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Dietary *Tenebrio molitor* larvae meal effects on cellular stress responses, antioxidant status and intermediate metabolism of *Oncorhynchus mykiss*

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Abstract

In the context of evaluating the impact of environmentally friendly and sustainably produced alternative protein sources in fish feed, the present study's aim was to examine the overall physiological stress response in one of the main fish species of European freshwater aquaculture, *Oncorhynchus mykiss* (rainbow trout), following the partial substitution of fish meal (FM) with a *Tenebrio molitor* (TM) (yellow mealworm) full-fat meal. In total, 222 rainbow trout individuals (115.2 ± 14.2 g) were allocated randomly into six tanks, three per dietary treatment, and were fed a formulated diet containing 60% yellow mealworm (TM60) compared to a control diet without insect meal (TM0). Both diets contained equal amounts of crude protein, dry matter and, lipid content, while the FM in TM60 was 100 g kg^{-1} corresponding to the one seventh of the TM0. Heat shock response (HSR), MAPK signalling, cell death pathways (apoptosis and autophagy), antioxidant defence mechanisms, and intermediate metabolism were evaluated. In general, HSR and MAPK signalling were activated in response to the inclusion of *T. molitor*. Moreover, triggering of apoptotic and autophagic processes and the onset of antioxidant defence mechanisms underlined the existence of physiological stress. Despite the apparent dietary-induced stress, rainbow trout in the present study exhibited no mortality and no significant effects regarding growth performance parameters. Specifically, TM60 dietary inclusion resulted in no changes in final body weight, weight gain, and specific growth rate. However, feed intake depicted a statistically significant decrease in TM60 fish compared to TM0 individuals. Nevertheless, nutrient stress should be considered a limiting factor regarding the utilization of *T. molitor* in *O. mykiss* diet due to the associated risks for health and welfare.

KEYWORDS

antioxidant defence, cell death, cell signalling, insect meal, metabolism

1 | INTRODUCTION

To date, there is a necessity for nutritionally appropriate, environmentally friendly, and sustainably produced alternatives to fish meal (FM) (Andreadis et al., 2021; Antonopoulou et al., 2022; Ferrer et al., 2019) to alleviate the constant exploitation of rapidly depleting wild fish stocks (FAO, 2020). Despite the nutrient-driven beneficial effects of fishmeal such as the improvement of feed efficiency, growth, and nutrient uptake, the price increase is inconsistent with the establishment of an economic and sustainable aquaculture (Hodar et al., 2020; Rombenso et al., 2013). However, exploring fishmeal alternatives is a complicated challenge considering that nutritional requirements of farmed species such as essential amino acids, lipids (especially polyunsaturated fatty acids), vitamins (e.g., vitamin C), and minerals must be met, and the required substitution level is often species-specific (Gasco et al., 2018; Hodar et al., 2020; Lovell, 1991).

The utilization of processed insect protein in aquafeeds resulting from reinforced scientific enquiry and validation studies regarding the insect meal's positive impact on fish, has recently been authorized by the EU (Commission Regulation EU, 2017). Insects constitute part of the feeding habit of several carnivorous and omnivorous fish species, as they provide adequate energy, macronutrients such as proteins and lipids, and micronutrients such as minerals and vitamins (Nogales-Mérida et al., 2019). Besides their high nutritional profile, insects are advantageous in terms of economic mass production due to the limited requirements of arable land and necessary energy, as well as the low greenhouse gas emissions associated with their production (Gasco et al., 2020; Oonincx & De Boer, 2012; Oonincx et al., 2010). In this context, the production and utilization of insects as protein sources contribute to the mitigation of both climate change and its environmental footprint. Furthermore, insects are able to efficiently convert miscellaneous substrates, including industrial and agricultural side flows and products into high-quality food and feed (Andreadis et al., 2021; Chia et al., 2019; Van Huis & Oonincx, 2017), which fit within the circular economy concept for the sustainable reduction of waste release (Jurgilevich et al., 2016). However, insects' inclusion in fish diet may be accompanied by limitations such as the fatty acid profile which highly depends on substrate composition and the presence of chitin in the exoskeleton (Henry et al., 2015; Makkar et al., 2014). Although the latter has been reported to prevent the enzymatic digestion and absorption of proteins and lipids, thus leading to a reduction in nutrient utilization and growth (Shiau & Yu, 1999), several studies have reported positive results related to dietary chitin and fish growth performance (reviewed in Henry et al., 2015).

The yellow mealworm (*Tenebrio molitor*) (Linnaeus, 1758), an insect species authorized by the recent EU regulation, has been studied for its suitability as fish feeds' ingredient in relation to various growth and welfare parameters in several reared fish species, including *Oncorhynchus mykiss* (Walbaum, 1792) (Chemello et al., 2020; Mastoraki et al., 2020, 2022; Panteli et al., 2021; Tran et al., 2015). The dietary inclusion of yellow mealworm (TM) generally generated positive effects on fish physiological parameters, such as body composition and growth performance (Mastoraki et al., 2020, 2022), However, it has been demonstrated that high inclusion levels (30%–50% TM) generate adverse effects on the digestibility of nutrients and growth rate (Piccolo et al., 2017; Sankian et al., 2018). Nevertheless, it should be highlighted that alterations in fish feed composition may lead to dietary deficiencies. The latter can subsequently lead to oxidative stress, which in turn triggers several signalling pathways and cellular processes (Lin & Shiau, 2007) such as protein denaturation, lipid peroxidation (and subsequent antioxidant defence), heat shock response (HSR), activation of members of the mitogen-activated protein kinases (MAPK) family, and cell death mediated by apoptosis, thus causing an overall deficit in fish physiological performance (Antonopoulou et al., 2017; Martínez-Álvarez et al., 2005).

Research regarding FM partial substitution with insect meal only continues to deepen, and several studies have explored the effects of yellow mealworm dietary inclusion on several parameters of rainbow trout including growth performance, nutrient digestibility, fillet chemical (e.g., amino acid) composition, and microbiota (e.g. Belforti et al., 2015; Chemello et al., 2020; Terova et al., 2021); however, the nature of this species' molecular, biochemical, and physiological responses in correlation to this supplementation remains to be elucidated. The latter is necessary due to the species- and tissue-specific responses to the insect meal (Bousdras et al., 2022; Mente et al., 2022). Several TM substitution levels have been examined (25%–100%) in rainbow trout in terms of growth and animal performance, nutrient digestibility, metabolic responses, and gut and skin microbiota, generally concluding that TM substitution over 50% affects the aforementioned physiological performances (Belforti et al., 2015; Chemello et al., 2020; Terova et al., 2021). Under this prism, the aim of the present study was to investigate the underlying molecular mechanisms when rainbow trout are fed with a 60% TM inclusion. The present study's results are valuable regarding insect meal utilization in the rearing and welfare of *O. mykiss*, one of the main freshwater fish species with significant market value reared in European aquaculture (Vasdravanidis et al., 2022).

2 | METHODOLOGY

2.1 | Dietary experiment

Detailed information regarding the dietary experiment is found in Mente et al. (2022). Briefly, a full-fat meal of *Tenebrio molitor* (TM) larvae was acquired from Gaobeidian Shannong Biology. The experimental facility of the Department of Agricultural, Forest and Food Sciences of the University of Turin in Italy housed the present trial (DGSFA 0019960-P) (02/11/2012), in which a control diet without TM (0%—TM0) and a high TM (60%—TM60) diet were formulated. The two experimental diets' ingredients are presented in Table 1 and full details are given in Mente et al. (2022), while the amino acid profile of the diets is provided in Table 2. The TM fatty acid profile is reported by Gasco et al. (2016). All ingredients were ground (0.5 mm sieve), mixed with fish oil, and later with water to ensure a proper pelleting

TABLE 1 Formulation and composition of the two experimental diets.

