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Effects of *Tenebrio molitor* larvae meal inclusion in rainbow trout feed: myogenesis-related gene expression and histomorphological features

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ABSTRACT

The effects of *Tenebrio molitor* larvae meal (TM) dietary inclusion in rainbow trout diet were evaluated on muscular growth using gene expression and histomorphological features of liver, spleen, kidney, anterior and posterior gut through histopathological analyses. Two hundred fifty-two grow-out rainbow trout were fed four experimental diets containing increasing levels of TM: 0% (TM0), 5% (TM25), 10% (TM50), and 20% (TM100) corresponding to different levels of fish meal replacement (0, 25, 50, and 100%, respectively). Muscular growth was evaluated analysing the expression of various genes involved in different steps of myogenesis. Among the analysed genes, only MyoD expression resulted significantly higher in fish fed TM100 compared to fish fed TM0. The gut histomorphology was not affected by TM dietary inclusion and villus height differs from anterior and posterior segments regardless of the fed diet. Histopathological alterations were observed in all the sampled organs for all the dietary treatments; however, dietary TM inclusion did not influence either development or severity of the observed histopathological changes. The results obtained confirmed the safe utilisation of TM as an alternative protein source in rainbow trout diets and highlighted the necessity to deepen the studies of TM effect on the myogenesis process, especially at a molecular level.

HIGHLIGHTS

- Rainbow trout can effectively be fed with a TM protein source.
- Total FM substitution by TM (TM100) decreased MyoD gene expression.
- Increasing TM dietary inclusion did not influence gut histomorphology.

Introduction

In recent years, a growing interest in insect meal as an alternative protein source in aquafeed has promoted investigations and related scientific production. The implications on growth, nutrient digestibility, fillet quality, and sensorial perception (Borgogno et al. 2017; Gasco et al. 2018; Lock et al. 2018; Ferrer Llagostera et al. 2019; Chemello et al. 2020; Caimi et al. 2020b), as well as the effects on gut health, immune system, and microbiota, have all been analysed in different marine and freshwater species (Elia et al. 2018; Henry et al. 2018a, 2018b; Antonopoulou et al. 2019; Rimoldi et al. 2019; Roques et al. 2020; Caimi et al. 2020a). The assessment of animal health status represents a crucial aspect when a novel feed ingredient is tested for its introduction in commercial

fish farming. Moreover, optimal growth performance is one of the features that reflects fish health and still represents a crucial aspect for the farmers.

Fish growth consists of muscle requirements. In most teleosts, the axial skeletal muscles represent more than 60% of body mass. Up to 90% of these muscles mainly consist of deep fast white fibres covered by a thin superficial layer of slow red muscle fibres are located under the skin, with an intermediate layer of pink fibres between them (Alami-Durante et al. 2010a). Changes in the somatic growth of fish are thus mainly attributable to changes in total white muscle growth, which is the result of muscle hypertrophy (i.e. increase in the size of existing muscle fibres) and/or hyperplasia (i.e. recruitment of new muscle fibres). In rainbow trout (*Oncorhynchus mykiss*),

as well as in other teleost species reaching a large adult body size, the myogenesis of white skeletal muscle occurs by both hyperplasia and hypertrophy not only before hatching but also over a large extent of post-hatching life (Rowlerson and Veggetti 2001).

The different steps of myogenesis, such as the commitment of muscle precursors, myoblast proliferation, migration, alignment, and fusion into myotubes – are controlled by the expression of different regulatory factors, including myogenic regulatory factors (MRFs) and growth factors. Therefore, identifying and studying these factors is of utmost importance to understand muscle development and, in turn, fish growth.

MRFs are selectively expressed during the determination, differentiation, activation, and proliferation of satellite cells (also referred to as myogenic precursor cells, MPCs) and in cells entering the terminal differentiation program. Primary myogenic regulatory factors, such as *MyoD* and *Myf-5*, play key roles in the specification of muscle lineage; while the secondary myogenic regulatory factors, myogenin (*Myog*) and *Myf-6*, control the differentiation of myoblasts into skeletal muscle fibres. Proliferative muscle precursor cells also express *PCNA* (proliferating cell nuclear antigen), which acts as a cofactor of DNA polymerase-d and is necessary for cell-cycle progression and cell proliferation (Sabourin et al. 1999; Sabourin and Rudnicki 2000; Buckingham and Rigby 2014).

