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Nile tilapia fed insect meal: Growth and innate immune response in different times under lipopolysaccharide challenge

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Abstract

Insects have been the subject of recent attention as a nutritious source and nutraceutical potential. Hence, we studied the effects of diets containing superworm larvae (*Zophobas morio*) meal on growth performance and innate immunity of Nile tilapia (*Oreochromis niloticus*). During 12 weeks, fish were fed with diets containing 0, 15% and 30% of superworm larvae meal (SWM) and then challenged with lipopolysaccharide (LPS). Cellular counts, lysozyme and complement system activity (HACS) were recorded over 0, 3, 6 and 9 hr post-challenge. The results revealed that dietary inclusion of SWM had no negative effects on growth performance of tilapia ($p < .05$). The moisture and lipid content increased ($p < .05$) in fish fed 30% SWM while ash and protein content decreased. However, there were no differences between fish fed 15% SWM and the control group. Thrombocytes and neutrophils showed increasing levels ($p < .05$) in those fed SWM diets mainly 6 hr after challenge. Increased lysozyme activity in serum and liver was registered in both groups fed SWM ($p < .05$). Fish fed SWM diets showed an increase in HACS in which higher haemolysis was registered at 0 hr and 3 hr ($p < .05$). The current study indicates that dietary inclusion of at least 15% SWM could influence selected innate immunity parameters of tilapia while maintaining growth performance and feed utilization. However, further investigations are needed to evaluate the effect of SWM on other immune parameters to better understand how this ingredient can improve the health of Nile tilapia.

KEYWORDS

cellular counts, growth performance, immune response, insect meal, Nile tilapia, *Zophobas morio*

1 | INTRODUCTION

Edible insects as an alternative protein source for human food and animal feed are interesting in terms of less water waste, low land use, a large number of offspring per reproduction, and their ability to transform low-value organic side streams into high-value protein products (van Huis et al., 2015; Makkar et al., 2014). In this perspective, given the strong interest shown for insect meals by insect producers and farmers, the European legislation expanded categories of novel foods, authorizing the incorporation of insect-based ingredients in the feed of animals for human consumption (Commission Regulation (EU) 2017/893/EC). However, as underlined by the EFSA Scientific Committee (2015), research is needed to clarify the biological and chemical aspects arising from the production and consumption of insects as feed.

Larvae from many insect species can be used for insect meal production. Among the species, superworm (*Zophobas morio*) is easily raised on low-nutritive plant products with high feed conversion efficiency (Jabir et al., 2012). The *Zophobas morio* is

a beetle considered a pest of grain, flour and other cereal products, found abundantly in tropical countries. The larvae stage of this insect can be harvested and used as a valuable nutritious feed source, due to its essential amino acids profile as well as its crude fat content, suitable for formulating fish diets (Henry et al., 2015; Jabir et al., 2012). Many studies have focused on a nutritional perspective, searching for ingredients which would promote the ideal development of fish, besides favouring the sustainability of the aquaculture production in economic and environmental level. Among the alternative sources evaluated, insect meal is promising as a nutritive and nutraceutical ingredient in animal feed (Barroso et al., 2014; Dietz & Liebert, 2018; Makkar et al., 2014; Su et al., 2017).

Nile tilapia is the third most farmed fish in the world with current production of 4.5 million tons in 2018 (FAO, 2020). More than 80% of global tilapia production is based on commercial aquafeed in which the predominant protein source is soybean meal (Dietz & Liebert, 2018; FAO, 2020). As an omnivorous fish, Nile tilapia has some advantages on chitin degradation because polysaccharides are part of the composition of insects which are a natural feed source for freshwater fish (Fontes et al., 2019; Molinari et al., 2007). Chitin, a natural polymer found in crustacean shells and exoskeletons of insects, has been shown to activate the immune response in mammals and fish (Gopalakannan & Arul, 2006; Henry et al., 2018; Jiang et al., 2019; Kumar et al., 2015, 2019). Furthermore, insects also contain antimicrobial peptides that have been reported to be active against Gram-positive and Gram-negative bacteria (Gasco et al., 2018). However, to the best of our knowledge, no report has described the effects of dietary *Zophobas morio* larvae meal on the immune response of Nile tilapia or any other fish.

The innate immune system of fish is considered to be the first line of defence against pathogens. Nutraceutical substances can increase resistance to infectious diseases by enhancing non-specific defence mechanisms (Zhang et al., 2009). Immunomodulatory substances are usually identified by their ability to activate leukocytes in vivo (Barros et al., 2014; Dotta et al., 2014). The inclusion of immunomodulatory substances in animal diets can provide resistance against pathogens during periods of high stress such as vaccination, grading, transport and stocking (Mouriño et al., 2012).

Most of the diseases in fish are caused by Gram-negative bacteria, and lipopolysaccharide (LPS) is a key immune component of these bacteria. Therefore, the use of isolated bacterial LPS from *Escherichia coli* is widely acknowledged in studying fish immunological response (Jiao et al., 2019; Li et al., 2020; Lulijwa et al., 2019; Paulsen et al., 2003), including several studies in Nile tilapia (Ha et al., 2017; Lazado et al., 2016; Liu et al., 2016). It can trigger fierce immune response in animals that leads to a signalling cascade including humoral and cellular components, for example lysozyme, complement factors, and the function and proportion of several kinds of cells, including blood cells (Li et al., 2020; Paulsen et al., 2003).

Applied research is now needed to fill the knowledge gaps by utilizing insect meal in fish diets. Since the immune system of fish is directly linked to its nutritional modulation for preventive health care, the present study aimed to determine the effects of using superworm larvae meal (SWM) as a partial and total replacement of soybean meal on growth performance and innate immune response of Nile tilapia after LPS challenge.

