated that the CD and TD diets exhibited overall higher feed digestibility compared to the OD diet. This effect is likely attributable to the presence of ANFs in camelina and linseed cakes which can impair productive performance, particularly when included at high concentrations (Aziza et al., 2014; Oryschak et al., 2020; Hajiazizi et al., 2024). Indeed, camelina and linseed cakes are known to contain glucosinolates, phytic acid, sinapine, and tannins (Tan et al., 2011; Pojić et al., 2015; Tangendjaja, 2022). Elevated levels of phytates can reduce amino acid digestibility and inhibit digestive enzyme activity. Likewise, tannins may decrease protein digestibility by forming indigestible protein-tannin complexes (Kahindi et al., 2014). This explains the reduced CP digestibility in the OD group compared to the CD. Consistent with these results, previous studies have reported decreased nutrient digestibility in chickens fed diets containing oilseed cakes or seeds (Rebolé et al., 2002; Amerah et al., 2015; Zając et al., 2020). In particular, the high levels of mucilaginous material in linseed have been shown to impair fat digestibility. These compounds belong to the non-starch polysaccharides (NSPs), a group of substances known to increase intestinal viscosity and thereby reduce nutrient absorption by limiting the diffusion of digestive enzymes and nutrients (Longstaff and McNAB, 1991; Choct et al., 1996).

Therefore, appropriate levels of camelina and linseeds products in poultry rations should be taken into consideration because of presence of these factors and their effects on feed consumption and assimilation (Thacker and Widyaratne, 2012; Pekel et al., 2015; Hajiazizi et al., 2024).

In contrast to the effects observed with oilseed cake inclusion, the incorporation of TM larvae into chicken diets did not negatively affect apparent nutrient digestibility, being the CP the only

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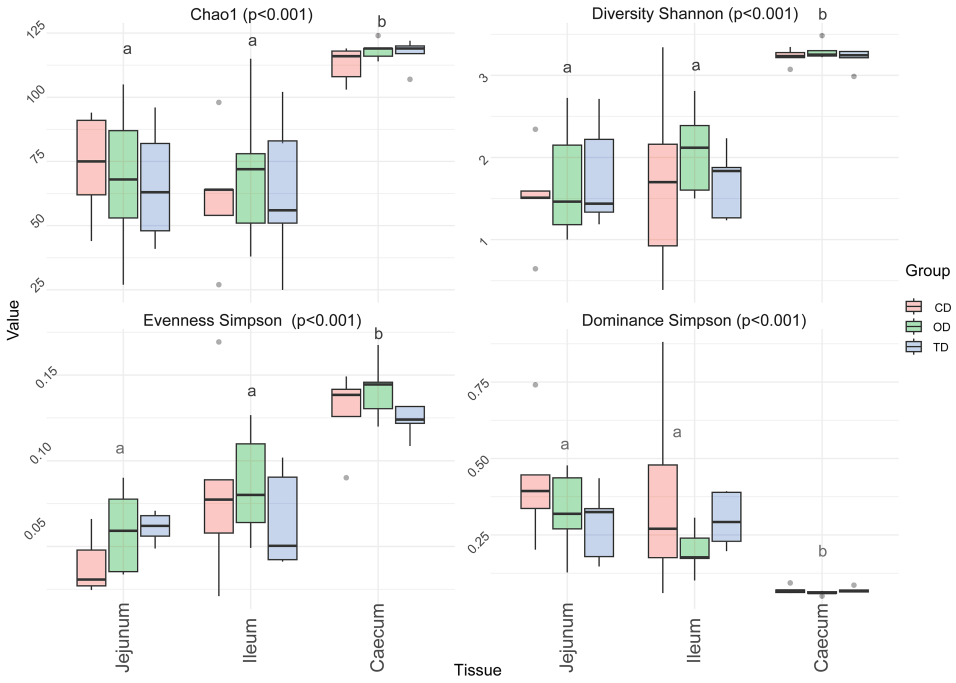


FIGURE 2
Alpha diversity indices of the intestinal microbiota in broilers fed different experimental diets. Boxplots represent the distribution of Chao1 richness, Shannon diversity, Simpson evenness, and Simpson dominance indices in the duodenum, ileum, and caecum across birds fed commercial (CD), oilseed (OD), or insect (TD)–based diets. Five birds for each experimental group were considered. Differences between groups were assessed using the Kruskal–Wallis test, followed by pairwise Mann–Whitney U tests with Bonferroni correction.