The complex mechanism of myogenesis varies between families and strains and it is influenced by different environmental factors, including the diet (Johnston 1999). To date, the effects of nutrients on white muscle development have been studied mainly in fish larvae and juveniles (Bjornevik et al. 2003; Ostaszewska et al. 2008; Silva et al. 2009; Alami-Durante et al. 2014; Vo et al. 2016; Canada et al. 2018; Alami-Durante et al. 2019). In particular, changes in dietary macronutrient source and composition have been reported to alter the mRNA level of myosin components in rainbow trout alevins and juveniles (Alami-Durante et al. 2014), in Atlantic salmon (Hevrøy et al. 2006), and Senegalese sole juveniles (Valente et al. 2016). In rainbow trout juveniles, the reduction of growth performances in animals fed a high level of plant protein sources has been linked to changes in the dynamics of white muscle growth and correlated with the differential expression of the Cathepsin D, a gene involved in lysosomal proteolysis in the white muscle (Alami-Durante et al. 2010a). Furthermore, white muscle cellularity (distribution of skeletal white muscle fibre size and diameter) has been influenced by changes in dietary protein source and amino acid

supplementation in fish juveniles (Alami-Durante et al. 2010b; Valente et al. 2016), as well as by changes in the dietary protein level (Alami-Durante et al. 2019). Unfortunately, the understanding of myogenesis regulation by specific nutrients in grown-out fish and the effects on the related genes is poorly documented or even absent, especially if the use of insect meal as an alternative protein source is considered. To our knowledge, no information on the effects of insect dietary inclusion on the expression of myogenesis regulatory genes in fish is currently available.

Since the digestion of the administered nutrients represents the first step of protein absorption and utilisation, the intestine plays a key role when novel protein sources are included in fish diets. Indeed, gut health status is the main determinant of fish health and performance (Caimi et al. 2020a, 2020b). Therefore, the parallel evaluation of selected mucosal morphometric features (such as the villus height) and inflammatory cell infiltration – along with the classical histological examination of the main organs – has nowadays become a routine approach. Previous studies have already assessed the effects of dietary *Hermetia illucens* meal inclusion on gut morphology and histological features of the main organs in rainbow trout (Renna et al. 2017; Elia et al. 2018), but data about the modulation of such aspects by TM utilisation in this fish species are still lacking.

Based on the above-reported background, the present study described, for the first time, the effects of total fish meal (FM) replacement by TM on MRFs gene expression in muscle tissue and both partial and total FM substitution by TM on histomorphological features in liver, spleen, kidney, anterior and posterior gut of rainbow trout. Our research aims to provide preliminary information on the process that coordinates muscle growth at the molecular level and the structural tissue integrity of fish fed with different dietary TM levels.

Materials and methods

All the samples analysed in this study were collected and processed at the end of a growth trial carried out at the Experimental Facility of the Department of Agricultural, Forest, and Food Sciences (DISAFA) of the University of Turin (Italy). The experimental protocol was approved by the Ethical Committee of the University of Turin (protocol n° 143811).

The experimental protocol, as well as diet preparation, ingredients, and proximate composition of the experimental diets, were thoroughly described in our

previous publication (Chemello et al. 2020). A summary illustrating diet features, and the experimental protocol is described below.

Fish diets

A partially defatted *Tenebrio molitor* larvae meal (TM) provided by Ÿnsect (Evry, France) was used to formulate four isonitrogenous (CP: about 42.5% as fed), isolipidic (EE: about 24.2% as fed), and isoenergetic (GE: about 23.8 MJ/kg) experimental diets based on a control diet containing 20% of FM. The extruded diets were prepared including, as fed basis, increasing levels of a partially defatted TM (0% – TM0, 5% – TM25, 10% – TM50, and 20% – TM100) in substitution of 0, 25, 50, and 100% of FM. The experimental diets were prepared by SPAROS LDA (Olhão, Portugal) as extruded pellets.

Because of the different chemical compositions of TM and FM, the amounts of some other dietary ingredients (i.e. wheat gluten, wheat meal, and sardine oil) were slightly modified with the dietary increase of TM inclusion (Table 1). Moreover, to ensure that the experimental diets fulfil the rainbow trout essential AA requirements, amino acid (AA) supplementation was included in diets. Details on the chemical composition of diets have been reported by Chemello et al. (2020).

Sampling

Two hundred and fifty-two grow-out rainbow trout (purchased from a private fish hatchery 'Troticoltura Bassignana'; Cuneo, Italy) were slightly anaesthetised (MS-222, PHARMAQ Ltd, UK; 60 mg/L), individually

weighed (78.3 ± 6.24 g) and randomly distributed into 12 400-L tanks (three replicate tanks per diet, 21 fish per tank).

At the end of a 154-day trial, spleen, kidney, liver, anterior and posterior intestine were sampled from 15 fish per each experimental diet (five fish per tank) for the histomorphological analysis. Simultaneously, fish for the MRFs gene expression analysis were randomly selected from tanks of the two extreme diets: TM0 and TM100 (100% FM and 100% TM, respectively) and killed by over anaesthesia (MS-222; PHARMAQ Ltd., UK; 500 mg/L). The same portion of dorsal white muscle was collected just below the dorsal fin of the left side (1 cm^3) from each fish and stored in RNA-later (Sigma–Aldrich, Milano, Italy) for molecular analysis, according to manufacturer's guidelines.