2 | MATERIAL AND METHODS

2.1 | Experimental diets

Three isonitrogenous and isoenergetic diets were formulated to meet the tilapia's nutritional requirements based on the National Research Council (NRC, 2011). The diets included 0 (Control diet), 15 and 30% inclusion of SWM in replacement of soybean meal and oil. A full-fat superworm larvae meal from the Laboratory of Entomoculture of the Federal University of Minas Gerais—ICA/ UFMG (Minas Gerais, Brazil) was used in the feeding trial. The larvae were reared on media containing plant-based material, killed by immersion in boiling water, dried in a forced-air oven (50°C), and milled in a screw meat grinder (Botini 1/3cv, Brazil). The diets were processed in an electrical pelletizing machine (CPM 2000), oven-dried (58°C for 24 hr) and stored −5°C. Ingredients composition and proximate analyses of the diets and SWM are shown in Table 1.

2.2 | Fatty acids determination

For fatty acid (FA) analysis of the diets, lipid extraction and fatty acid (FA) profile were analysed according to Araújo et al. (2017). For each sample, total lipid was extracted with chloroform and methanol using the Folch method (Folch et al., 1957) with minor modifications. FA profile was analysed is a gas chromatograph (GC 2010) equipped with an auto-sampler (Shimadzu, Kyoto, Japan), flame ionization detector (FID) and a SP-2560 fused silica capillary column (Supelco, Sigma Aldrich; 100.0 m long, 0.25 mm, 0.20 mm thickness) filled with helium gas (28 cm/s). Fatty acid peaks were integrated and quantified using chromatographic GC solution software (version 4.02), and peaks were identified by comparison to known standards (Supelco, Sigma Aldrich; 37 Component FAME Mix).

2.3 | Fish and rearing conditions

Animal procedures were performed under the guidelines of the Ethics Committee of Animal Welfare of the Federal University of Lavras, protocol number 031/2018. The indoor recirculation system consisting of 12 circular fibreglass tanks (water volume: 100 L). Each tank was provided with continuous aeration. During the trial, water quality was monitored daily and maintained within optimal conditions for Nile tilapia; water temperature was maintained at 28 ± 2°C, dissolved oxygen 5.5 ± 0.3 mg/L, total ammonia 0.3 ± 0.03 mg/L, pH: 7.5 ± 0.2 and the light:dark cycle was 12D:12L with the light period from 6:00 to 18:00 hours.

TABLE 1 Ingredients and proximate composition of SWM and experimental diets

Ingredient (% dry weight)	SWM	Control	15% SWM	30% SWM
Menhaden fishmeal[a]	–	13.00	13.00	13.00
SWM[b]	–	0.00	15.00	30.00
Soybean meal[c]	–	19.50	9.75	0.00
Corn meal[d]	–	17.40	17.40	17.40
Wheat Flour[e]	–	15.00	15.00	15.00
Rice Flour[f]	–	10.00	10.00	10.00
Corn gluten[g]	–	9.50	9.50	9.50
Inert (Kaolin)	–	5.80	5.00	4.00
Soybean oil[i]	–	8.70	4.25	0.00
Antioxidant BHT [j]	–	0.05	0.05	0.05
Premix[k]	–	1.00	1.00	1.00
Proximate composition (% dry weight)				
Dry matter	94.57	88.60	88.90	89.90
Corrected protein[l]	30.43	28.91	28.66	28.41
Crude fat	33.05	13.06	13.37	13.88
Ash	2.77	12.2	12.2	11.1
Energy (MJ/Kg)	26.8	14.1	14.1	14.2
Chitin	22.56	0.00	1.93	3.86

[a]Crude protein 67.0%, crude fat 15%, dry matter 92%.

[b]Superworm larvae meal from Laboratory of Entomoculture of the Federal University of Minas Gerais (ICA/ UFMG), MG, Brazil.

[c]Crude protein 46%, crude fat 3%, dry matter 89% Cargill, SP, Brazil

[d]Crude protein 7.9%, crude fat 3%, dry matter 90% Bioquima, MG, Brazil.

[e]Crude protein 14%, crude fibre 8%, crude fat 4%, dry matter 90.5% Bioquima, MG, Brazil.

[f]Crude protein 12%, crude fat 14%, dry matter 92% Cargill, SP, Brazil.

[g]Crude protein 62%, crude fat 4%, dry matter 90% Cargill, SP, Brazil.

[i]Commercial refined soybean oil, MG, Brazil.

[j]Butylated hydroxytoluene (antioxidant).

[k]Mix Vita/Min Omnivorous fish 5 kg/ton Cargill, SP, Brazil

[l]Corrected crude protein calculated by applying a nitrogen-to-protein conversion factor of Kp = 4.76 (Janssen et al., 2017).

Nile tilapia (*Oreochromis niloticus*) were obtained from the Fish Laboratory of Federal University of Lavras and acclimated to the rearing conditions for 7 days. After the acclimation period, 144 Nile tilapias (3.00 ± 0.20 g) were randomly distributed into 12 tanks for 12 fish per tank. Fish were fed twice a day until apparent satiation, and feed consumption was recorded. Each diet treatment was randomly assigned to four groups, and the trial lasted 12 weeks.

After the feeding trial, all fish were counted, measured and weighed after 24 hr of fasting. Three fish per tank (n = 12/treatment) were randomly sampled after being euthanized with an overdose of 250 mg/L benzocaine, followed by spinal cord sectioning to determinate whole-body composition. Additionally, six fish per tank were anesthetized (benzocaine 100 mg/L) and injected with *Escherichia coli* LPS (3 mg/kg of body weight; L2880, Sigma, USA) at swim bladder as previously described by Matushima and Mariano (1996). As a control group, 2 fish per tank were injected with saline solution (NaCl 0.65%) without *E. coli* LPS.