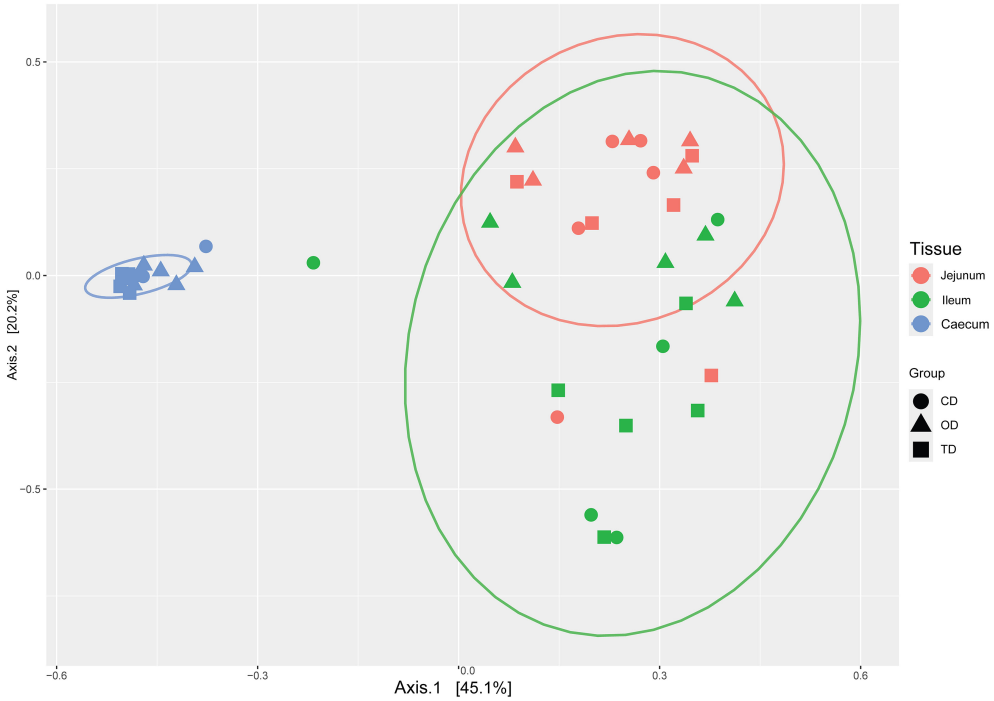


FIGURE 3
Principal coordinates analysis (PCoA) of the intestinal microbiota composition in broilers fed different diets. The ordination is based on beta-diversity distances, showing sample clustering according to intestinal segment (jejunum, ileum, and caecum) and dietary treatment. CD, Control Diet; OD, Oilseed Diet and TD, Tenebrio Diet. Five birds for each experimental group were considered.