	TM0	TM60
Ingredients (g kg^{-1})		
Herring fish meal[a]	700	100
TM[b]	0	600
Cod liver oil	150	53
Corn gluten meal	0	37
Wheat meal	40	40
Wheat bran	57	50
Gelatinized starch, D500	33	100
Mineral mixture[c]	10	10
Vitamin mixture[d]	10	10
Proximate composition[e]		
DM (g 100 g^{-1})	95.6	94.9
CP (g 100 g^{-1}, as fed)	42.4	41.3
EE (g 100 g^{-1}, as fed)	21.3	21.1
Ash (g 100 g^{-1}, as fed)	10.7	8.2
Gross energy (MJ kg^{-1}, as fed)	22.8	23.1

Abbreviations: CP, crude protein; DM, dry matter; EE, ether extract.

[a]Herring fish meal purchased by FF Skagen A/S.

[b]*Tenebrio molitor* larvae meal purchased from Gaobeidian Shannong Biology.

[c]Mineral mixture (g or mg kg^{-1} diet): dicalcium phosphate, 500 g; calcium carbonate, 215 g; sodium salt 40 g; potassium chloride, 90 g; magnesium chloride, 124 g; magnesium carbonate, 124 g; iron sulphate, 20 g; zinc sulphate, 4 g; copper sulphate, 3 g; potassium iodide, 4 mg; cobalt sulphate, 20 mg; manganese sulphate, 3 g; and sodium fluoride, 1 g (purchased from Granda Zootecnica).

[d]Vitamin mixture (U or mg kg^{-1} diet): DL-α tocopherol acetate, 60 U; sodium menadione bisulphate, 5 mg; retinyl acetate, 15,000 U; DL-cholecalciferol, 3000 U; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg; and choline chloride, 2000 mg (purchased from Granda Zootecnica).

[e]Values are reported as means of duplicate analyses.

consistency. A 3.5 mm grinder was used for obtaining the pellets which were subsequently dried (50°C, 48 h).

To balance nutrients and energy while partially substituting FM with TM meal, the amounts of some other ingredients needed to be altered (e.g., starch which was gelatinized to increase its digestibility; Romano & Kumar, 2019).

2.2 | Sampling

A total of 222 rainbow trout (*O. mykiss*) individuals were purchased from a private fish hatchery (Troticoltura Bassignana). They were individually weighed [115.2 ± 14.2, mean ± standard deviation—the initial body weight was chosen to allow us to obtain fish of commercial size (about 350 g) at the end of the trial] and were divided randomly into six fibreglass tanks with the following dimensions: width 0.5 m; length 0.5 m; height 0.4 m. The tanks were filled with water from an artesian well (13 ± 1°C, mean ± standard deviation), and water inflow was maintained at 8 L min^{-1} due to an open water (flow-throughout) system throughout the trial. Each tank was assigned to a dietary treatment (three replicates per diet).

TABLE 2 Amino acid (AA) composition of the diets and the trout AA requirements, expressed as g 100 g^{-1} CP.

	TM0	TM60	Requirements[a]
Essential AA			
Arginine	7.6	5.0	5.0
Histidine	3.0	3.1	1.8
Isoleucine	5.1	4.4	2.0
Leucine	8.8	8.2	3.5
Lysine	9.1	5.5	4.5
Methionine	3.3	2.7	
Cysteine	1.0	0.8	
Methionine + Cysteine	4.3	3.5	3.5
Phenylalanine	4.8	3.9	
Tyrosine	3.8	6.2	
Phenylalanine + Tyrosine	8.6	10.1	4.5
Threonine	5.0	3.8	2.0
Valine	6.0	5.5	3.2
Non-essential AA			
Alanine	7.6	6.8	
Aspartic acid	11.1	7.3	
Glutamic acid	8.5	10.5	
Glycine	1.1	4.0	
Proline	15.3	7.6	
Serine	5.2	6.2	

Abbreviations: CP, crude protein; TM, *T. molitor* larvae meal.

[a]Reported by NRC National Research Council (1993).

Fish feeding until satiation (via visual observation) was performed twice daily, six days a week, for a total of three months (90 days). Each day, the exact quantity of feed distributed to each tank and fish mortality were recorded. After the 3-month period, 12 fish from each dietary group (following fasting for 24 h) were sacrificed by tricaine methanesulfonate-MS222 overdose (Sigma-Aldrich). Their body weight was measured, and tissue samples from the intestine, heart and muscle were dissected and stored at −80°C until further biochemical analyses.

The experimental protocols were conducted at the registered experimental facility of the DISAFA following the approval by the Ethic Committee (DM no. 182/2010). All procedures were in accordance with both the European Directive (2010/63/EU) and the ARRIVE guidelines (Du Sert et al., 2020) for the protection of animals used for scientific purposes.

2.3 | SDS-PAGE/immunoblot and dot blot analysis

The preparation of heart, intestine, and muscle samples for SDS-PAGE/immunoblot and dot blot analyses is based on well-established protocols, such as the ones described in detail in Feidantsis et al. (2021). Briefly, the antibodies which were employed for the present study were: anti-heat shock protein 70 kDa (H5147) and mouse anti-heat shock protein 90 kDa (H1775) (Sigma) for the HSR, anti-phospho-p38 MAP kinase (9211), anti-phospho p44/42 MAPK (4376) and anti-phospho-SAPK-JNK (9252) (Cell Signaling) for the MAPK pathway, anti-p62/SQSTM1 (5114), anti-LC3B (3868), and anti-ubiquitin (3936) (Cell Signaling) for autophagy, and anti-Bcl2 (7973) (Abcam), anti-Bax (2772) and anti-cleaved caspase (8698) antibody (Cell Signaling) for apoptosis. Quality of protein loading, transfer immunoblot, and dot blot were evaluated by staining with Ponceau and determining levels of actin using the antibody anti-β actin (3700) (Cell Signaling). Finally, enhanced chemiluminescence and laser-scanning densitometry with GelPro Analyser Software (GraphPad) were used for the detection of blots.

2.4 | Antioxidant enzymatic defence

The measurement of antioxidant enzyme activity (V_{max}) in the examined tissues was determined spectrophotometrically according to the protocols described in Feidantsis et al. (2021). The activity of total superoxide dismutase (mitochondrial Mn- and cytosolic Cu/Zn-superoxide dismutase, SOD EC 1.15.1.1) (expressed as units per mg protein) was evaluated by observing the oxidation of NADH at 340 nm per min ($\varepsilon = 6.22\ mM^{-1}\ 1\ cm^{-1}$ at 340 nm). The activity of catalase (CAT, EC 1.11.1.6) (expressed as μmoles per minute per mg protein) was evaluated by observing the changes in the absorbance of H_2O_2 at 240 nm ($\varepsilon = 0.0394\ mM^{-1}\ 1\ cm^{-1}$ at 240 nm). Finally, the activity of glutathione reductase (GR, EC 1.8.1.7) (expressed as μmoles per minute per mg protein) was evaluated by observing the changes in the absorbance of NADPH per min at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ 1\ cm^{-1}$).

2.5 | Intermediate metabolism

The measurement of intermediate metabolism enzyme activity (V_{max}) (expressed as μmoles per minute per mg protein) in the examined tissues was spectrophotometrically determined according to the protocols described in Feidantsis et al. (2009). The activities of L-lactate dehydrogenase (L-LDH; E.C. 1.1.1.27.) and malate dehydrogenase (MDH; E.C. 1.1.1.37) enzymes were evaluated by observing the changes following the oxidation of NADH at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ 1\ cm^{-1}$). L-LDH and MDH were chosen as key enzymes in the cycle of glycolysis. The activity of citrate synthase (CS; E.C. 4.1.3.7.) enzyme was evaluated by observing the changes following the reaction of free coenzyme A with 5,5V dithio-bis(2-nitrobenzoic acid) (DTNB) at 412 nm ($\varepsilon = 13.6\ mM^{-1}\ 1\ cm^{-1}$). CS was chosen since it stands as the pace-making enzyme in the first step of the citric acid cycle.