Blood was obtained from the caudal vein prior to *E. coli* LPS challenge (0 hr, injected with saline solution) and then, 3, 6 and 9 hr after *E. coli* LPS challenge (n = 8/treatment). The collected blood sample was divided into two sets. The first blood set was added to the tube with EDTA as an anticoagulant for haematological procedures. The second blood set was added to the EDTA-free tube and centrifuged at 4,800 *g* for 5 min at room temperature for blood serum. Head kidney, spleen and liver tissues were removed from all fish, immediately weighed and frozen in liquid nitrogen and stored at −80°C until analysis of innate immune responses.

2.4 | Growth performance

Growth performance and body condition indexes data were computed using the following calculations (a) FW: final weight (g); (b) SGR: Specific growth rate, % = [100 × (ln final body weight (g) – ln initial body weight (g)/days of feeding trial)]; (c) FI: Feed intake, % = (100 × [dry feed intake/square root of initial body weight − final body weight (g))/days on feeding trial]; (d) FE: Feed efficiency ratio, % = (weight gain/dry feed intake); (e) Survival rate, % = [(number of fish at the end of the experiment/ number of fish at the beginning of the experiment) × 100].

2.5 | Proximate chemical analysis

Chemical analysis of the experimental diets and fish whole-body were analysed according to AOAC methods (AOAC, 2012). The samples were dried to a constant weight at 105°C for 24 hr to determine the dry matter content. Crude protein was determined by the Kjeldahl method after acid digestion. The nitrogen-to-protein correction factor of 4,76 was used for a more correct estimate of the insect meal protein content as reported by Janssen et al. (2017) in which the percentage of chitin and its nitrogen content is not considered. Crude fat was carried out according to Folch, Lees, & Stanley's method (1957) and chitin according to Clark et al. (1993). Ash content was determined by incineration in a muffle furnace at 550°C for 12 hr.

2.6 | Cellular counts

Total red cell count was performed in a Neubauer chamber, using whole blood diluted in formaldehyde citrate buffer 1:200. The total and differential count of leukocyte was performed using an optical microscope (CH30 Olympus) at 100× in immersion oil on blood smears (two slides per fish, 5–10 μl blood drop), fixed in methanol, and coloured with May–Grunwald–Giemsa as previously described by Rosenfeld (1947). The leukocytes were measured by the indirect method, which considers the number of leukocytes

and thrombocytes for 2000 erythrocytes counted. Additionally, to the differentiation of leukocytes, 200 cells were counted and the amount of each cell type was identified and expressed as cells/μl.

2.7 | Lysozyme activity

Lysozyme activity was determined in pooled serum (LYZ-SE), spleen (LYZ-SP), head kidney (LYZ-HK) and liver (LYZ-L) using a turbidimetric assay as previously described by Jørgensen et al. (1993) with an adjustment of the pH of the *Micrococcus lysodeikticus* (M0508, ATCC No 4698, Sigma Aldrich) to 6.2 to maximize the activity (Ellis, 1990; Milla et al., 2010; Pereira et al., 2017). The LYZ–SP, LYZ-HK and LYZ-L were determined in extracts of the organs homogenized in four volumes (w/v) of 0.1 M Tris/HCl Buffer (pH 7.8) and centrifuged at 13,000 × *g* for 30 min at 4°C (Pereira et al., 2017). Following the centrifugation, the supernatant was collected and used as a crude enzyme solution. Briefly, 10 μl of the sample was mixed with 200 μL *Micrococcus lysodeikticus* suspension in PBS at pH 6.2. Lysozyme activity (units/mL) was calculated using the following formula: $[(\Delta_{absorbance(4\text{-}1\ min)}/3)/0.001] \times 100$. One unit of lysozyme activity was defined as the quantity of enzyme that caused a 0.001 decrease in absorbance per minute measured at 450 nm.

2.8 | Haemolytic activity of the alternative complement system

Haemolytic activity of the alternative complement system (HACS) was measured using sheep red blood cells as targets (Sutili et al., 2016). Briefly, tilapia serum (10 μl) was incubated at room temperature for 1 hr with 2%-sheep blood (25 μl). After the incubation time, 100 μl of cold-PBS was added and centrifuged at 2,500 × *g* for 5 min at 4°C. Following this, 100 μl of supernatant was transferred to 96-well microplates and the absorbance of the samples was read at 405 nm. The percentage of haemolysis of the saline solution for each sample was calculated using the absorbance of a total haemolysis control (distilled water + sheep blood) and spontaneous lysis (PBS 0.1 M + sheep blood) according to the following calculation: % haemolysis = $[(A_{405}\ sample - A_{405}\ no\text{-}haemolysis)/(A_{405}\ total\ haemolysis - A_{405}\ no\text{-}haemolysis)] \times 100$.

2.9 | Statistical analysis

Data are presented as mean ± pooled standard error of the mean. Data analysis was performed by one-way and two-way analysis of variance (ANOVA) with treatment and time as independent variables after testing for normality and homogeneity of variances with Shapiro–Wilk and Levene tests respectively. Significant differences among means were determined by the Tukey HSD test. A probability level of 0.05 was used for rejection of the null hypothesis. Statistical analysis was done using IBM SPSS Statistics for Windows, Version 23.0 (Armonk, NY, USA).