Molecular analysis

RNA extraction and cDNA synthesis

Total RNA was extracted from rainbow trout dorsal muscle using Trizol (TRIzol^{TM} reagent, Thermo Fisher Scientific) and following the manufacturer's protocol. The isolated RNA was eluted in 60 μl RNase-free water (Sigma Aldrich) and treated with DNase I (Thermo Fisher Scientific) to remove potential DNA contamination. Final RNA concentration and quality were assessed as OD$_{260/280}$ ratio (≥ 2.0) by Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Reverse transcription was performed in a final reaction volume of 20 μl starting from 50 ng of total RNA using the Verso cDNA synthesis kit (Thermo Fisher Scientific) and following the producer's instructions. A mixture of random hexamer (400 ng/μl) and anchored oligo dT (500 ng/μl) in the ratio 3:1 was used to provide a flexible RNA priming method for cDNA synthesis. RT enhancer was also included in the cDNA reaction mixture to ensure the total lack of contaminating DNAs not degraded by DNAse I treatment.

Real-time PCR

Quantitative PCR was performed according to Gu et al. (2020) using 2X QuantiTect SYBR Green PCR Master Mix in presence of ROX passive reference dye (Qiagen), 5 ng/μl of cDNA, and 0.5 μM of each primer. Analyses were carried out with a MiniOpticon Real-time PCR System (Bio-Rad) in triplicate for each RT product.

The thermal profile for all reactions was 3 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at

Table 1. Ingredients (g/kg) of the experimental diets.

	TM0	TM25	TM50	TM100
Fishmeal 65 (Peruvian)	200.00	150.00	100.00	–
Tenebrio molitor larva meal	–	50.00	100.00	200.00
Soy protein concentrate	180.00	180.00	180.00	180.00
Wheat gluten	77.50	74.00	74.00	70.60
Corn gluten	80.00	80.00	80.00	80.00
Soybean meal 48	70.00	70.00	70.00	70.00
Wheat meal	142.30	140.00	142.30	138.00
Sardine oil	43.00	42.60	42.00	41.00
Soybean oil	86.00	85.20	84.00	82.00
Rapeseed oil	86.00	85.20	84.00	82.00
Soy lecithin	5.00	5.00	5.00	5.00
Vit-Min premix	10.00	10.00	10.00	10.00
Antioxidant	2.00	2.00	2.00	2.00
Sodium propionate	1.00	1.00	1.00	1.00
Monocalcium phosphate	5.20	9.20	9.20	17.20
L-arginine	–	–	–	1.00
L-lysine	–	3.00	3.00	6.00
L-tryptophan	0.50	0.80	1.00	1.20
DL-methionine	1.50	2.00	2.50	3.00
Celite®	10.00	10.00	10.00	10.00

TM: *Tenebrio molitor* larvae meal.

Table 2. Primers sequences and accession numbers.

Gene	Accession no.		Sequence 5′ > 3′	Size (bp)	References
MyoD	X75798	For	GATAGCACCTACTTCAACGA	91	Alami-Durante et al. 2010a
		Rev	CCACGATGTTTGACAGGCAG		
Myf5	AY751283.1	For	GCTTACCTTCTCGCCCTCCA	149	Alami-Durante et al. 2014
		Rev	TCAAAGCGGTATGCGGTTGA		
Myf6	EF450079	For	AGCAATTGCAGGACCTGTTG	343	Moghadam et al. 2007
		Rev	GCTGAGGTCTGCCAGTTTTG		
Myog	Z46912	For	CAGTACATTGAGAGGCTGCAGGCA	123	Alami-Durante et al. 2010a
		Rev	CTCACTCGACGACGACACCCTG		
PCNA	BT046966	For	CCACGTCTCCCTAGTCCAGC	198	Alami-Durante et al. 2010a
		Rev	TCCTGGTTGAGTGTCTCAA		
fMHC	Z48794	For	AGAAACTGGAGTCCAGGGTG	114	Gauvry and Fauconneau 1996
		Rev	TGAGCTCCTTGACTCTGCGC		
CathD	U90321	For	GCCTGTCATCACATTCAACCT	102	Alami-Durante et al. 2010a
		Rev	CCACTCAGGCAGATGGTCTTA		
18S	AF308735	For	CGGAGGTTCGAAGACGATCA	62	Alami-Durante et al. 2010a
		Rev	TCGCTAGTTGGCATCGTTTAT		
EF1-α	AF498320.1	For	TCCTCTTGGTCGTTTCGCTG	159	Fontagné-Dicharry et al. 2014
		Rev	ACCCGAGGGACATCCTGTG		

55 °C (annealing) and 30 s at 72 °C (extension). Fluorescence was monitored at the end of each cycle. Amplification specificity was checked using melting curves made from 55 °C to 95 °C by increasing 0.5 °C every 10 s. The genes selected were all important markers of myogenic determination and differentiation [myoblast determination protein (MyoD), myogenic factor 5 (Myf5), myogenic factor 6 (Myf6), and myogenin (Myog)], cell proliferation [proliferative cell nuclear antigen (PCNA)], involved in muscle structure and function [fast-myosin heavy chain (fMHC)] and proteolysis [cathepsin D (cathD)].