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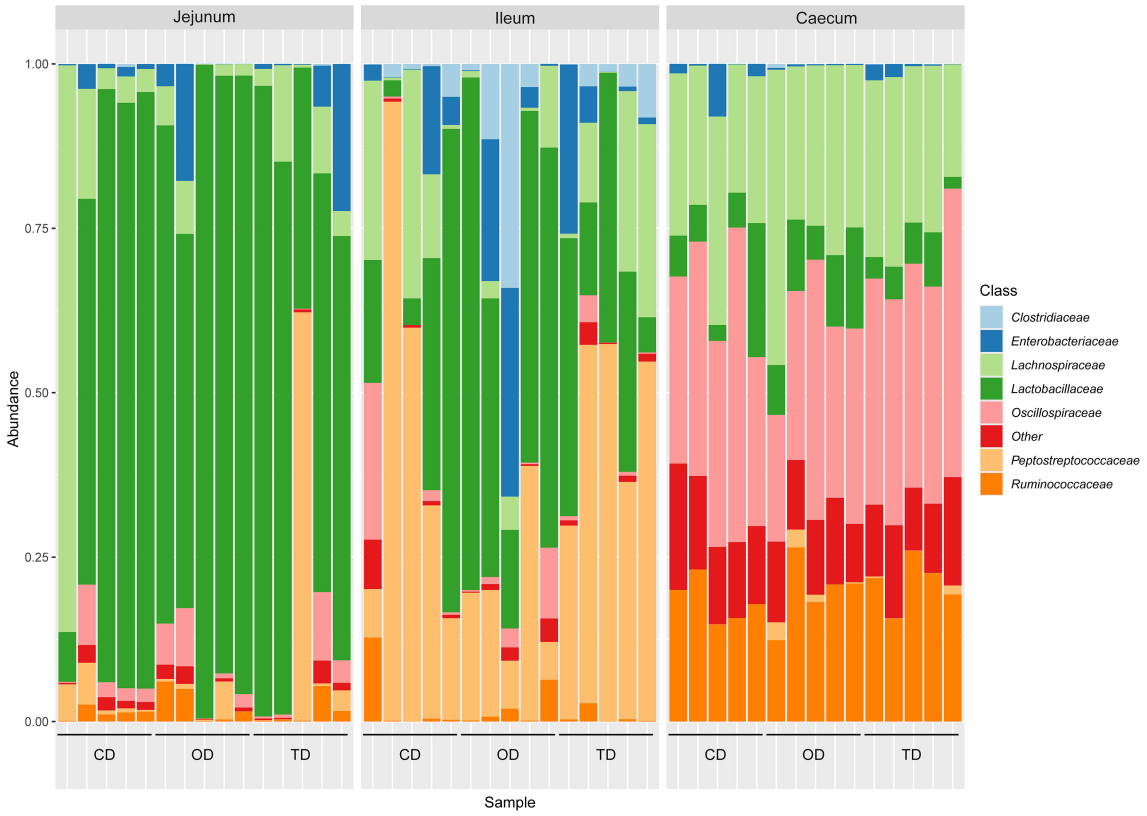


FIGURE 4
Relative abundance of bacterial families in the intestinal microbiota of broilers fed different experimental diets. Stacked bar plots show the taxonomic composition in the jejunum, ileum, and caecum of chickens receiving the control (CD), Camelina sativa–based (OD), or Tenebrio molitor–based (TD) diets. Each column corresponds to one of the five birds included in each group.