TABLE 2 Fatty acid composition (% of total fatty acids) of the experimental diets

Fatty acid	Control	15% SWM	30% SWM
C14:0	0,3	0,9	0,9
C16:0	14,3	18,7	23,5
C16:1	0,0	0,7	0,8
C18:0	4,3	5,8	7,7
C18:1n9	26,6	32,6	36,2
C18:2n-6 LNA	45,6	34,7	26,3
C18:3n-3 ALA	3,9	2,5	1,2
C18:3n-6	0,9	0,0	0,0
C20:0	0,4	0,0	0,4
C20:1n-9	0,4	0,6	0,2
C20:3n-6	0,4	0,3	0,2
C20:4n-6 ARA	0,1	0,3	0,2
C20:5n-3 EPA	0,3	0,4	0,2
C22:1n-9	0,2	0,6	0,3
C22:6n-3 DHA	0,7	0,8	0,8
Σ SFA	41,7	52,1	60,9
ΣMUFA	27,2	34,5	37,5
ΣPUFA	52,0	39,0	28,9
Σ LC-PUFA	1,5	1,7	1,4
Σ n-6	47,0	35,3	26,7
Σ n-3	4,9	3,6	2,1
Σ n-3/ Σ n-6	0,1	0,1	0,08

Note: Σ SAFA: sum of saturated fatty acids; Σ MUFA: sum of monounsaturated fatty acids; Σ PUFA: sum of polyunsaturated fatty acids; LC-PUFA: sum of long chain polyunsaturated fatty acids.

3 | RESULTS

To evaluate the immunomodulatory effect of SWM on the induction of fish immune response, Nile tilapia juveniles were fed 12 weeks with the experimental diets before being inoculated with *E. coli* LPS. Biochemical and cellular indicators were evaluated just before inoculation (0 hr) and 3, 6 and 9 hr after inoculation.

3.1 | Diets composition

All diets were comparable in terms of dry matter and other main nutrients. The fatty acid (FA) composition is concerned (Table 2), oleic acid (C18:1n9) was by far the most represented FA in SWM diets. Also, monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA) increased following the increased inclusion level of SWM. Consequently, polyunsaturated fatty acids (PUFA) and the n-3/n-6

balance decreased following the increased inclusion of SWM. The γ-linolenic acid (C18:3n6) was not detected in SWM diets.

3.2 | Growth measurements

After the 12 weeks feeding period, there were no effects of SWM inclusion on final weight, specific growth rate, survival, or any of the growth or feed intake parameters (SGR, FI, FE) ($p > .05$). Also, the treatments did not interfere with fish mortality values before or after challenge ($p > .05$) (Table 3).

3.3 | Whole-body composition

Whole-body moisture and lipid contents of Nile tilapia fed 30% SWM were higher than in fish fed 15% SWM and control group ($p < .05$) (Table 4). Ash and protein contents of fish fed 30% SWM were lower than in the other groups ($p < .05$).

3.4 | Cellular counts

Total leukocyte and the numbers of lymphocytes, monocytes and erythrocyte were not changed by the diet ($p > .05$; Figure 1). However, fish fed 30% SWM diet showed a higher number of neutrophils at 6 hr after challenge compared to the control group ($p < .05$). Also, fish fed 15% and 30% SWM diet showed a higher number of thrombocytes mainly 6 hr after challenge compared to the control group ($p < .05$). Comparing the hours (0, 3, 6, 9 hr), there was an increase in WBC in all treatments, where the highest values were observed between 3 and 6 hr, gradually returning to the baseline values after 9 hr in most counts ($p < .05$) (Figure 2). Differential leukocyte counts were characterized by the predominance of lymphocytes.

3.5 | Lysozyme activity

Liver lysozyme activity (LYZ-L) increased at 3h in fish fed with 15% and 30% SWM ($p < .05$; Figure 3). Head kidney (LYZ-HK) and spleen lysozyme activity (LYZ-SP) did not show differences among the treatments ($p > .05$). Higher serum lysozyme activity (LYZ-S) was observed in fish fed 15% and 30% SWM in all times after challenge compared to the control group ($p < .05$).

3.6 | Haemolytic activity of the alternative complement system

Fish fed diets containing 15% and 30% SWM showed an increase in haemolytic activity of the alternative complement system (HACS) ($p < .05$) (Figure 4). In addition, higher haemolysis percentages were registered at 0 and 3 hr after challenge for all feeding trials ($p < .05$).

4 | DISCUSSION

In the current study, dietary inclusion of full-fat *Zophobas morio* larvae meal in replacement of soybean meal and soybean oil did not affect the growth performance parameters. The fish promptly consumed all tested diets and no differences in feed intake were registered, indicating that SWM was acceptable and well-digested by Nile tilapia juveniles. These results are in agreement with previous studies on the inclusion of black soldier fly larvae meal in diets for salmonid in which no differences on the growth parameters were reported (Belghit et al., 2019; Lock et al., 2016). Similarly, the inclusion of *Tenebrio molitor* larvae meal and *Musca domestica* meal did not affect the fish growth performance of gilthead sea bream (*Sparus aurata*) and barramundi (*Lates calcarifer*) respectively (Lin & Mui, 2017; Piccolo et al., 2017). Belforti et al. (2016), reported the inclusion of full-fat *Tenebrio molitor* in diets for rainbow trout did not affect the final fish weight and the weight gain but improved feed efficiency and specific growth rate.

In contrast, other studies observed decreased growth performance at higher inclusion of *Tenebrio molitor* for African catfish, (*Clarias gariepinus*) and European sea bass (*Dicentrarchus labrax L.*) (Gasco et al., 2016; Ng, 2001). Also, Jabir et al. (2012), in a study using full-fat SWM in diets for Nile tilapia, showed that inclusion of SWM up to 50% adversely affects growth and feed utilization, probably due to either the essential amino acids deficiency or low

TABLE 3 Growth performance of juvenile Nile tilapia fed with experimental diets

	Control	15% SWM	30% SWM	S.E.M.	C.V.	Pr > F*
Final weight (g)	68.90	66.43	69.60	1.02	9.89	0.4318
Specific growth rate (%/day)	3.73	3.69	3.74	0.45	8.96	0.4202
Feed intake (%)	2.25	2.19	2.31	0.75	2.88	0.4189
Feed efficiency (%)	0.89	0.88	0.91	0.93	5.05	0.1912
Survival (%)	91.66	91.66	93.75	1.25	4.29	0.4389

Note: Initial fish average weight was of 3.00 ± 0.20.