2.6 | Statistics

Significance at the 5% level was examined in growth performances, protein expression and enzyme activity values (means ± SD) by employing two-tailed p value t test (GraphPad Instat 3.10; GraphPad Instat Software). Moreover, for the determination of the correlation between the examined variables, principal component analysis (PCA) was applied using the FactoMineR package in R (Lê et al., 2008).

3 | RESULTS

3.1 | Growth performance

The growth performance parameters of rainbow trout, including weight gain, specific growth rate, feed conversion rate, protein efficiency ratio and feed intake, are depicted in Table 3.

As far as the growth parameters are concerned, no differences appeared between the groups, except for the feed intake parameter that resulted lower in the TM60 group. A lower feed intake in this group could be due to the presence of chitin, a non-protein nitrogen, source in the cuticle of insects.

3.2 | Heat shock response

Regarding Hsp70 expression, no changes were observed in response to TM60 diet in the three examined tissues of rainbow trout (Figure 1a). On the other hand, Hsp90 levels in the muscle exhibited a statistically significant decrease in the TM60 feeding regime, compared to the TM0. However, the heart and the intestine exhibited no differentiation in Hsp90 expression between TM0 and TM60 (Figure 1b).

TABLE 3 Growth performance parameters (mean ± SD) of rainbow.

	TM0	TM60
Survival rate (%)	87.80 ± 13.50	84.50 ± 10.70
IBW (g)	113.80 ± 3.00	115.90 ± 2.00
FBW (g)	324.40 ± 18.70	285.30 ± 23.50
WG (g)	210.60 ± 17.20	169.40 ± 25.60
SGR (% day^{-1})	1.01 ± 0.19	0.81 ± 0.12
FCR	1.19 ± 0.29	1.24 ± 0.24
PER	1.76 ± 0.41	1.77 ± 0.24
FI (g kg^{-1} ABW day^{-1})	12.81 ± 0.66	11.27 ± 0.71*

Note: Asterisk (*) denotes statistically significant differences ($p < 0.05$) between TM0 (0%) and TM60 (60%). $N = 5$ preparations from different animals for each group.

Abbreviations: ABW, average body weight; FBW, individual final body weight; FCR, feed conversion ratio; FI, feed intake; IBW, individual initial body weight; PER, protein efficiency ratio; SD, standard deviation of the mean; SGR, specific growth rate; WG, weight gain.

3.3 | MAPKs phosphorylation

The phosphorylation of MAPKs exhibited a differential pattern concerning both the MAPK members and the examined tissues (Figure 2). Regarding p38 MAPK phosphorylation, the changes which were statistically significant were observed in the heart (Figure 2a). Specifically, in the latter tissue, TM60 provoked a decrease when compared to the TM0 regime. Although a similar pattern of phosphorylation was observed concerning JNKs (Figure 2c), p44/42 MAPK phosphorylation in the examined tissues displayed a different response. In specific, a decreased p44/42 MAPK phosphorylation was apparent in the intestine of the TM60 regime compared to TM0, while significantly increased levels were observed in the muscle. On the contrary, no statistically significant differences between the two diets were observed in the heart (Figure 2b).

3.4 | Apoptosis

Apoptotic indicators (Bax/Bcl-2 ratio and cleaved caspases) followed a similar pattern in the examined tissues. Specifically, in the heart and the intestine, Bax/Bcl-2 ratio levels exhibited a significant increase in the TM60 diet (Figure 3a). Caspase levels decreased in the muscle of TM60 group compared to TM0, while in the heart and the intestine

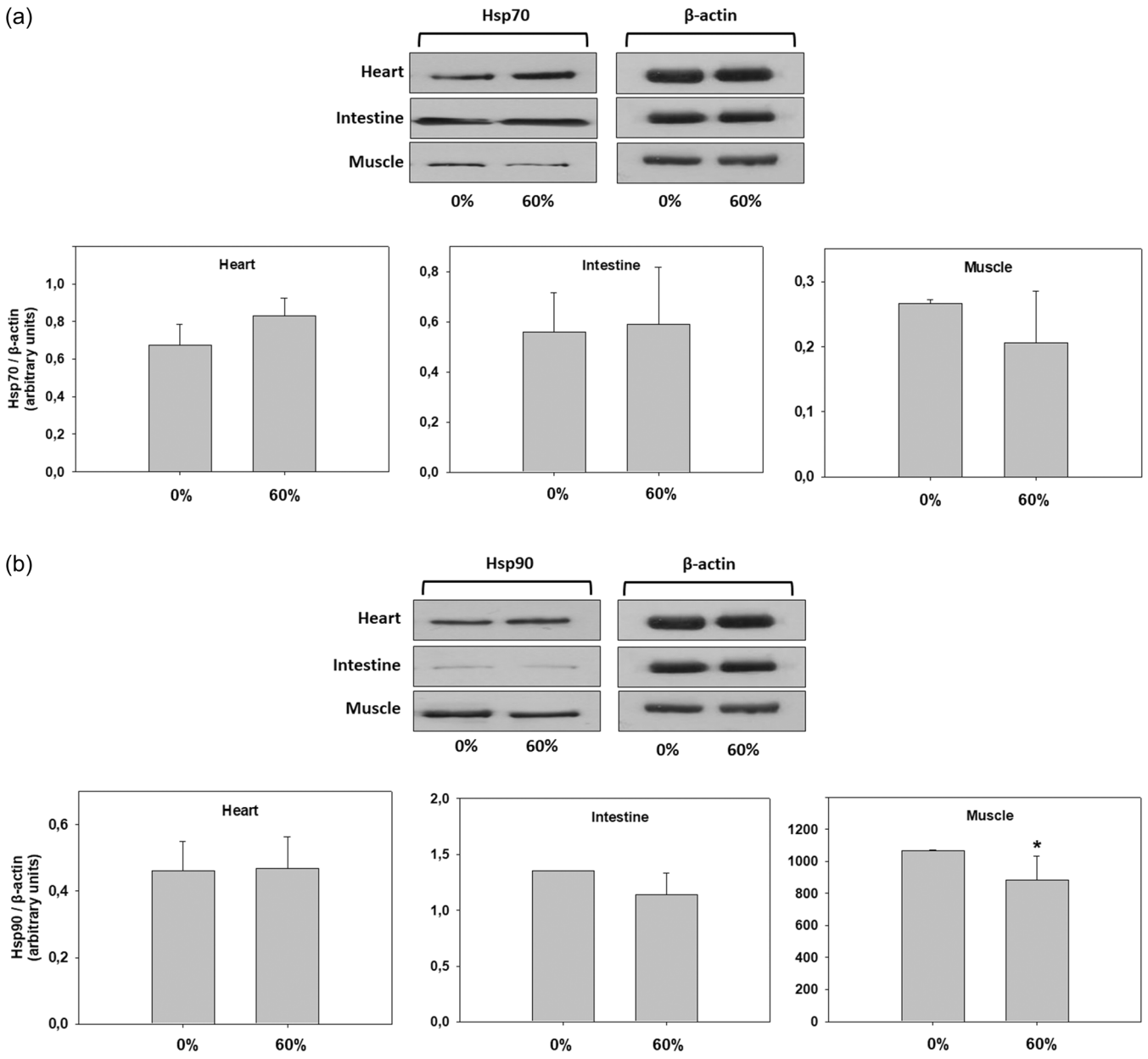

FIGURE 1 (a) Hsp70 and (b) Hsp90 levels (mean ± SD) in the heart, the intestine and the muscle of *Oncorynchus mykiss* under the TM0 (0%) and TM60 (60%) dietary inclusion. $N = 5$ preparations from different animals for each group. Representative western blot images are provided. Asterisk (*) denotes statistically significant differences ($p < 0.05$) between TM0 (0%) and TM60 (60%).