Relative quantification of the expression of the selected genes was determined according to Vandesompele et al. (2002) using two [18S and elongation factor1-alpha (EF1-α)] out of three tested housekeeping genes [18S, EF1-α, and β-actin (ACTB)] as endogenous controls and the geometric mean values for normalisation as follows:

$$(E_{GOI})^{\Delta CtGOI}/GeoMean[(E_{REF})^{\Delta CtREF}]$$

where E_{GOI} is the efficiency of the gene of interest; ΔCt GOI is the difference between the threshold cycle of the gene of interest; GeoMean is the geometric mean; E_{REF} is the efficiency of the reference gene; ΔCt REF is the difference between the threshold cycle of the reference gene.

Primer sequences were selected from previous publications (Table 2) and synthesised by Microsynth GmbH (Vienna, Austria).

Histomorphological analysis

Anterior and posterior gut samples of ∼5 cm in length were excised and flushed with 0.9% saline to remove all the content. The liver, spleen, and kidney were also collected. All the samples were fixed in 10% buffered formalin solution, embedded in paraffin wax blocks, sectioned at 5 μm thickness, mounted on glass slides, and stained with Haematoxylin & Eosin for histomorphological investigations.

A total of three serial sections were prepared for each intestinal segment and examined using light microscopy. The same slide among the serial sections was considered for morphometric analysis and then captured with a Nikon DS-Fi1 digital camera coupled to a Zeiss Axiophot microscope using a 2.5× objective lens. NIS-Elements F software was used for image capturing. The villus height (Vh) was evaluated on 10 well-oriented and intact villi per captured field (10 villi/sample) by Image®-Pro Plus software, according to Renna et al. (2017). Following the same procedures firstly adopted by Biasato et al. (2020) in pigs, for each gut segment, the mucosa, submucosa, and muscular layers were also separately assessed for the presence of inflammatory cells infiltrates using a semiquantitative scoring system from 0 (absence of inflammatory cells infiltration) to 3 (severe inflammatory cells infiltration). The total score of each intestinal segment was obtained by adding the mucosa, submucosa, and muscular layer scores, while the total score of each fish was represented by the mean value of the anterior and posterior gut scores. All the slides were blind assessed by three observers and the discordant cases were reviewed, using a multi-head microscope, until unanimous consensus was reached.

Liver, kidney, and spleen sections were also examined using light microscopy to identify histopathological alterations. In particular, a total of three serial sections were prepared for each sampled organ too, with the most representative one per fish being considered for histopathological examination. The observed histopathological findings were evaluated
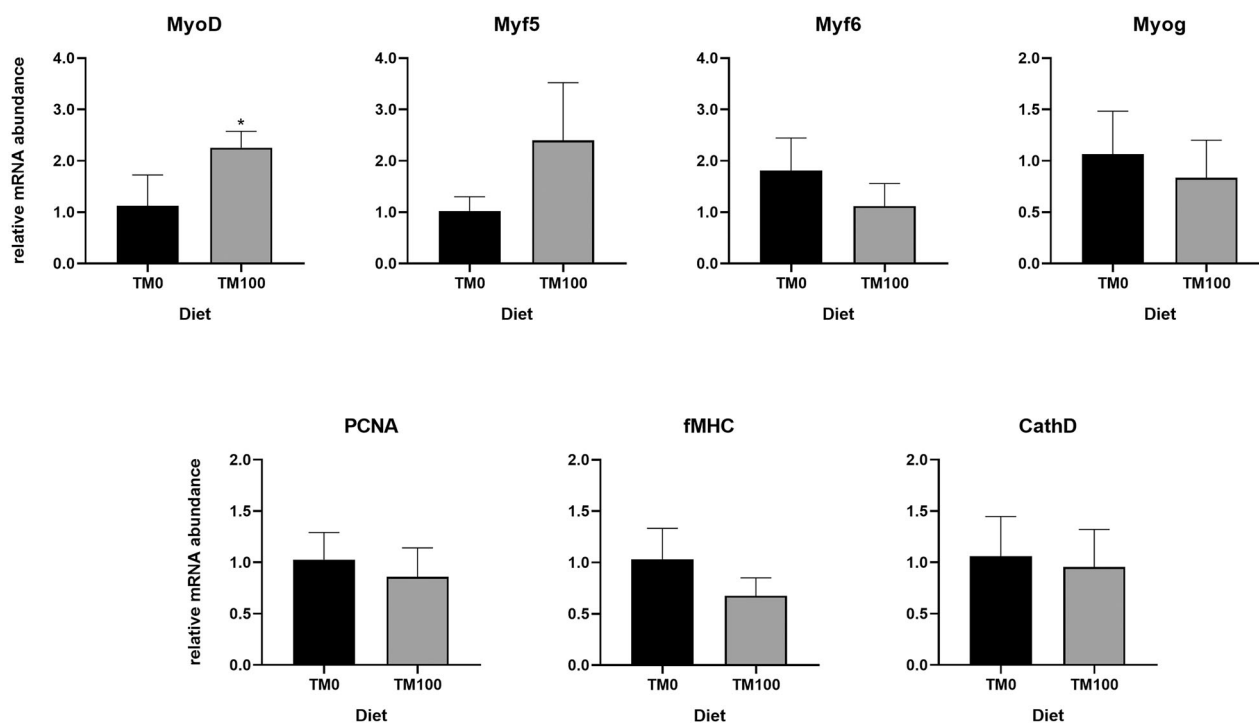
Figure 1. Relative mRNA levels of genes involved in fish myogenesis (*MyoD*, *Myf5*, *Myf6*, *Myog*, and *fMHC*), cell proliferation (*PCNA*), and proteolysis (*cathD*) analysed in the muscles of fish fed with TM0 and TM100. Values are presented as mean ± SEM. Asterisks indicate significant differences ($p \leq 0.05$).