The values are means of four replicate tanks, $n = 12$ individuals/treatment. SEM is pooled of standard error.

TABLE 4 Whole-body composition of Nile tilapia fed with experimental diets

	Control	15% SWM	30% SWM	S.E.M.	C.V.	Pr > F*
Moisture, %	72.47[b]	72.46[b]	74.55[a]	0.28	1.38	0.0004
Crude Protein, %	21.62[a]	21.3[a]	19.28[b]	0.29	1.38	0.0171
Crude Fat, %	5.53[b]	5.72[b]	6.06[a]	0.11	0.52	0.0352
Ash, %	1.72[a]	1.87[a]	1.28[b]	0.09	0.40	0.0028

Note: The values are means of four replicate tanks, $n = 12$ individuals/treatment. SEM is pooled of standard error. Means in the same row with letters indicate significant differences ($p < .05$).

feed intake. Generally, fluctuations in crude protein and lipid contents are linked with life stages or feeding habits of fish used for the trials. Hence, it is possible that these inconsistent results in growth performance could be due to a difference in the tolerance level of insect ingredients between fish species, or also because of the insect processing techniques. Moreover, there may be particularities among the range of insects used in animal feed that is hitherto unknown.

The inclusion of insect meal in Nile tilapia diet affected the whole-body composition in our study. The results showed higher moisture and lipid, and lower protein content of fish fed 30% SWM. These results are in agreement with previous studies in which *Clarias gariepinus* fed diets containing *Tenebrio molitor*, showed an increase in total fat level without significant changes in protein levels compared to control fish (Ng, 2001). In contrast, Belforti et al. (2016), registered a significant decrease of dry matter and lipid contents, and an increase of protein content with increasing inclusion of *Tenebrio molitor* larvae meal in rainbow trout diets. The replacement of fish meal by black soldier fly maggot meal did not alter the protein content in the whole-body of Nile tilapia as reported by Muin et al. (2017).

The higher concentration of lipids in fish carcass could be explained by the concentration of saturated fatty acids and the balance of n-3/n-6 of SWM, which may induce lipogenesis. The insect *Zophobas morio* is one of the species with the highest proportion of fat, which varies depending on the life stage, normally ranging between 32% and 42% (Barroso et al., 2014; Finke, 2002). According to Barroso et al., (2014) *Z. morio* has higher ratios of omega 6, saturated and monounsaturated fatty acids, as registered in the fatty acid analysis in the diets of our study. Further, it has been reported that fatty acid imbalance of n-3/n-6 could lead to lipid deposition of fish (Mu et al., 2018; Paulino et al., 2020). However, it is possible to modify the insect fatty acids profile manipulating the rearing substrate as reported by several authors (Belforti et al., 2016; Gasco et al., 2018; St-Hilaire et al., 2007). In general, the diets fatty acid composition is reflected in the body composition, however supplementary studies are needed.

White blood cells (WBC) count is an important parameter for assessing the immune system of fish and it may vary according to the fish species, age, sex, nutrition and season (Fazio, 2019; Tripathi et al., 2004). Three types of leukocytes, namely lymphocytes, monocytes and neutrophils, were identified in the circulating blood of juveniles Nile tilapia in this study. The obtained reference intervals for differential WBC count of Nile tilapia were similar to those reported for this species (Barros et al., 2014; El-Boshy et al., 2010; M. Martins et al., 2008), channel catfish, hybrid tilapia and koi (Lloyd-Evans et al., 1994; Tavares-Dias & Moraes, 2007; Tripathi et al., 2004). Moreover, the number of leukocytes is known to highly increase when infections occur, as one of the primary lines of defence of the body (Sahu et al., 2007). Hence, the increase in WBC count in fish fed SWM diets support the fact that SWM contains immunological properties for juvenile Nile tilapia. This finding is consistent with other works that found an increase in WBC count in African catfish (*Clarias gariepinus*) when fed cricket meal (*Gryllus bimaculatus*) and fruit fly pupae (*Drosophila melanogaster*) (Okore et al., 2018; Taufek et al., 2018).

Erythrocyte counts were not changed by the diet and the highest value, in all treatments, was registered 6 hr after challenge. Total leukocyte counts as well the number of lymphocytes and monocytes in the Nile tilapia blood also were not changed by the diet, but these cells were seen to be, at least, two to four times the initial value mainly 6 hr after LPS challenge. Nevertheless, circulating lymphocytes were the most abundant type of leukocytes in all treatments. Although the function of the lymphocytes remains partially unclear (Scapigliati et al., 2018), we observed a reduction in the number of lymphocytes in fish blood 9 hr after challenge, suggesting the migration of these cells to the inflammation focus recruited by the defence mechanism as verified by Lamas et al., (1994) in *Oncorhynchus mykiss* blood and Garcia et al. (2009) in *Piaractus mesopotamicus* blood.