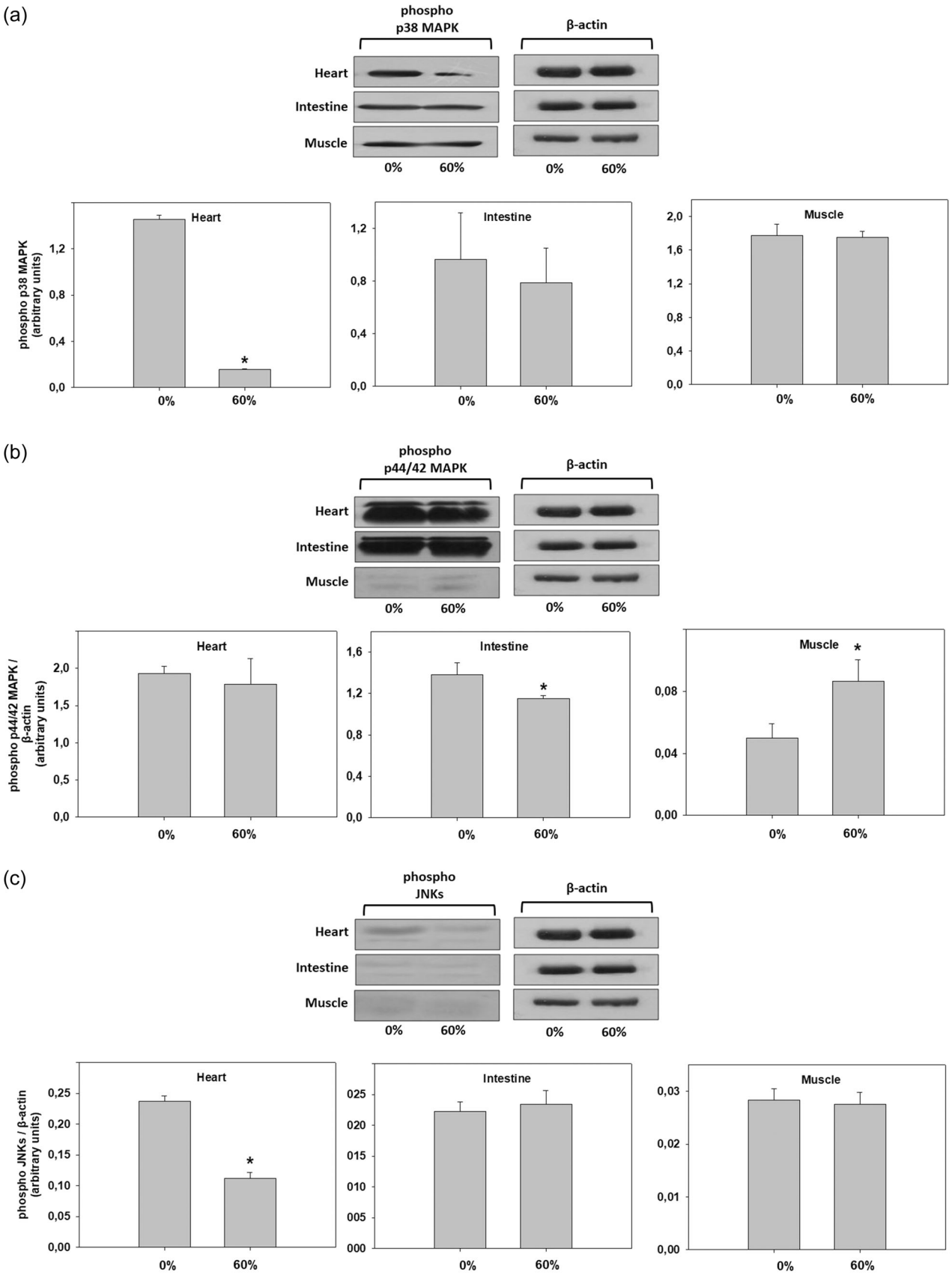

FIGURE 2 (a) phosphorylation of p38 MAPK, (b) p44/42 MAPK and (c) JNKs levels (mean ± SD) in the heart, the intestine and the muscle of *Oncorynchus mykiss* under the TM0 (0%) and TM60 (60%) dietary inclusion. $N = 5$ preparations from different animals for each group. Representative western blot images are provided. Asterisk (*) denotes statistically significant differences ($p < 0.05$) between TM0 (0%) and TM60 (60%).

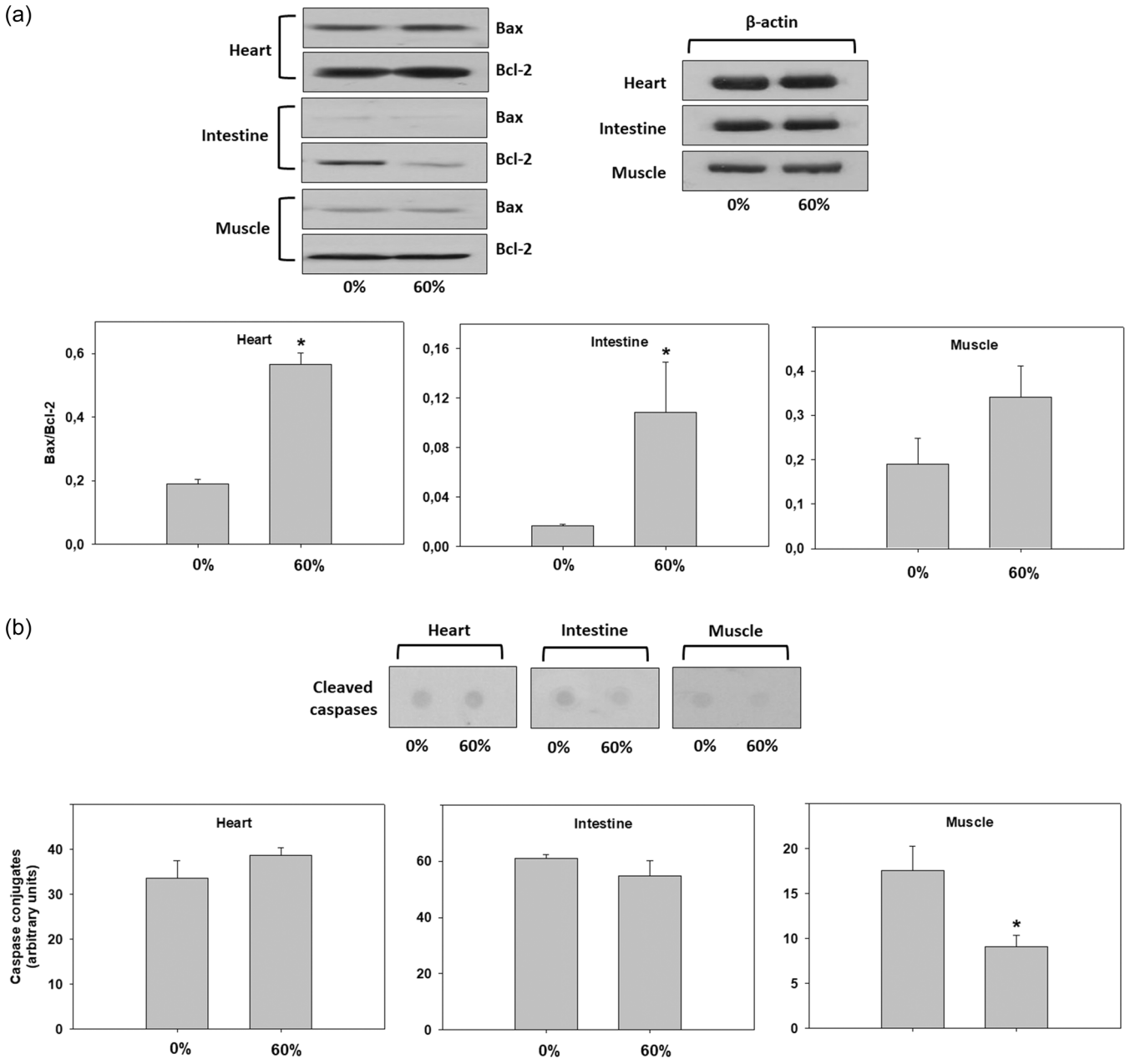

FIGURE 3 (a) Bax/Bcl-2 ratio and (b) cleaved caspases levels (mean ± SD) in the heart, the intestine and the muscle of *Oncorhynchus mykiss* under the TM0 (0%) and TM60 (60%) dietary inclusion. $N = 5$ preparations from different animals for each group. Representative western blot and dot blot images are provided. Asterisk (*) denotes statistically significant differences ($p < 0.05$) between TM0 (0%) and TM60 (60%).

no statistically significant changes were exhibited between the two feeding regimes (Figure 3b).