using a semiquantitative scoring system based on their distribution and severity according to Elia et al. (2018): absence of alterations (score 0); focal to multifocal, mild alterations (score 1); multifocal, moderate alterations (score 2); multifocal to diffuse, severe alterations (score 3).

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics v. 25.0 for Windows. The assumption of normality was checked using the Kolmogorov–Smirnov test. Relative quantification of gene expression was analysed by Student's t-test. Levene's homogeneity of variance test was used to assess homoscedasticity. If such an assumption did not hold, the Brown–Forsythe statistic was applied to test the equality of group means instead of the F one.

The intestinal morphometric findings (10 measurements/slide, one slide/gut segment, two gut segments/fish, 15 fish/treatment) were analysed by fitting a general linear mixed model (GLMM). The GLMM allowed the morphometric index (Vh) to depend on three fixed factors (diet, intestinal segment, and the interaction between the diet and intestinal segment). The animal was included as a random effect to account for repeated measurements on the same fish. The interactions between the levels of the fixed

factors were evaluated using pairwise comparisons. The histopathological scores (one score/slide, one slide/organ, four organs/fish, 15 fish/treatment) were analysed using the Kruskal-Wallis test (*post-hoc* test: Dunn's Multiple Comparison Test).

The results were expressed as the mean (molecular and histopathological data) or least square mean (intestinal morphometric findings) and pooled standard error of the mean (SEM). Significance was set at $p \leq 0.05$.

Results

Real-time PCR results

Real-time PCR analysis was performed on genes involved in fish muscle growth. Results showed that total FM replacement with TM (TM100) affected *MyoD* RNA level, which was significantly ($p < .05$) higher in TM100 samples compared to the TM0 control diet (Figure 1). No further differences in gene expression were observed for the other analysed genes.

Histomorphological analysis

The Vh in the gut of the fish (Figure 2) significantly depended on the gut segment ($p < .001$), with the posterior intestine showing higher Vh when compared
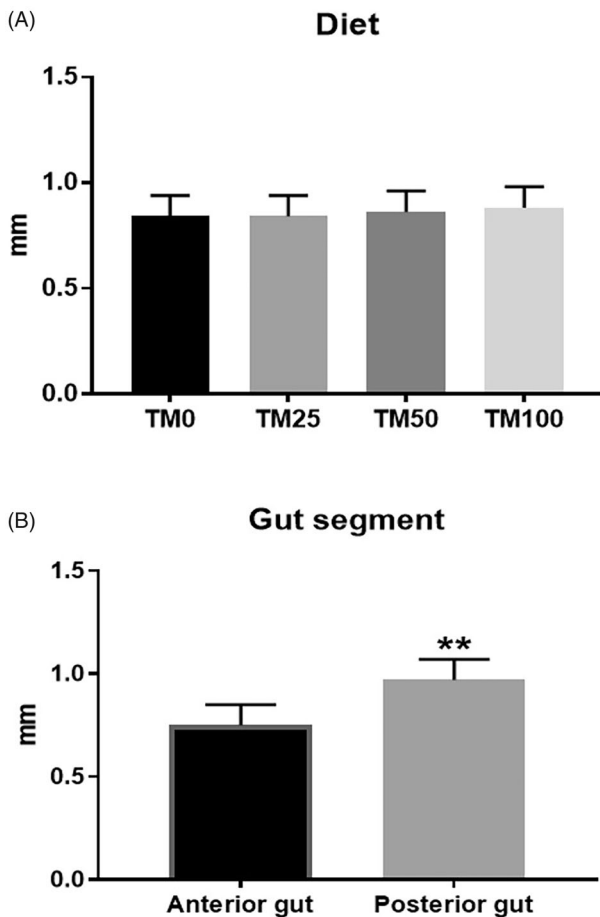
(A)

(B)

Figure 2. Effects of dietary Tenebrio molitor larvae meal (TM) inclusion on rainbow trout gut morphology. (A) Villus height (Vh) (expressed in mm) in the four dietary treatments for the two intestine segments (anterior and posterior). (B) Vh (in mm) in the anterior and posterior intestine, independently of the dietary treatment considered. ** indicates p < 0.001.

to the anterior one ($p < .001$). On the contrary, no influence of dietary TM inclusion or interaction between the diet and the gut segment was observed on the histomorphological findings (Figure 2).