Fish fed 15% and 30% SWM diet showed the highest number of thrombocytes mainly 6 and 9 hr after challenge. Thrombocyte is an important cell involved in fish defence, representing a link between innate and adaptive immunity (Bozzo et al., 2007; Martins et al., 2006; Passantino et al., 2005; Tavares-Dias & Moraes, 2007). Bozzo et al. (2007), studying pacu (*Piaractus mesopotamicus*) and Corrêa et al. (2017), studying *O. niloticus* reported thrombocytes as the main cells present in the inflammatory exudate of fish, suggesting that thrombocytes act as defence cells in fish, besides presenting haemostatic functions. Nine hours after LPS challenge, the SWM was found to incite more production of thrombocyte in the circulation blood while leukocytes were returning to the baseline. According to Passantino et al. (2005), it is likely that thrombocytes release several inflammatory mediators with transfer information to other responding innate immune cells and antigen presenting cells in order to generate an adaptive response. It suggests that thrombocytes are also involved in the resolution phase of inflammation as it has already been registered recently in mammals (Slaba et al., 2015).

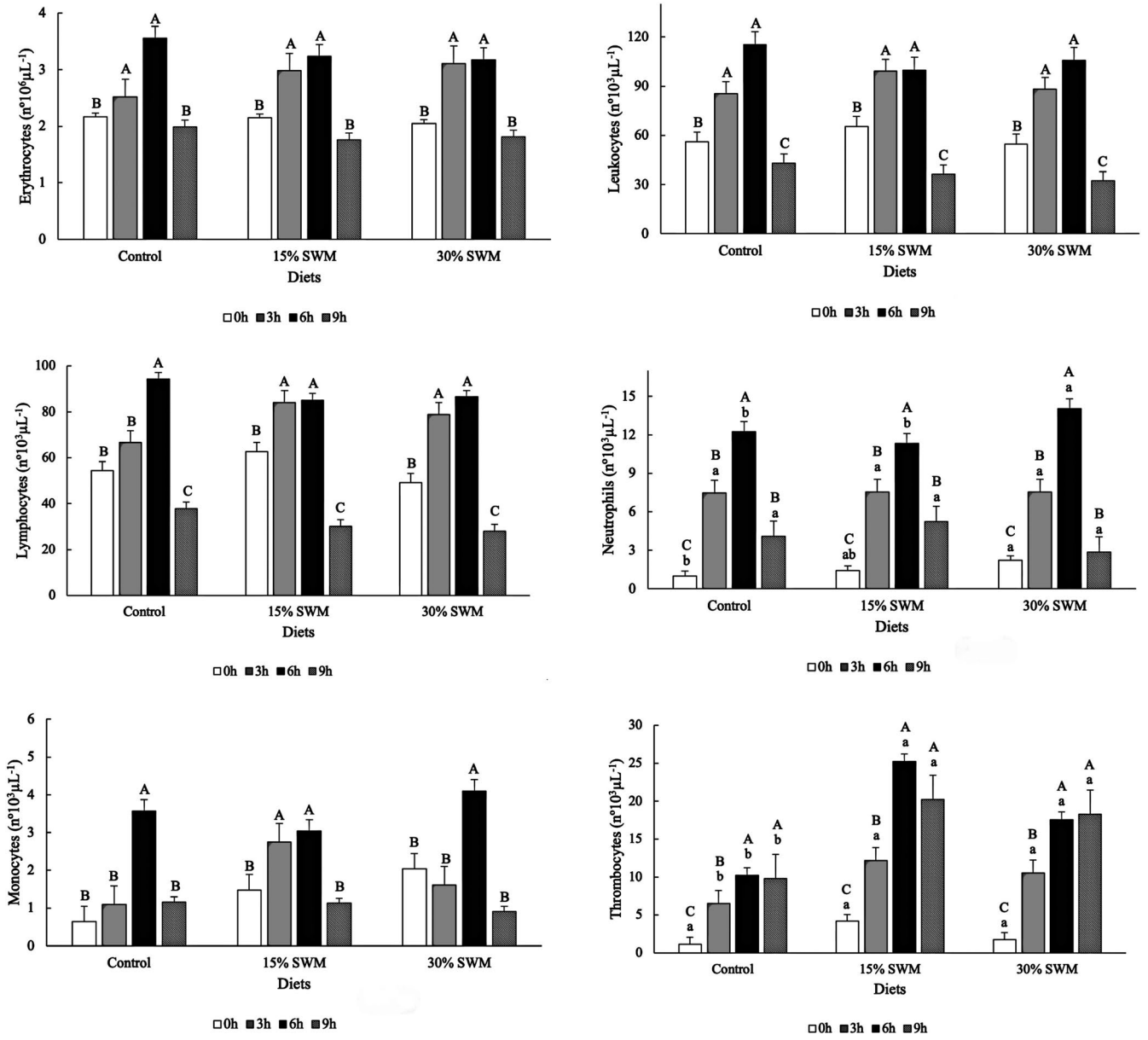

FIGURE 1 Number of circulating erythrocytes, leukocytes, lymphocytes, neutrophils, monocytes and thrombocytes in Nile tilapia at the end of the feeding trial (pre-challenge – time 0) and 3, 6 or 9 hr post-challenge with E. coli LPS ($n = 8$/treatment). Data are reported as the mean ± *SEM* with their standard deviation represented by vertical bars. a, b, c: significant difference ($p < .05$) among different treatment within the same period. A, B, C: significant difference ($p < .05$) within the same treatment among different periods. Letters were omitted when there was no statistical difference

The assessment of neutrophils migration in the blood system is an important health status indicator of fish population. Neutrophils are defence cells critical to the maintenance of homeostasis, and their values may vary according to the diet composition of the fish (Flajnik & Du Pasquier, 2004). Upon initiation of inflammation, neutrophils are the first cells recruited into the site of infection, followed by other inflammatory cell populations (Kourtzelis et al., 2017). According to Sebastião et al. (2011), fish infected with bacteria *Flavobacterium columnare* showed an increase in the number of lymphocytes and neutrophils. Our results confirmed these observations since the highest values of neutrophils were observed in fish fed 30% SWM diet 6 hr after challenge. This result may be related to the efficacy of fish that received SWM diets to mobilize circulating WBC faster than fish receiving the control diet to combat the infection.