3.5 | Autophagy and ubiquitination

Regarding ubiquitination, TM60 provoked a statistically significant increase in the heart and the intestine, while a decrease compared to TM0 was observed in the muscle (Figure 4a).

Increased levels of autophagy were apparent in the muscle of fish fed with the TM60 compared to TM0, as indicated by the increased LC3 II/I ratio and decreased levels of SQSTM1/p62, while the other tissues exhibited no relation between the autophagic indicators. Specifically, TM60 exhibited a statistically significant decrease regarding the LC3 II/I ratio in the heart tissue, while no changes were observed in SQSTM1/p62 levels. On the contrary, in the intestine, TM60 led to no change in SQSTM1/p62, while an increase in LC3 II/I ratio was observed compared to TM0 (Figure 4b,c).

3.6 | Antioxidant defence

SOD activity, a key enzyme of the antioxidant defence mechanism, significantly increased under the TM60 feeding regime in the heart

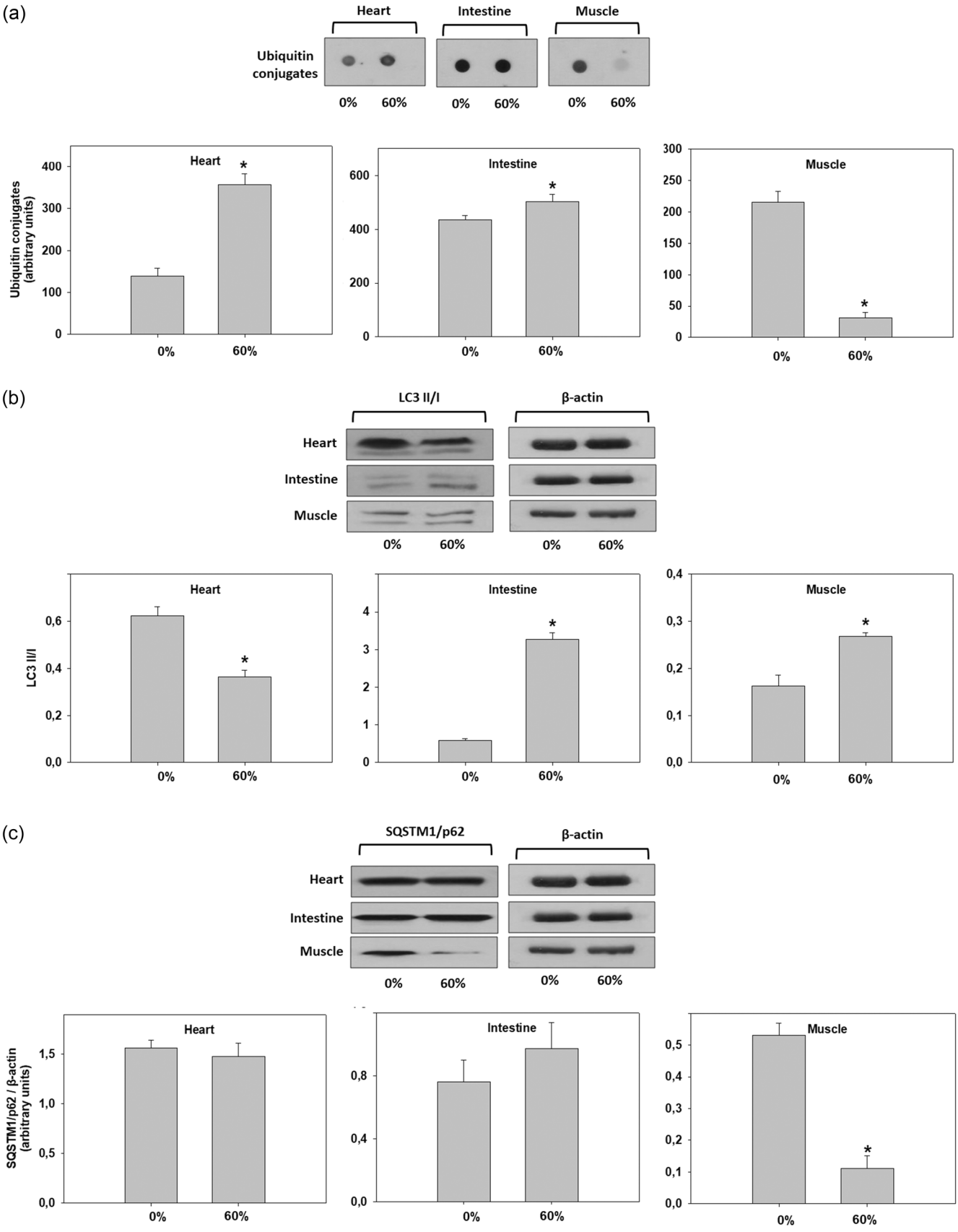

FIGURE 4 (a) Ubiquitin conjugates, (b) LC3BII/LC3BI ratio and (c) SQSTM1/p62 levels (mean ± SD) in the heart, the intestine and the muscle of *Oncorhynchus mykiss* under the TM0 (0%) and TM60 (60%) dietary inclusion. $N = 5$ preparations from different animals for each group. Representative western blot and dot blot images are provided. Asterisk (*) denotes statistically significant differences ($p < 0.05$) between TM0 (0%) and TM60 (60%).

and the muscle and decreased in the intestine compared to TM0 (Figure 5a). Regarding GR activity, a statistically significant increase was exhibited in the intestine and the heart of TM60 fed fish. In contrast, no statistically significant changes in the GR activity were exhibited in the muscle of TM60 fish (Figure 5b). Catalase exhibited a different pattern of activity: while changes observed in the heart between TM0 and TM60 were not statistically significant, muscle and intestine displayed a statistically significant increase and a decrease, respectively, in the catalase levels of TM60 fish compared to TM0 (Figure 5c).