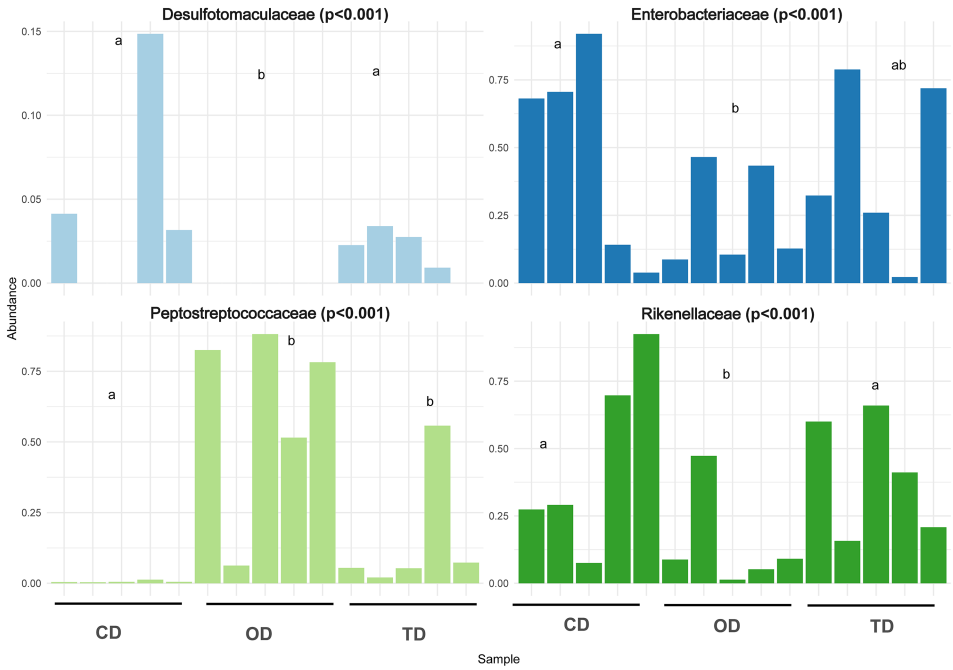


FIGURE 5
Relative abundance of selected bacterial families identified in the caecal microbiota of broilers fed different experimental diets. Bar plots show interindividual variability in the relative abundance of Desulfotomaculaceae, Enterobacteriaceae, Peptostreptococcaceae, and Rikenellaceae across samples from the control (CD), Oilseed (OD), and *Tenebrio molitor* (TD) dietary groups. Each column corresponds to one of the five birds included in each group.

exception. Schiavone et al (Schiavone et al., 2014). reported a reduction in CPd when 25% TM larvae meal was included in chicken diets, and a similar effect was observed by Bovera et al (Bovera et al., 2016). following complete replacement of soybean meal with TM meal. Accordingly, in the present study, a reduction in CPd was observed with the 10% inclusion of TM in the diet of broilers, most likely due to the presence of chitin. Chitin, indeed, is an indigestible polysaccharide that can negatively affect nutrient digestibility in poultry. Although chickens possess some chitinase activity, it is insufficient to effectively degrade chitin (Tabata et al., 2017). The indigestible chitin may increase digesta viscosity or slow intestinal transit, thereby limiting enzyme–substrate interaction and reducing nutrient absorption (Abenaim and Conti, 2025). The inclusion level used in the present study was sufficient to avoid compromising the apparent ileal digestibility of most nutrients, although it already caused a reduction in CPd. In contrast, the higher inclusion levels adopted in other studies may have been excessive, leading to a more pronounced decrease in nutrient utilization (Belluco et al., 2013; Schiavone et al., 2014). Thus, selecting an appropriate inclusion level may help mitigate the negative effects of chitin present in the larvae exoskeleton.

The administration of TM larvae did not significantly affect gut morphometric parameters, as no statistically significant differences were observed compared to the control diet with the exception of crypt depth in the ileum. However, a general trend toward increased villus length (except in the ileum) and reduced crypt depth was noted. These morphological features—longer villi and shallower crypts—are associated with improved nutrient absorption capacity in the gastrointestinal tract (Aziza et al., 2014). Previous studies have reported both no effect of TM administration in broilers and improved intestinal morphology in Japanese quails (Zadeh et al., 2019; Malematja et al., 2023). Conversely, reduced villus height has been observed in laying hens fed high levels of black soldier fly larvae and in Ross broilers fed high levels of mealworm (Biasato et al., 2018; Moniello et al., 2019). Interestingly, such negative effects were not observed with lower dietary inclusion levels. This evidence suggests a dose- and/or host-dependent effect, or more broadly, the contribution of multiple interacting factors. The intermediate dose provided in the present study may have not led to detrimental effect described for high dose, although causing lower, not statistically significant, positive effect of villi structure. Furthermore, the limited processing steps may have preserved the native structure and concentration of nutrients, potentially affecting their bioavailability (Elahi et al., 2022).

Overall, the insect-based diet can be considered beneficial, leading to improved or at least comparable intestinal morphometric features, which suggest a similar absorptive potential. Likewise, the observed pattern in crypt depth indicates an equal or reduced need for tissue regeneration.