Lysozyme is a widely expressed enzyme and an important index of the innate immunity of fish. Several studies indicate the supplementation of chitin and its derivatives could elevate lysozyme activity in fish (Chen & Chen, 2019; Shanthi Mari et al., 2014; Taufek et al., 2018). In fish, lysozyme is expressed in the hematopoietic organs such as spleen, liver, kidney, gills and mucosal tissues (Kim & Nam, 2015; Saurabh & Sahoo, 2008). The lysozyme activity is dependent on the intensity of stress, infection conditions, and/or

nutrition (Saurabh & Sahoo, 2008; Yildiz, 2006). However, to date, no studies have been found evaluating the immunomodulatory capacity of SWM against an immunological challenge for fish. In the present study, dietary inclusion of SWM influenced the innate immune responses of Nile tilapia. The results of LYZ-L showed higher lysozyme activity at 3h in fish fed 15% and 30% SWM compared to the control group. The results of spleen and head kidney lysozyme activity (LYZ-SP and LYZ-HK) had a high degree of variability among different times and treatments, probably due to the difference of lysozyme levels in all fish tissues. Furthermore, fish that were not challenged by *E. coli* LPS showed relevant lysozyme rates (time 0 hr). This was probably due to the presence of the monosaccharide N-acetyl-D-glucosamine present in the insect meal and its immunostimulatory potential (Kumar et al., 2019). It may have, in addition to the stress of the saline inoculation, stimulated the production of lysozyme.

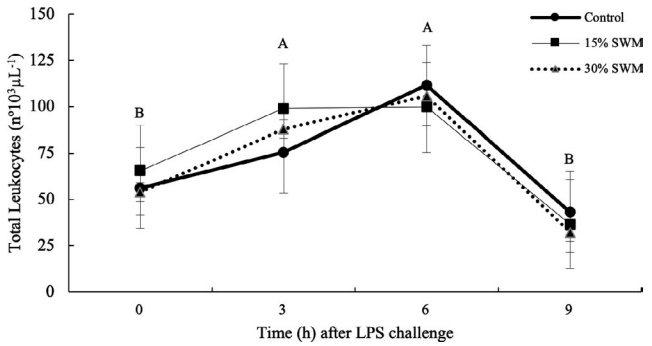

FIGURE 2 Total leukocytes in Nile tilapia at the end of the feeding trial (pre-challenge – time 0) and 3, 6 and 9 hr post-challenge with E. coli LPS ($n = 8$/treatment). The peak was reached 3 to 6 hr after LPS challenge gradually returning to the baseline values. Data are reported as the mean ± *SEM* with their standard deviation represented by vertical bars. A, B, C: significant difference ($p < .05$) within the same treatment among different periods

The serum lysozyme (LYZ-S) is used to measure the innate immune response on fish due to the bacterial activity and opsonin effects by activating the complement system (Bergljot, 2006). The highest serum lysozyme activity was observed in fish fed with both SWM diets mainly 3 hr after challenge. In addition, the levels of LYZ-S remained between 120 – 150 U/mL, similar to those reported for Nile tilapia (Aly et al., 2008; Yin et al., 2006). Jeong et al. (2020), has been reported in rainbow trout (*Oncorhynchus mykiss*) fed with 0%, 7%, 14%, 21% and 28% of a full fat *Tenebrio molitor* meal. Their findings showed that the serum lysozyme activity was higher compared with those fed control diets. Superworm contains chitin, which is the major structural component of insect exoskeleton and has been reported to exhibit immune stimulatory activity in fish (Gopalakannan & Arul, 2006; Henry et al., 2018; Powell & Rowley, 2007; Shanthi Mari et al., 2014).