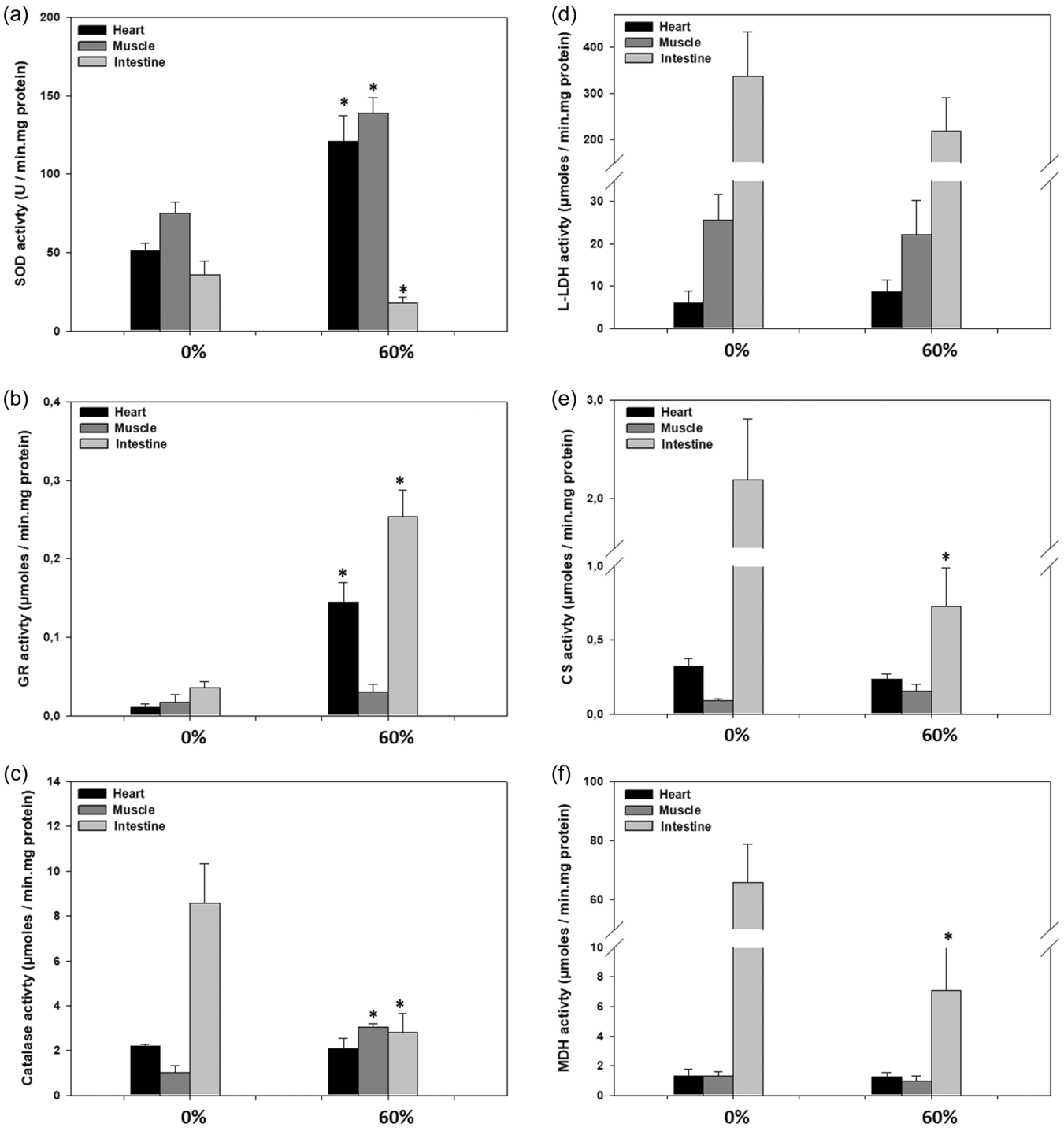

FIGURE 5 (a) SOD, (b) GR, (c) catalase, (d) L-LDH, (e) CS and (f) MDH activity levels (mean ± SD) in the heart, the muscle and the intestine of *Oncorhynchus mykiss* under the TM0 (0%) and TM60 (60%) dietary inclusion. $N = 5$ preparations from different animals for each group. Asterisk (*) denotes statistically significant differences ($p < 0.05$) between TM0 (0%) and TM60 (60%).

3.7 | Intermediate metabolism

The activities of intermediate metabolism enzymes are depicted in Figure 5. In the intestine, a different metabolic potential was apparent, where activities of metabolic enzymes were higher compared to all other examined tissues. In the other tissues, L-LDH activity showed the following ranking: muscle > heart, and CS and MDH: heart > muscle (Figure 5).

Concerning L-LDH, in all examined tissues, the observed changes between TM0 and TM60 were not statistically significant. In contrast, TM60 resulted in decreased CS and MDH levels in the intestine compared to TM0. The muscle and the heart exhibited no significant changes between TM0 and TM60 with regard to the CS and MDH activities (Figure 5).

3.8 | Multivariate component analysis

Tissue specificity is reflected in the PCA analysis results (Figure 6). In the heart, Hsp70, caspases, Bax/Bcl-2, ubiquitin, SOD, GR and L-LDH formed a cluster closer to the TM60 feeding regime, while all other parameters to the TM0 (PC1 explained 28.28% and PC2 29.97% of the variance). The TM60 regime in the muscle formed a cluster comprising of Bax/Bcl-2, p44/42 MAPK, LC3 II/I, SOD, GR, catalase and CS (PC1 explained 28.28% and PC2 11.51% of the variance). On the other hand, in the intestine, this regime formed a cluster including GR, Hsp70, pJNKs, Bax/Bcl-2, ubiquitin, SQSTM1/p62 and LC3 II/I (PC1 explained 28.28% and PC2 27.97% of the variance) (Figure 6).

4 | DISCUSSION

4.1 | Heat shock response

The present results have shown that the most pronounced changes regarding HSR were evident only in the muscle and only concerning Hsp90 induction levels, which decreased under the TM60 feeding regime. Recruitment of Hsps under both normal and stress conditions may occur to mediate several processes of protein and amino acid metabolism, including protein folding and translocation of newly synthesized proteins (Ryan & Jensen, 1995). Evidently, the composition of the TM60 diet did not seem to trigger such molecular mechanisms. According to the review of Moura et al. (2018), only a significant change in the quantity of dietary amino acids is necessary for a change in the Hsps, and specifically Hsp70, levels, while amino acid deprivation can lead to Hsps deficiency. Considering that all amino acid requirements were met in the present study, we can assume that differences between TM0 and TM60 were not significant in order to provoke changes in Hsp70 expression. However, the decrease of Hsp90 levels under the effect of TM60 could be attributed to the involvement of this Hsp member in specific functions, including hormone signalling and ATPase activity (Hoter et al., 2018; Jackson, 2013). Since hormones are not addressed in the present study, the correlation between Hsp90 changes and hormone signalling cannot be concluded. However, since ATPases are involved in cellular energy production, and enzyme activities in the present study are decreased under the TM60 dietary inclusion, the Hsp90 decreased levels may be linked with the overall decreased metabolic processes. Moreover, we can assume that since the cellular processes examined in the present study are energetically demanding, HSR has been downregulated in favour of other cytoprotective mechanisms such as antioxidant defence. In compliance with these results, black soldier fly larvae meal as FM substitute (66% and 100% of the fishmeal protein were replaced with insect meal for eight weeks) reportedly decreased the *hsp* gene expression in the head kidney leucocytes of Atlantic salmon (*Salmo salar*) (Linnaeus, 1758) (Stenberg et al., 2019). Additionally, in the latter species, 600 g kg^{-1} inclusion of defatted *Hermetia illucens* insect meal has shown no effect on the hepatic *hsp70* gene expression levels (Belghit et al., 2019). However, in the Jian carp (*Cyprinus carpio* var. Jian) (Linnaeus, 1758) and the rainbow trout, the *hsp70* gene is reported to be up-regulated under the effect of *H. illucens* insect meal substitution (25% and 50%), which has been

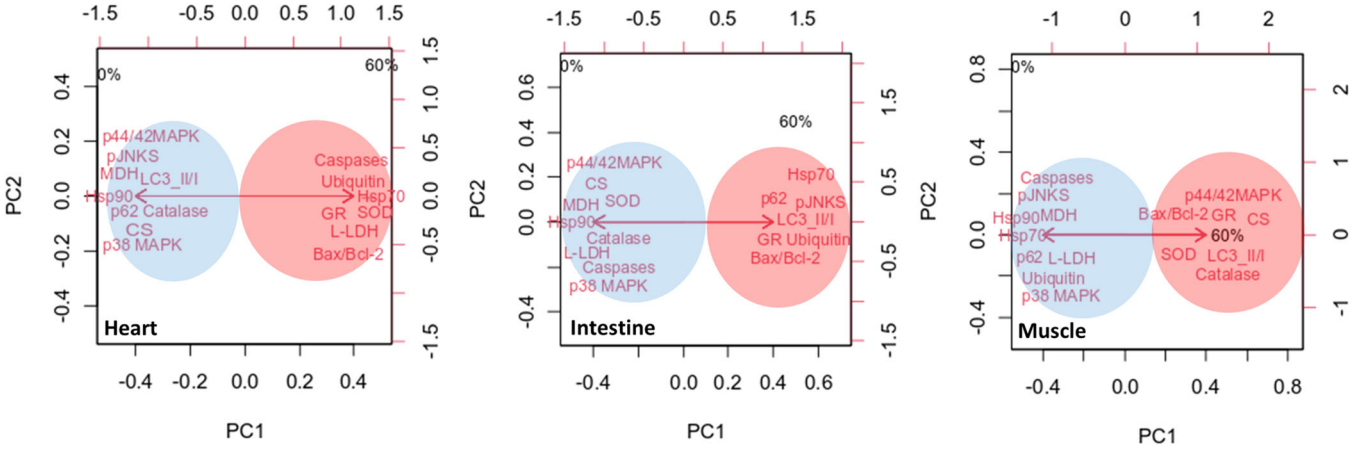

FIGURE 6 Multivariate component analysis depicting biochemical parameters' correlations with the PC1 and PC2 (principal components) in the examined tissues of *Oncorhynchus mykiss*. [Color figure can be viewed at wileyonlinelibrary.com]