The results of the oilseed-based diet appear more surprising, as despite its known beneficial properties, the presence of ANFs was also expected to play a role. A statistically significant increase in villus length and reductions in crypt depth were herein observed in most intestinal segments, resulting in a significantly higher villus-to-crypt ratio (V:C). However, a shortening of villus length had previously been reported in association with camelina-based diets (Aziza et al., 2014). Various factors may account for these findings, as previous studies on camelina administration have often yielded contradictory results, which have been attributed to differences in diet composition, feed formulation, bird genotype, and other experimental conditions. An increase in villus length has been reported in diets enriched with components such as dietary fibre, which is known to decrease overall feed digestibility (Jha and Mishra, 2021), and may represent a compensatory response to increase absorptive capacity (Liebl et al., 2022). The doses also often have a main role in the observed results, since previous studies revealed a detrimental effect associated particularly with high doses. A 5% inclusion of camelina seed in poultry diets had no adverse effects on growth performance. However, 10% inclusion was associated with reduced body weight (BW) and BW gain (Juodka et al., 2022). The effects of camelina cake supplementation are more variable: some studies reported that 8–16% inclusion enhanced BW and BW gain while others attributed reduced growth performance (Ryhänen et al., 2007; Oryschak et al., 2020). Moreover, the content and bioavailability of ANFs can be influenced by the specific camelina cultivar and processing methods applied (Singh et al., 2023). Therefore, it can be hypothesized that these variables also played a role in the present study. The direct effect of PUFA and antioxidants, as γ-tocopherol present in camelina and linseed and α-tocoferol abundant in TM larvae, or the indirect modulation of the microbiome could have favored enterocyte differentiation and maturation, improved villi development and reduced regeneration need. Different studies have confirmed the many favorable effects of dietary ω-3 PUFA, including anti-inflammatory or inflammation-reducing properties (Alagawany et al., 2019; Malematja et al., 2023). The higher expression of BCL-2 in both treatment diets might support their beneficial effects in reducing stresses and damages to the intestinal cells.

No effect on mucin composition has herein been reported, comparably to Colombino et al (Colombino et al., 2021). However, evidence of a dose dependent effect on mucus layer, potentially mediated by altered microbiota, was also described (Biasato et al., 2018; Biasato et al., 2019; Biasato et al., 2020). An analogous dose of TM of 10% was associated with lower mucin staining intensity (Biasato et al., 2019). A 10% camelina meal also resulted in reduction of goblet cell number (Aziza et al., 2014). The lack of effects in the present study may thus be due to other concomitant factors.

Despite the absence of significant histological differences in immune cell infiltration, some inflammatory mediators exhibited differential expression profiles among groups. The higher expression of IL-10, an anti-inflammatory cytokine, might reflect the beneficial effects of PUFAs or other anti-inflammatory and antioxidant compounds provided by camelina, linseed and TM. A reduced stimulation of the immune system by microbial components is also plausible. The presence of antimicrobial molecules, which are well documented in TM larvae, could also have contributed to this response. However, the higher expression of IL-1β, along with several other inflammatory mediators—although not statistically significant—in the camelina-fed group still suggests the presence of an inflammatory stimulus, which was not observed in the CD or TD groups. Certain ANFs, or their metabolites, may compromise the integrity of the mucosal barrier,

thereby triggering an inflammatory response. In particular, some toxic glucosinolate derivatives, in addition to affecting feed palatability and nutrient assimilation and exerting systemic and hormonal effects, may directly irritate the gastrointestinal mucosa (Bischoff, 2021). However, extensive literature also provides evidence of the anti-inflammatory effects of several such molecules, complicating results interpretation (Bischoff, 2021).The specific—and sometimes conflicting—findings observed in the present and other studies may result from complex interactions between the specific composition of the feed and host-related factors, including the microbiota. Comparably, chitin, a component present in TM larvae is known for its positive effect on inflammation modulation, stimulating cytokine production (Swiatkiewicz et al., 2015; Elieh Ali Komi et al., 2018; Elahi et al., 2022; Malematja et al., 2023). However, in the present study, no marked pro-inflammatory pattern was observed. Comparably, a downregulation of IL-2, another pro-inflammatory mediator, has been reported by Colombino et al. (2021), in poultry fed with live TM, and was attributed to their lower chitin content (Colombino et al., 2021). A similar conclusion can be proposed in the current context, where a relatively low TM dose was administered. An alternative, non-contradictory hypothesis might be attributed to the reduced stimulation of the immune system resulting from the exclusion or limitation of pathogenic agents—possibly induced by antimicrobial peptides (AMPs), competitive exclusion by other bacteria, and short-chain fatty acid production (Zhang et al., 2023).