Histopathological alterations were observed in all the sampled organs for all the dietary treatments (Figure 3). Gut showed mild, multifocal mucosal/sub-mucosal lymphoplasmacytic infiltrates. Mild to moderate, multifocal lymphoplasmacytic infiltrates, as well as mild to severe, multifocal to diffuse steatosis were identified in the liver. Kidneys showed mild to moderate, multifocal interstitial lymphoplasmacytic infiltrates, while mild to moderate, focal to multifocal white pulp hyperplasia and haemosiderosis were observed in the spleen. However, dietary TM inclusion did not influence either development or severity of the observed histopathological alterations ($p > .05$, Table 3).

Discussion

Expression of genes involved in muscles growth

Studying the effects of insect meal-containing diets on fish growth is crucial to understanding how nutrients are involved in myogenesis regulation mechanisms.

Data presented in our previous study reporting the results of growth and digestibility trials in rainbow trout revealed that TM dietary inclusion did not negatively affect nutrients digestibility and somatic growth (Chemello et al. 2020). However, the lack of detrimental consequences at the somatic level may not be representative of the molecular dynamics regulating muscle growth (Chapalamadugu et al. 2009; Alami-Durante et al. 2010b). Indeed, the inclusion of 20% TM (TM100) in the rainbow trout diet significantly increased the relative expression of *MyoD*. It has been proved that *MyoD* has a specific role in post-juvenile growth in fish promoting the differentiation of activated satellite cells (Sabourin et al. 1999). Quiescent satellite cells display no detectable levels of MRFs expression, whereas, upon satellite cell activation, *MyoD* expression is rapidly up-regulated before other MRFs involved in the myogenesis process (Sabourin and Rudnicki 2000). Our results may suggest that TM dietary inclusion could have affected *MyoD* expression and indirectly, its regulatory function on satellite cells differentiation into myocytes.

It was established that changes in dietary macronutrients could alter the expression of some MRFs (Chapalamadugu et al. 2009; Campos et al. 2010; Alami-Durante et al. 2010b; Valente et al. 2016). Alami-Durante et al. (2010b) observed reduced levels of *MyoD* expression in rainbow trout fed diet rich in soybean meal; whereas Chapalamadugu et al. (2009) found that rainbow trout fed with 15 and 25% carbohydrate-containing diets had an increased transcription of *MyoD*, *Myf5*, and myogenin.