interpreted by the authors as indicative of a physiological stress response activation (Cardinaletti et al., 2019). Similarly, Bousdras et al. (2022) have observed Hsps induction in the digestive tract following TM25 inclusion, as well as in the muscle of gilthead seabream (*Sparus aurata*) (Linnaeus, 1758) and European sea bass (*Dicentrarchus labrax*) (Linnaeus, 1758) in response to higher inclusion levels (TM50). The aforementioned Hsps upregulation in both gene and protein levels may derive from changes in the feed composition and the subsequent induced cell stress responses (Jiang et al., 2019) due to the TM inclusion-related influx in free amino acids which need to be metabolized (Kari et al., 2022). Contrary to the above, in the heart of both European sea bass and gilthead sea bream, and in the liver of *Amphiprion ocellaris* (Cuvier, 1830), no changes have been observed with regard to Hsps' protein and gene expression respectively (Bousdras et al., 2022; Vargas-Abúndez et al., 2019).

4.2 | MAPK activation

Regarding MAPKs phosphorylation, a tissue-specific pattern (p38 MAPK and JNKs decrease in activation in the heart, p44/42 MAPK decrease in the intestine, and increase in the muscle of TM60 fish) was evident herein, which is consistent with previous results in European sea bass and gilthead sea bream following the partial substitution of FM with TM (Bousdras et al., 2022). Moreover, the observed reduction of JNKs and p38 MAPK in the heart and p44/42 MAPK in the intestine is in line with a previously reported down-regulation of *p38 MAPK* in Atlantic salmon' head kidney leucocytes following the inclusion of black soldier fly larvae meal (Stenberg et al., 2019). However, the action of MAPKs signalling pathways, which are activated by miscellaneous stress stimuli to regulate several cellular processes (Wada & Penninger, 2004) in response to the insect based-meal, needs to be elucidated since literature remains scarce. Previous studies have reported a regulatory role of MAPK pathways in cellular metabolism, including gluconeogenesis, lipolysis and protein and glycogen synthesis (Atalay & Hänninen, 2010; Gehart et al., 2010). Considering that in the present study, the protein efficiency ratio was stable among the two diets, a protein-sparing effect did not occur, and thus, glucose may not be used for energy purposes instead of protein. However, TM60-induced differentiations in the phosphorylation of MAPK members among the examined tissues highlight the tissue-specific requirements in metabolic processes such as protein synthesis. Therefore, from the obtained results, it appears that the muscle tissue is MAPK-oriented metabolically reorganized in order to better exploit amino acids under the effect of a TM meal with amino acids stimulating growth performance (Mastoraki et al., 2020, 2022).

4.3 | Apoptosis and autophagy

Similarly, the apoptotic responses (either examined by the ratio of Bax/Bcl-2 or the levels of cleaved caspases) in the rainbow trout fed with TM60 exhibited a tissue-specific pattern in this study, where apoptotic processes increased in the heart and the intestine and decreased in the muscle. The observed results in the heart and the intestine correspond with the increased apoptosis (both in mRNA and protein levels) denoted in the liver of largemouth bass *Micropterus salmoides* (Lacépède, 1802) when fed with 75% TM meal fishmeal replacement (Su et al., 2017). In contrast to the above but consistent with the results observed in the muscle, Mente et al. (2022) showed that the gradual inclusion of TM meal triggers a decrease in apoptosis in the muscle of gilthead seabream. Autophagy and apoptosis are two cellular processes that may act either concurrently or antagonistically (Mukhopadhyay et al., 2014). For instance, autophagy may exert a protective role through the inhibition of apoptosis, which is achieved via the recycling of dysfunctional cellular components (Kobayashi, 2015). However, the present results showcased a similar pattern between the two cellular processes. Previous studies have demonstrated that when stress is intensified, autophagy (similar to apoptosis) can lead to cell death (Dodson et al., 2013). Thus, the obtained results suggest that TM inclusion acted strongly on the cell death mechanisms since both processes were triggered in tandem.

Nevertheless, according to the present results as well as existing literature, it is evident that gene expression levels are not always consistent with the respective post-translational ones (Ingolia et al., 2009). Therefore, for an in-depth analysis of all these biochemical processes, all biological organization levels should be considered.

4.4 | Antioxidant defence

According to the PCA analysis, indicators of antioxidant defence, apoptosis and autophagy form clusters in the examined tissues, which reinforced the fact that dietary oxidative stress resulting from nutrient deficiencies (Birnie-Gauvin et al. (2017)) is implicated in apoptotic (Kannan & Jain, 2000) and autophagic processes (Kiffin et al., 2006). However, the relationship between food, oxidative damage, and antioxidant defence mechanisms should be further examined. In general, the TM60 regime seemed to trigger the rainbow trout's antioxidant enzymatic defence. Specifically, levels of SOD activity were increased in the muscle and the heart, while GR increased in the heart and the intestine. Similarly, the TM50 regime has been previously reported to increase SOD and GR activity in the rainbow trout's proximal intestine (Henry et al., 2018). However, the present results showcased a decrease in GR activity levels in the intestine under the effect of the TM60 regime. Increased antioxidant capacity in the kidney and the liver of rainbow trout and Siberian sturgeon (*Acipenser baerii*) (Brandt, 1869) has also been reported after dietary substitution of FM by 25% and 50% black soldier fly meal (Caimi et al., 2020; Elia et al., 2018). Regarding the catalase activity levels observed herein, significant changes compared to TM0 were evident only in the muscle and the intestine. Likewise, black soldier fly larvae meal resulted in decreased expression of antioxidant defence genes in the head kidney leucocytes of Atlantic salmon (Stenberg et al., 2019). Overall, the present results demonstrated an

enhanced antioxidant capacity due to the TM inclusion. However, this capacity seemed to be tissue- and enzyme-oriented in the rainbow trout.

4.5 | Intermediate metabolism

Energy production in several fish species under modified biotic and abiotic conditions is achieved by adjusting the intermediate metabolism (Somero, 2010). Notably, the dietary inclusion of TM60 did not exert significant effects regarding enzymes of the intermediate metabolism. The latter is consistent with the observations in the study of Chemello et al. (2020) regarding hepatic enzymes' activities which are implicated in the synthesis of lipids and amino acid metabolism of the same species. Likewise, insect meal has shown no apparent effects on head kidney leucocytes' mitochondrial metabolism of Atlantic salmon (Stenberg et al., 2019) and on the gilthead seabream's hepatic intermediate metabolism (Fabrikov et al., 2021). However, the TM60 in this study triggered a decrease in the MDH and CS activities in the intestine. The latter implies a downregulation of both lipid and protein metabolism in the intestine. In addition, L-LDH activity levels remained unchanged in both TM0 and TM60 feeding regimes, which suggests that carbohydrate anaerobic metabolism equally contributes to rainbow trout's energy requirements. Nevertheless, considering that insect meal differentially activates genes of the fatty acid metabolism (Basto et al., 2021), such species- and tissue-specific nutrient-metabolic processes should be further investigated.

