ANGÉLICA PRISCILA DO CARMO ALVES

EFFECTS OF DIETARY *ZOPHOBAS MORIO* LARVAE MEAL ON THE GROWTH PERFORMANCE AND INNATE IMMUNE RESPONSE OF NILE TILAPIA JUVENILES (*OREOCHROMIS NILOTICUS*)

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Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração Produção e nutrição de animais não ruminantes, para obtenção do título de Mestre.

Prof Dr. Priscila Vieira e Rosa
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“The purpose of this glorious life is not simply to endure it, but to soar, stumble and flourish as you learn to fall in love with existence. We were born to live, my dear, not to merely exist.” - **Becca Lee**

“Cada dia que amanhece assemelha-se a uma página em branco, na qual gravamos os nosso próprios pensamentos, ações e atitudes. Na essência, cada dia é a preparação de nosso próprio amanhã.” - **Chico Xavier**

Abstract

Among plant origin feed, soybean meal is the most used protein source in aquaculture due to its high protein level and essential amino acid profile. However, sustainability in the use of soybean meal has been criticized due to uncontrolled deforestation and the excessive use of pesticides. In this way, alternative protein sources have been studied in order to replace, totally or partially, the use of this ingredient in aquaculture. Super worm larvae meal (*Zophobas morio*) therefore, appears as a promising protein source, due to the high content of proteins, essential amino acids, lipids, and because they need small areas for their production, especially when compared to other crops such as soybean. In addition, it is a chitin source, a polysaccharide present in the exoskeleton of insects, linked to modulation of the immune system. From this, the influence of diets containing super worm larvae meal in total or partial replacement of soybean meal was evaluated on the growth performance and innate-immune response of juveniles of Nile tilapia (*Oreochromis niloticus*) in the present study. Three diets containing 0 (control diet), 15 and 30% super worm larvae meal (SWM) were formulated. Nile tilapia were randomly divided into 3 groups with 4 replicates in each group. Fish from each group were fed one of three experimental diets for 12 weeks. The growth performance, hematological parameters and immune response of juveniles were determined at the end of the feeding period, as well as 0, 3, 6 and 9 h after the challenge of E. coli LPS. This dissertation is divided into two parts; the first part is presented in the theoretical reference form that was formulated in order to provide basic information to support the understanding of this research. The second part relies on the manuscript, where we detail the research, the material and methods used as well as its results and the discussion of the data obtained.

Keywords: Insect meal, *Zophobas morio,* Nile tilapia, Growth performance, Immune response.

RESUMO

Dentre os alimentos de origem vegetal, o farelo de soja é a fonte proteica mais utilizada em aquicultura em função do seu alto nível proteico e perfil de aminoácidos essenciais. Contudo, a sustentabilidade no uso do farelo de soja vem sendo criticada devido ao desmatamento desenfreado e ao uso excessivo de pesticidas. Desta forma, fontes proteicas alternativas vem sendo estudadas a fim de substituir de forma total ou parcial o uso deste ingrediente em aquicultura. A farinha de larvas de tenébrio gigante (*Zophobas morio*) surge, portanto, como fonte proteica promissora, devido ao alto teor de proteínas, aminoácidos essenciais, lipídios e por necessitar de pequenas áreas para sua produção, principalmente quando comparamos à outras culturas como a soja. Além disso, é fonte de quitina, um polissacarídeo presente no exoesqueleto dos insetos, ligado a modulação do sistema imunológico. A partir disso, no presente estudo avaliou-se a influência de dietas contendo farinha de larvas de tenébrio gigante (*Zophobas morio*) em substituição total ou parcial de farelo de soja no desempenho e resposta imune inata de juvenis de tilápia do Nilo (*Oreochromis niloticus*). Para isso, foram formuladas três dietas contendo 0 (dieta controle), 15 e 30% de farinha de larvas de tenébrio gigante (SWM). As tilápias do Nilo foram divididas aleatoriamente em 3 grupos com 4 repetições em cada grupo. Os peixes de cada grupo foram alimentados com uma das três dietas experimentais por 12 semanas. O desempenho, os parâmetros hematológicos e a resposta imune dos juvenis foram determinados no final do período de alimentação, bem como 0, 3, 6 e 9 h após o desafio de LPS de *E. coli*. Este trabalho de dissertação está dividido em duas partes; a primeira está apresentada em forma de referencial teórico que foi formulado a fim de fornecer informações básicas que apoiem o entendimento desta pesquisa. A segunda parte conta com o artigo, onde nós detalhamos a pesquisa, o material e métodos utilizado bem como seus resultados e a discussão a parti dos dados obtidos.