Unfortunately, neither study identified with certainty the mechanisms behind such alteration of *MyoD* and other MRFs expressions. Alami-Durante et al. (2010b) hypothesised that the decrease of *MyoD* expression may be triggered by the isoflavones normally present in soybean meal that are known to inhibit the proliferation of muscle cells in mammals *in vitro* (Pan et al. 2001). While Chapalamadugu et al. (2009) were not able to clarify whether dietary carbohydrates directly affected *MyoD* expression or if their positive impact on trout growth were mainly due to the protein-sparing effect. The up-regulation of *MyoD* expression has been related to the dietary amino acid deficiency as reported by several studies for different
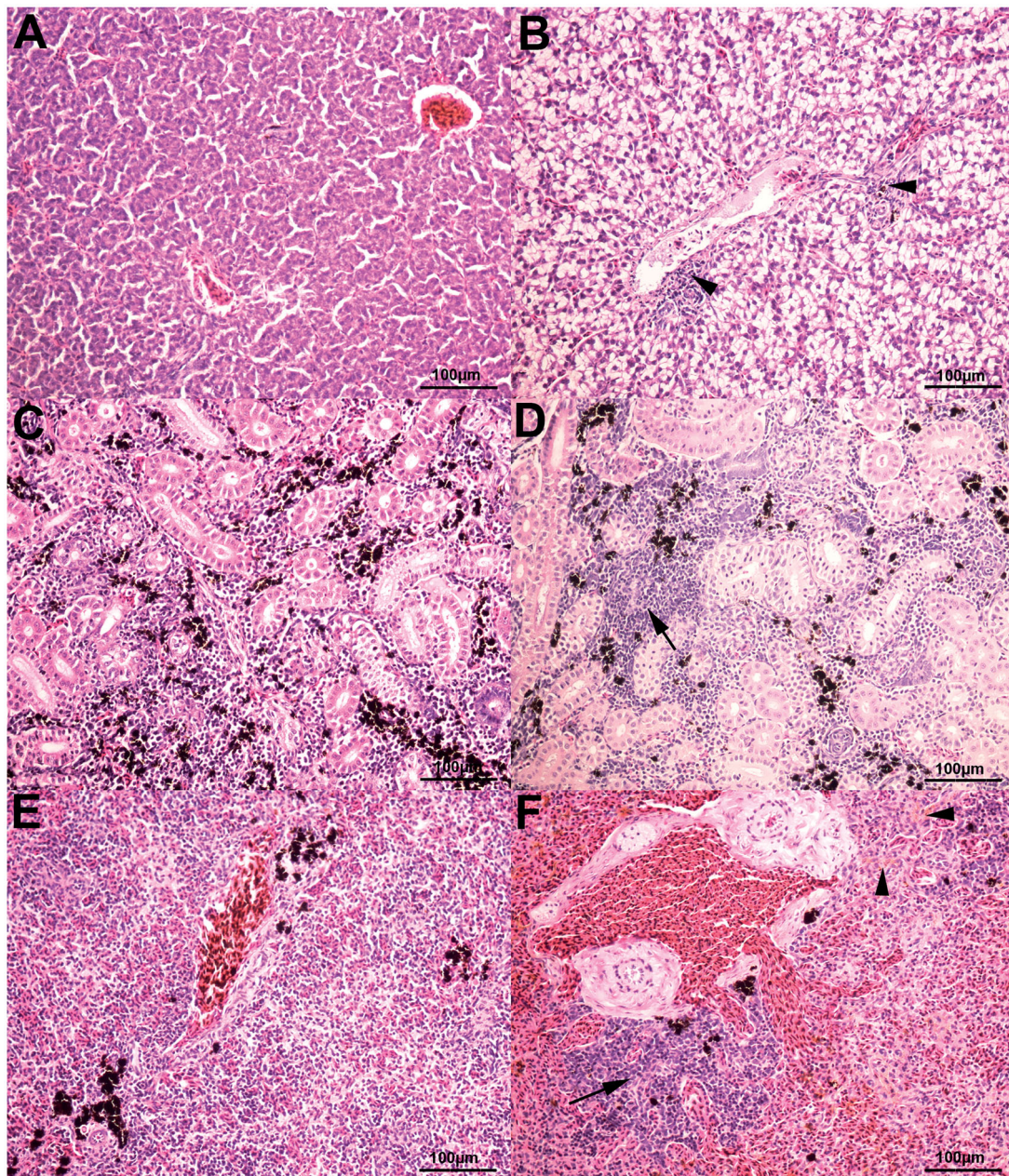
Figure 3. Rainbow trout histological features stained with Haematoxylin & Eosin stain. (A) Normal liver, 20× magnification. TM25 diet. (B) Liver, 20× magnification. Diffuse, moderate to severe steatosis, as well as mild, multifocal lymphoplasmacytic perivascular infiltrates (arrowheads), are observed. TM0 diet. (C) Normal kidney, 20× magnification. TM0 diet. (D) Kidney, 20× magnification. Focal, moderate interstitial lymphoplasmacytic infiltrates (arrow) are identified. TM50 diet. (E) Normal spleen, 20× magnification. TM100 diet. (F) Spleen, 20× magnification. Focal, moderate white pulp hyperplasia (arrow), along with mild, multifocal hemosiderosis (arrowheads), are observed. TM0 diet.

species (Michelato et al. 2017; Alami-Durante et al. 2018; Cai et al. 2018; Alami-Durante et al. 2019). However, the diets administered in our study were formulated to be isonitrogenous, isolipidic, and isoenergetic and amino acid supplementation was added to meet the fish essential amino acid requirement (Chemello et al. 2020). Therefore, the alteration of *MyoD* expression could not be related to an amino

acid deficiency and the role of TM in *MyoD* up-regulation is still unclear and deserves further investigation.

There is documentary evidence that the expression of genes involved in myogenesis is also influenced by feed intake as reported by Alami-Durante et al. (2010b). However, the results described by Chemello et al. (2020) highlighted no differences in the feed intake among the experimental diets.

	TM0	TM25	TM50	TM100	SEM	p
Liver						
Inflammatory cells infiltration	0.00	0.27	0.40	0.33	0.07	.134
Steatosis	1.67	1.90	1.50	1.87	0.10	.311
Kidney						
Inflammatory cells infiltration	0.90	1.00	0.67	0.93	0.10	.599
Spleen						
White pulp hyperplasia	0.10	0.33	0.13	0.20	0.03	.523
Hemosiderosis	0.00	0.00	0.13	0.07	0.06	.284
Intestine						
Inflammatory cells infiltration	0.43	0.53	0.43	0.33	0.06	.555

SEM: standard error of the mean.
The data are expressed as the mean of the scores (0 = absence of alterations; 1 = mild alterations; 2 = moderate alterations; 3 = severe alterations). The gut scores resulted from the mean of the two gut segments scores (in turn obtained from the adding of the mucosa, submucosa and muscular layers scores).