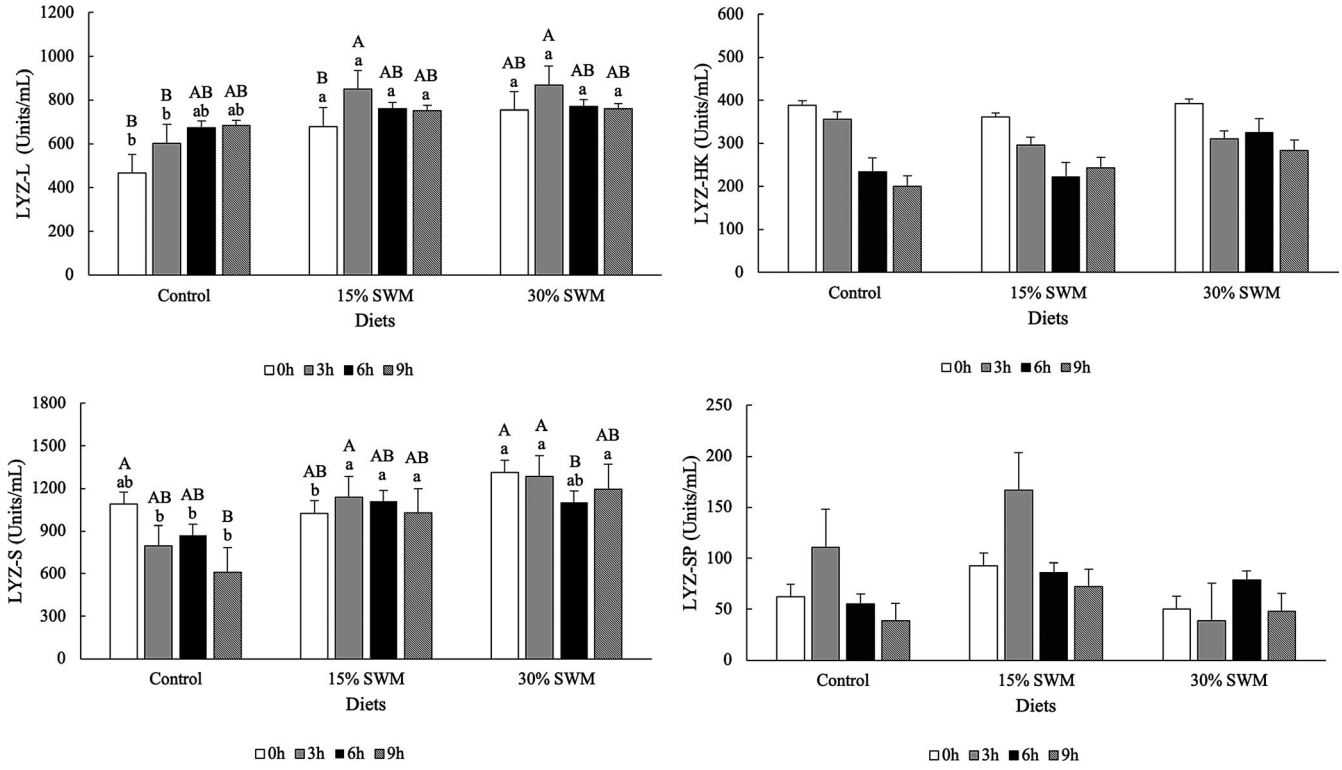

FIGURE 3 Lysozyme activity on head liver, kidney, serum and spleen in Nile tilapia at the end of the feeding trial (pre-challenge – time 0) and 3, 6 or 9 hr post-challenge with E. coli LPS ($n = 8$/treatment). Data are reported as the mean ± *SEM* with their standard deviation represented by vertical bars. a, b, c: significant difference ($p < .05$) among different treatment within the same period. A, B, C: significant difference ($p < .05$) within the same treatment among different periods. Letters were omitted when there was no statistical difference

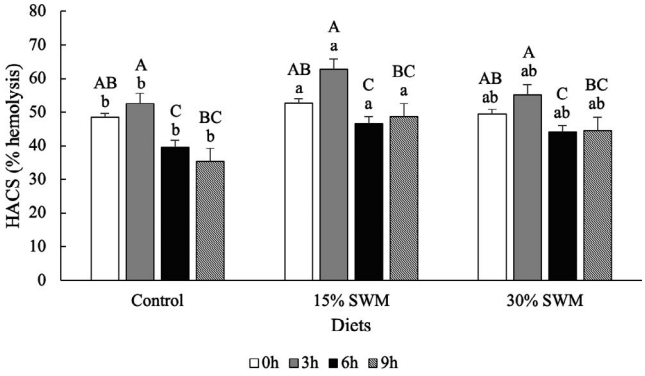

FIGURE 4 Serum haemolytic activity of alternative pathway of complement system in Nile tilapia at the end of the feeding trial (pre-challenge–time 0) and 3, 6 or 9 hr post-challenge with *E. coli* LPS ($n = 8$/treatment). Data are reported as the mean ± *SEM* with their standard deviation represented by vertical bars. a, b, c: significant difference ($p < .05$) among different treatment within the same period. A, B, C: significant difference ($p < .05$) within the same treatment among different periods

The complement system plays an essential role in innate and adaptive immunity alerting the host of the presence of pathogens. In this regard, the evaluation of the alternative pathway of the complement system is widely used as an immune indicator in teleost due to its function of the organism defence, such as cellular activation, phagocytosis, inflammatory response and lysis of bacterial cell (Biller-Takahashi et al., 2012). The activation of the complement system also contributes to the action of antimicrobial enzymes such as lysozyme and the development of an acquired immune response (Boshra et al., 2006). In turn, we are the first to investigate the haemolytic activity of complement system against *E. coli* LPS in the serum of Nile tilapia fed SWM diets. The results of this research showed that the SWM highly activated the alternative complement pathway of Nile tilapia compared to the control group. Furthermore, the highest values were detected at 3 hr after challenge for all feeding trials. Comparing the hours, there is no difference between 0 and 3 hr, the highest percentage of haemolysis. It is therefore likely that the peak of haemolysis occurred between these times.

Complement-mediated haemolytic activity has been reported to be significantly enhanced in fish fed with immunostimulants (Boshra et al., 2006). Lin et al. (2012) suggested the administration of nutraceutical substances may activate different parts of the immune system of fish and take advantage of different substances to enhance the immunity continuously. It may have the potential for counteracting stress-induced immunosuppression and render fish more resistant to disease. Our results are the first evidence that SWM can modulate lysozyme activity and the alternative complement system before and after challenge. The chitin from SWM would be recognized as a stimulator of the innate immune response. When chitin binds to receptors, it can be similar to pathogen stimuli that lead to producing a variety of cell surface receptors including macrophage mannose receptor, toll-like receptor 2 (TLR-2), interferon-γ (IFN-γ) and Dectin-1 (Lee et al., 2008, 2011). However, further studies are necessary to isolate and characterize the active compounds in SWM including its chitin content.

In summary, the present study provided some evidence that dietary superworm larvae meal could modulate the innate immune response of Nile tilapia. Combined considering the effects on growth performance, haemato-immunological response, and the bacterial resistance of fish, superworm larvae meal is a promising alternative protein source and at least 15% of SWM is recommended to be included in feeds for Nile tilapia. However, the innate immunity of fish is a complex subject and further studies need to be carried out to isolate and characterize the compounds in SWM that modulates the non-specific humoral immunity in Nile tilapia.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

No additional unpublished data are available.

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