It is generally accepted that the control of growth and metabolism is mediated by the endocrine system. Specifically, growth and intermediate metabolism are interrelated and many of the endocrine factors that are involved in the regulation of lipid and protein metabolism are also involved in nutrient utilization, immune system function, and somatic growth (Mommsen, 1998, 2001; Pérez-Sánchez & Le Bail, 1999). The extent to which insect meal is suitable as a substitute for FM seems to depend on parameters such as fish species and processes of fish feed (Reyes et al., 2020). Herein, the dietary inclusion of TM60 did not exert significant effects regarding growth parameters. Specifically, under the TM60 dietary inclusion, FBW, WG and SGR exhibited a decreasing but not statistically significant trend. However, FI exhibited a decrease under the TM60 diet. Despite the linkage of insects' proteins in chitin fibres (Nogales-Mérida et al., 2019), which may hamper the proper digestion/utilization, *T. molitor* meal seems to have provided an adequate nutrient content for the growth of rainbow trout. Similarly, no differentiation in weight gain was observed in rainbow trout fed TM larvae meal at a 50% inclusion level, while simultaneously, an increase was apparent in both the protein efficiency ratio and SGR (Belforti et al., 2015). In contrast, previous reports have shown that inclusion at high levels of dietary insect meals may adversely affect growth and nutritive parameters. For instance, high inclusion of 30% TM in the diets of European sea bass and mandarin fish *Siniperca scherzeri* (Steindachner, 1892) diets has resulted in worsening of specific growth rate (SGR) and weight gain (Sankian et al., 2018). Similarly, *H. illucens* and TM dietary inclusion at high levels of 50% substitution has decreased nutritional indices in European sea bass, including feed intake and protein efficiency ratio respectively (Reyes et al., 2020). However, since metabolic procedures and requirements of nutrients vary between fish species and largely depend on their ecology and/or biology, the TM inclusion may apply differentially to each fish species (Moraes & de Almeida, 2020).

5 | CONCLUSION

Collectively, rainbow trout's biochemical responses to the dietary inclusion of yellow mealworm meal showcased a differential pattern regarding the processes and the tissues examined herein (Table 4). In general, TM feeding regime did not affect HSR in most tissues, while activation of p44/42 MAPK due to TM was prominent in the muscle. Moreover, the triggering of apoptotic and autophagic processes and the onset of antioxidant defensive mechanisms indicated the induction of oxidative stress due to TM inclusion (Figure 7). Despite the apparent dietary-induced stress, rainbow trout in the present study exhibited no mortality. However, it should be highlighted that the dietary inclusion of TM60 did not exert significant effects regarding metabolic indexes and growth parameters. Specifically,

TABLE 4 Effect of 60% *Tenebrio molitor* (TM60) dietary inclusion on the heart, muscle and intestine of the rainbow trout (*Oncorhynchus mykiss*).

Oncorhynchus mykiss	Heart 60%	Muscle 60%	Intestine 60%
Hsp70	-	-	-
Hsp90	-	↓	-
phospho p38 MAPK	↓	-	-
phospho 44/42 MAPK	-	↑	↓
phospho JNKs	↓	-	
Bax/Bcl-2	↑	-	↑
LC3 II/I	↓	↑	↑
SQSTM1/p62	-	↓	
Ubiquitin conj.	↑	↓	↑
Cleaved Caspases	↑	↓	↓
GR	↑	-	↑
SOD	↑	↑	↓
CAT	-	↑	↓
L-LDH	-	-	
CS	-	-	↓
MDH	-	-	↓

Note: Up-pointed arrows (↑) indicate increase, down-pointed arrows (↓) indicate decrease and stroke (-) indicates no change compared to the FM – control (0% TM dietary inclusion – TM0).

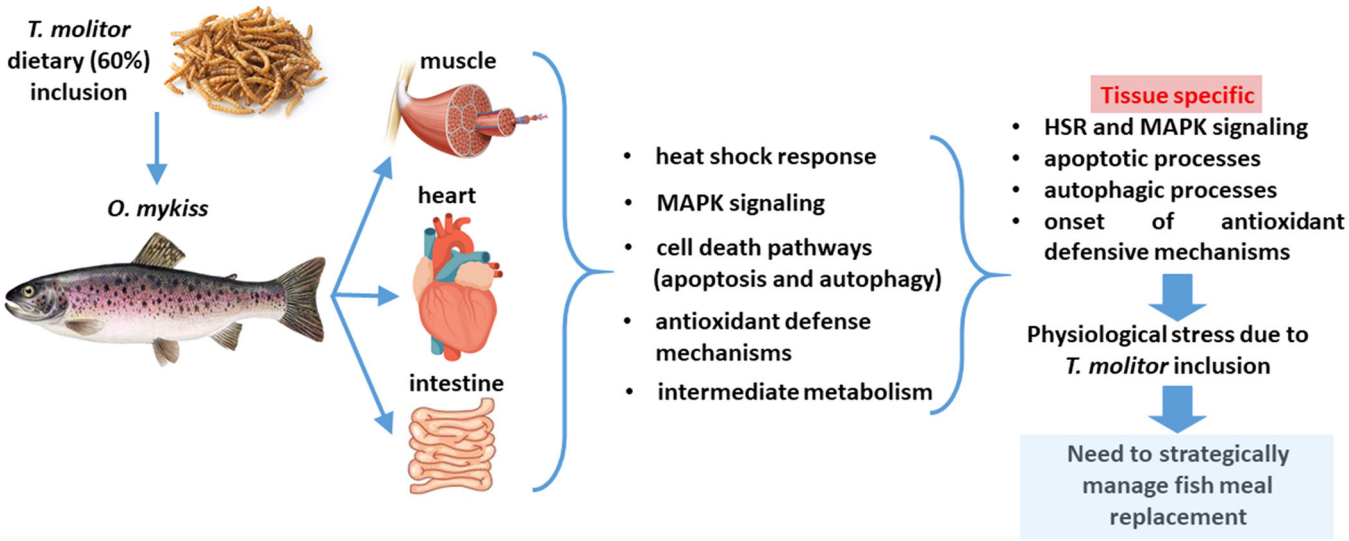

FIGURE 7 Graphical model of *Tenebrio molitor* (TM) dietary inclusion effect on cellular stress responses, antioxidant defence and metabolism of *Oncorhynchus mykiss*. [Color figure can be viewed at wileyonlinelibrary.com]

under the TM60 dietary inclusion, FBW, WG, and SGR exhibited a decreasing but not statistically significant trend. However, FI depicted a statistically significant decrease in TM60 fish compared to TM0 individuals. Although a precise interpretation regarding the impact of insect meal on rainbow trout's physiology cannot be concluded yet due to the varied species- and tissue-specific responses, the present results can be utilized in an attempt to shed light on the underlying mechanisms in response to TM feeding stimuli in this highly economical species. Therefore, future studies should be species-oriented to achieve optimal inclusion levels for the sustainable rearing and management of fish. The impact of insect meal on the physiological indexes of rainbow trout remains understudied, and therefore, knowledge regarding biochemical processes in response to insect meal substitution is needed for this highly economic fish species. Nevertheless, the present findings enriched the current knowledge at the cellular level concerning the adverse effects due to high levels of insect meals' dietary inclusion.

AUTHOR CONTRIBUTIONS

Nikolas Panteli: Investigation of the study, methodology, data curation, software, formal analysis, visualization, conception and design, writing of the first draft of the manuscript and revision of the final draft of the manuscript. Konstantinos Feidantsis: Investigation of the study, methodology, data curation, software, formal analysis, visualization, conception and design, writing of the first draft of the manuscript and revision of the final draft of the manuscript. Thomas Bousdras: investigation of the study, methodology, data curation, software, formal analysis and revision of the final draft of the manuscript. Francesco Gai: Investigation of the study, methodology, data curation, software, formal analysis, visualization, conception and design, funding and resources for the study and revision of the final draft of the manuscript. Laura Gasco: investigation of the study, methodology, data curation, software, formal analysis, visualization, conception and design, funding and resources for the study, funding and resources for the study, revision of the final draft of the manuscript; EA: investigation of the study, methodology, data curation, software, formal analysis, visualization, conception and design, supervision of the study, funding and resources for the study, writing of the first draft of the manuscript, revision of the final draft of the manuscript. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data sets generated and/or analyzed during the current study are not publicly available due to the nature of this research. Accompanying data remain unpublished to date, but are available from the corresponding author on reasonable request.

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