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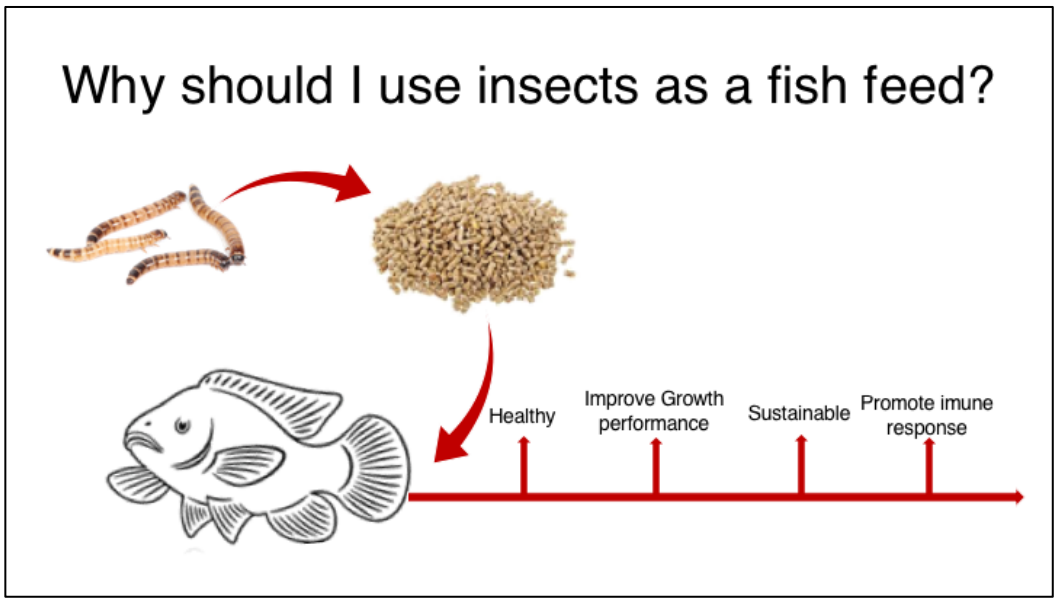
Palavras-chave: Farinha de inseto, *Zophobas morio*, Tilápia do Nilo, Desempenho, Resposta imune.

INTERPRETIVE SUMMARY AND GRAPHICAL ABSTRACT

Legitimate concerns are being raised about aquaculture future and its actual and finite protein sources. As an alternative to fish and soybean meal, the objective of this study was to investigate the effect of insect meal (*Zophobas morio*) in juvenile Nile tilapia diets in growth performance and also, investigate hematological parameters and immune response after *E. coli.* LPS challenge. Our results provided the first indication of positive immune system modulation by *Z. morio* in Nile tilapia diets by the higher activity of the complement system in serum and in liver lysozyme. Also, fish fed with the insect meal showed an improvement of growth performance compared to the control group.



The super worm larvae meal could effectively replace soybean meal in Nile tilapia diets with positive effects on growth performance and immunological parameters.

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LIST OF ABBREVIATIONS AND ACRONYMS

AOAC	Association of Official Agricultural Chemists
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization of the United Nations
IBGE	Instituto Brasileiro de Geografia e Estatística
LPS	Lipopolysaccharides
NRC	National Research Council
ONU	Organização das Nações Unidas
SWM	Super worm larvae meal

SUMMARY

1. OVERVIEW

Nile tilapia (*Oreochromis niloticus*) was chosen in this research due to its importance in global aquaculture. Nile tilapia is the most important fish cultured in Brazil accounting for 50% of the national aquaculture production (FAO, 2018). The importance is due to its favorable characteristics as rusticity, high growth rate, annual spawning and good quality of the meat with great acceptance in the national market.

The use of insects on the aquaculture production is a promising alternative due to its nutritional value, efficient breeding system, nutraceutical properties (antimicrobial peptides and chitin), and its sustainability, since it requires less water, space and uses by-products as substrate. Moreover, the insect's antimicrobial peptides are effective against many pathogens and, also they act on the modulation of the immune response and because of it, is becoming notorious.

The present work provides research findings about the nutritional aspects of super worm larvae meal in Nile tilapia diets and also discuss its immunomodulatory function against the challenge by LPS E. coli at different times of administration. We also discuss how insect meal present in diets acts on the leukocyte mobilization in the bloodstream in response to the infectious process.

The aim of this theoretical framework is to provide a basic background information in order to support the understanding about the manuscript discussed below.