It is also important to specify that the existing literature concerning the effects of nutrients on the myogenesis process has been focussed mainly on alevins and juveniles fish. The grow-out rainbow trout used in our study are most likely characterised by a slower growth rate compared to younger fish and therefore muscle growth may be characterised by different regulatory mechanisms. Finally, it is interesting to consider that our results also highlighted a trend of increased *Myf5* RNA level in fish fed the TM100 diet although there is no evidence of a significant alteration ($p = .08$). As already mentioned, *Myf5* is involved in the first steps of myogenesis together with *MyoD* and myogenin, supporting the proliferation of satellite cells (Chapalamadugu et al. 2009). This observation may sustain the hypothesis of TM potential influence on myogenesis process activation.

The other genes studied—*cathD*, *PCNA*, *Myf6*, *Myog*, and *fMHC*—did not differ between diets, thus suggesting that dietary TM inclusion does not impact the subsequent steps of myogenesis in which these genes are involved.

Gut morphology and histopathological analysis

Dietary TM inclusion did not affect the gut morphology of the rainbow trout of the present study, thus suggesting that insect meal utilisation does not alter the nutrient digestion and absorption processes. This agrees with previous researches assessing the effects of insect-based diets on the intestinal morphometric features of the fish (Renna et al. 2017; Józefiak et al. 2019; Caimi et al. 2020a). Indeed, unaffected Vh was observed in rainbow trout (Renna et al. 2017) and juvenile Siberian sturgeon (Józefiak et al. 2019; Caimi et al. 2020a) fed different inclusion levels of HI (20–40%, Renna et al. 2017; 18.5–37.5%, Caimi et al.

2020b ; 15%, Józefiak et al. 2019) or TM (15%, Józefiak et al. 2019) meals when compared to the control diets. The identification of unaffected gut morphology is also in line with the unaffected growth performance previously reported by Chemello et al. (2020), as a confirmation of the already mentioned influence of the gut health status on the animal performance.

Independently of TM utilisation, the posterior intestine also showed longer villi than the anterior segment. This appears to be in contrast with Khojasteh et al. (2009), which reported progressively shorter villi towards the posterior intestine. However, Verdile et al. (2020) recently reported a detailed characterisation of the epithelial cells lining the intestinal mucosa in rainbow trout, simultaneously observing short and long villi in both the anterior and the posterior gut. The same authors also reported that the long villi length in the proximal intestine increased when the fish reached their highest weight (with the short villi length remaining constant), while the distal intestine showed similar villi length along with the fish development (Verdile et al. 2020). Therefore, the identification of differences between the two gut compartments may be related to either the simultaneous presence of short and long villi or the villi length changes throughout the fish cycle. Another interesting aspect to underline is that Verdile et al. (2020) also observed a higher mucosa volume in the posterior than in the anterior intestine, thus herein suggesting a potential role of the villi (which are the major component of the gut mucosa) that deserves future investigations.

Dietary TM inclusion did not influence the histological features of the gut, the liver, the kidney, and the spleen of the fish of the current trial, thus confirming that TM meal utilisation up to 20% of dietary inclusion does not cause the development of specific pathologies. Indeed, the histopathological alterations were identified in both the control- and the TM-fed fish, also resulting to be predominantly mild to moderate. Furthermore, similar histopathological findings were also observed in rainbow trout fed either the FM- or the HI-based diets (Elia et al. 2018). The identification of histopathological alterations in the control group is not indicative of altered health status of the fish, as liver, gut, and spleen lesions were considered paraphysiological (attributable to the intensive feeding and farming of rainbow trout), and kidney findings were too non-specific to be related to any infectious and/or metabolic disorders. Furthermore, the fish fed the basal diet more than triplicated their initial weight, also displaying a condition's factor indicative of a good physiological state of well-being, and either the

hepatosomatic index or the hepatic enzymatic activity suggestive of unaltered liver functionality (Chemello et al. 2020).

Conclusions

The present study reported, for the first time, preliminary results describing the influence of TM dietary inclusion on rainbow trout myogenesis. Although the mechanism through which TM influenced the expression of *MyoD* and satellite cell activation needs to be further analysed, we obtained a first insight into the complex mechanism that regulates fish myogenesis. These results may represent the starting point for further studies that could consider the muscle growth dynamics (such as distribution of muscle fibres size) through histomorphological analyses. Moreover, after observing *MyoD* up-regulation in fish fed TM100, it will be interesting to detect the threshold level of TM dietary inclusion that affects *MyoD* expression and may influence muscle morphology.

Finally, our results highlighted the absence of variation in gut morphology and other organs (liver, kidney, and spleen) histopathology, confirming the safe utilisation of TM as an alternative protein source in rainbow trout diets.

Ethical approval

The experimental protocol was approved by the Ethical Committee of University of Turin (protocol n° 143811) and was designed according to European and Italian guidelines on the care and use of experimental animals (European directive 86 609/EEC, put into 119 law in Italy with D.L. 116/92).

Disclosure statement

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

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