No remarkable differences were observed in the microbiome among the different diets in terms of alpha diversity (i.e., within-sample variability), and only minimal differences were detected in beta diversity (i.e., between-group variability). The proximal intestinal tract was dominated by *Firmicutes*, particularly *Lactobacillaceae*, *Clostridiaceae*, and *Peptostreptococcaceae* in the ileum, although some inter-individual variability and the presence of other bacterial groups were also observed; Proteobacteria were represented mainly by *Enterobacteriaceae*, in line with other studies (Oakley et al., 2014). As expected, the caecum displayed a much higher microbial heterogeneity (Rychlik, 2020). However, differently from what is often reported, Firmicutes remained largely dominant. Previous studies have shown that in the caecal microbiota of healthy adult hens, Gram-positive Firmicutes and Gram-negative Bacteroidetes are usually equally represented, each accounting for approximately 45% of the total microbial community (Rychlik, 2020). In the present study, regardless of the diet, Firmicutes still represent approximately 95% of the detected species. The analysis of rarefaction curves indicated adequate coverage of the taxa at the sequencing depth considered, supporting the reliability of the observed microbial composition rather than suggesting an artifact. Nevertheless, microbiome composition appears variable and other studies have also reported a much lower contribution of Bacteroidetes to the caecal microbiota (Wei et al., 2013; Oakley et al., 2014; Huang et al., 2021; Yin et al., 2023). Moreover, age, chicken genotype, geographical origin, and diet related factor, as well as colonization pathways, play a role (Singh et al., 2014; Siegerstetter et al., 2017; Huang et al., 2021; Yin et al., 2023). It has been reported that relative abundance shifts between Firmicutes and Bacteroidetes may be linked to feed efficiency. In humans, the Firmicutes/Bacteroidetes (F/B) ratio has been correlated with obesity (Bervoets et al., 2013) and a higher Firmicutes/Bacteroidetes ratio has been observed in poultry exhibiting greater feed conversion efficiency (Huang et al., 2021). Firmicutes are known to degrade host-indigestible polysaccharides, thereby promoting nutrient digestion and absorption. The balance among different taxa determines the overall metabolic output, including the production of short-chain fatty acids (SCFAs), which have a profound impact on host metabolism and physiology. Firmicutes are mainly butyrate producers, contributing to a butyrate-dominant energy supply in the gut. Lachnospiraceae family, significantly present in the caeca, is a well-known butyrate producer, along with *Ruminococcaceae* (Biasato et al., 2020). Accordingly, Loponte et al. (2019) found an increased amount of total VFAs (+45.6%), acetate (+40.3%), and butyrate (+64.6%) in broilers fed a TM larvae meal as a complete replacement of soybean meal (Loponte et al., 2019). Butyrate is considered the primary energy source for enterocytes (Bovera et al., 2011) and is also essential for the proper development of Gut-Associated Lymphoid Tissue (GALT), which may have contributed to the well-developed intestinal mucosa observed in this study. Moreover, van der Wielen et al. (2000) reported that increased butyrate concentrations were associated with reduced levels of Enterobacteriaceae (van der Wielen et al., 2000) and the high concentration of butyrate produced in the caeca of broilers fed the TD diet may play a key role in inhibiting E. coli and Salmonella colonization. Therefore, the detected microbiome composition can be considered beneficial for both animal and, indirectly, human health.

Among the individual taxonomic groups that were differentially represented compared to the diet control, Peptostreptococcaceae were significantly overrepresented in both the OD and TD diets. Peptostreptococcaceae contributes substantially to the butyrate pool in the chicken caecum. Several studies have reported that increased intestinal butyrate levels enhance gut barrier function, as evidenced by reduced translocation of bacterial endotoxins (LPS) from the lumen into the bloodstream and improved mucosal regenerative capacity. An anti-inflammatory effect has also been reported (Zhang et al., 2023). Desulfotomaculaceae, which were more abundant in the control diet group compared to the other experimental groups, produce hydrogen sulfide (H_2S) as the final product of their metabolism. At high concentrations, H_2S is cytotoxic to intestinal epithelial cells, as it inhibits mitochondrial enzymes and can induce apoptosis or cell death. An increase in Desulfotomaculaceae in the poultry microbiota is generally considered unfavorable from an immunological perspective, as it tends to promote a pro-inflammatory state (Onrust et al., 2015). Therefore, the decrease in this population in OD—and in TD group compared to the control, although not reaching statistical significance—might have contributed to the observed anti-apoptotic status, the reduced expression of some pro-inflammatory cytokines, and the higher levels of the anti-inflammatory IL-10. However, a higher presence of this taxon in TD compared to OD was also observed, suggesting that they only play a part in the overall regulation of caecum health and immunity regulation. Overall, the complexity of the potentially underlying determinants and the presence of multiple confounding factors require caution in the assessment of causal associationns. Finally,

a reduction in *Enterobacteriaceae* was observed in the OD group compared to the control. This family, belonging to the phylum *Proteobacteria*, includes Gram-negative facultative anaerobes such as *Escherichia coli*, *Salmonella*, *Klebsiella*, and other genera relevant to the poultry microbiota. Unlike many obligate anaerobes, they do not produce appreciable amounts of propionate or butyrate. Consequently, a bloom of *Enterobacteriaceae* in the caecum does not enhance the production of short-chain fatty acids (SCFAs) beneficial to the host; on the contrary, it is often associated with a reduction in butyrate-producing fermenters and decreased energetic yield from the diet. *Enterobacteriaceae* are also known to exert predominantly pro-inflammatory effects in the intestinal tract. Their lipopolysaccharide (LPS) is among the most potent immune activators (Xi et al., 2023). Therefore, a reduction in their abundance might be considered beneficial and desirable.

Overall, the inclusion of oilseed cakes significantly reduced the digestibility of most nutrients, likely due to the higher fiber content and the presence of residual antinutritional factors which can interfere with nutrient absorption. In contrast, the inclusion of TM larvae affected mainly CPd. These findings suggest that the levels of inclusion of such raw materials may need to be carefully evaluated to avoid negative effects on nutrient digestibility. Despite these reductions, the camelina and linseed-based diet, led to improvements in intestinal morphometry and seems to positively affect immune parameters. Similarly, the TM-based diet appeared to promote a lower inflammatory status. Although no striking changes were observed in the microbiome composition, the main differences included an increase in taxonomic groups associated with butyric acid production in the experimental diets compared to control, along with a reduction in less beneficial or potentially pathogenic bacteria. Therefore, a likely combination of increased availability of antioxidants, polyunsaturated fatty acids (PUFAs), and other bioactive compounds—together with a modest modulation of the microbiome and its short-chain fatty acid (SCFA) metabolism—may have contributed to enhanced intestinal functionality and modulation of immune-related gene expression. These findings, combined with broader socio-economic considerations, suggest that such raw ingredients may offer advantages for poultry health and, indirectly, for human health, supporting their suitability for more consistent inclusion in poultry feed.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/sra, PRJNA1393451.

Ethics statement

The animal study was approved by Ethical Committee for Animal Experimentation (Organismo Preposto al Benessere degli Animali, OPBA) of the University of Padova, Italy (project 49/2024; Prot. n. 100806 approved on May 2024). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

GF: Data curation, Writing – review & editing, Validation, Conceptualization, Software, Methodology, Funding acquisition, Resources, Formal analysis, Writing – original draft, Project administration, Visualization. MB: Resources, Writing – original draft, Writing – review & editing, Formal analysis, Conceptualization, Project administration, Validation, Data curation, Investigation, Supervision. BP: Writing – review & editing, Investigation, Writing – original draft. GD: Formal analysis, Writing – review & editing. MCe: Writing – review & editing, Investigation. LN: Investigation, Writing – review & editing. MCu: Writing – review & editing, Investigation. EP: Investigation, Writing – review & editing. ADZ: Methodology, Project administration, Validation, Supervision, Conceptualization, Writing – original draft, Investigation, Funding acquisition, Writing – review & editing. DP: Investigation, Conceptualization, Writing – review & editing, Funding acquisition.

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Conflict of interest

Author LN was employed by company Private Veterinary Laboratory "MyLav".

The remaining author(s) declared that this work 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fanim.2026.1778929/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Relative abundance of bacterial phyla in the intestinal microbiota of broilers fed different diets. Stacked bar plots represent the taxonomic composition (at phylum level) in the jejunum, ileum, and caecum of chickens receiving the control (CD), Camelina sativa–based (OD), or Tenebrio molitor–based (TD) diets.

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