2. THEORETICAL FRAMEWORK

2.1 Nile tilapia

Nile Tilapia (*Oreochromis niloticus*) belongs to the order of the Perciformes, Cichlidae family, and Pseudocrenilabrinae subfamily. This species is originated in Africa Middle East and it is widespread in tropical and subtropical regions (CARVALHO, 2006). In Brazil, the species was introduced in 1970 through a program of the Departamento Nacional de Obras Contra a Seca (DNOCS) called the "Programa Brasileiro de Produção de Juvenis" with the objective of populating public reservoirs in the Northeast region. Due to the high prolificity and sexual precocity of the species, its dissemination occurred in a disordered way, which caused problems of genetic anomalies due to the difficulty in avoiding mating among parental individuals (OLIVEIRA et al., 2011).

The expansion of tilapia production in Brazil occurred only in 1990 and, according to FAO (2016), this is the most produced species of fish in Brazil reaching in 2015 the annual production of 219 thousand tons. The importance of the production of tilapia in this country is due to its zootechnical characteristics, such as the acceptance of balanced diets, rusticity under the breeding condition, high growth rate, annual spawning and good quality of the meat with great acceptance in the national market (POPMA & MASSER, 1999).

The cultivation of tilapia occurs in practically all Brazilian states, with the highest production recorded in the southern region of the country (IBGE, 2016). The species presents an omnivorous habit with great acceptance of artificial feeds at all stages of life (Santiago et al., 1987; Schwarz et al., 2010). Moreover, among the most cultured fish species, it is the one that best resists temperature variations, low dissolved oxygen concentration and high ammonia concentration in water (POPMA & PHELPS, 1998). Among these reasons, Nile tilapia is highly indicated for intensive breeding in Brazil.

Technification and diversification of tilapia cultivation, however, constitute one of the major problems of the aquaculture sector (FAO, 2018). The occurrence of diseases due to the presence of pathogens in the environment has a negative impact on supply and demand in trade, as well as the commitment of ecosystems and also, people working directly with these organisms (LINO, 2012). The growing interest in the cultivation of these species has led to an increase in research regarding management, prophylaxis and adequate nutrition of these organisms. However there is still a need for more information about the immunological functions of these teleosts, as well as the mechanisms that involve the protection of the body and its link to nutritional aspects. Thus, the development of diets that would promote increased resistance of fish to infections through modulation of the immune system and manipulation of nutrients are fundamental for the development of aquaculture.

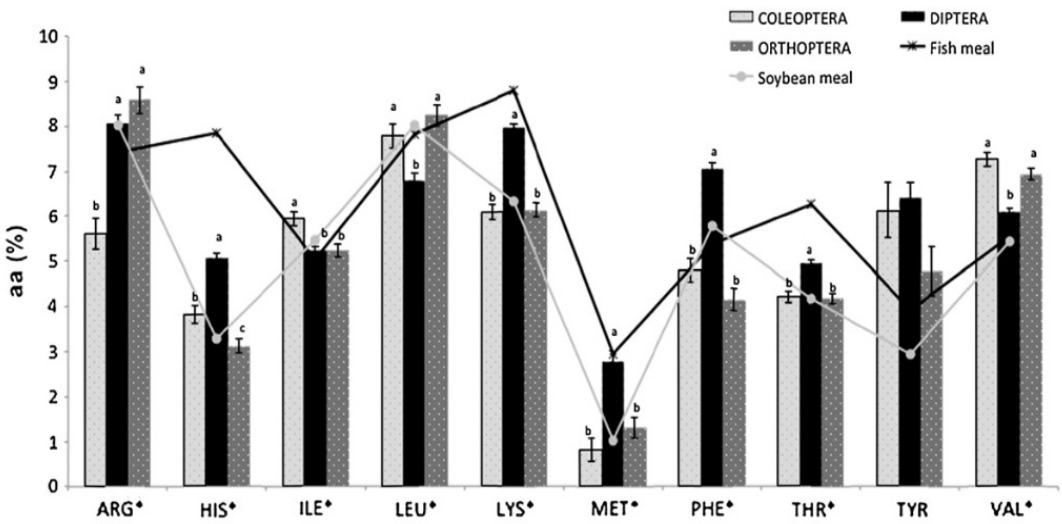
2.2 Insect meal as a fish feed

The world population is estimated to reach almost 10 billion by 2050 with a 60-70% increase in consumption of animal products (UNITED NATIONS, 2017). This increase in consumption will require different resources, with food being the most challenging problem. The availability of conventional feed such as fishmeal will be a limited resource that will not be produced in the future in sufficient quantities to support production growth. Also, the soybean meal, one of the most important sources of protein, is criticized due to the lack of sustainability and excessive use of pesticides.

Aquaculture is a sector with the greatest expansion in the production of animal protein and this is due to the concern in the ingestion of food beneficial to the health and by the financial contribution made in the sector in the last years in order to leverage their production (FAO, 2018). Studies have been done to use soybean meal as a primary source of fish feed protein (ESPE et al., 2006; GATLIN et al., 2007), however, the presence of antinutritional factors (COLLINS, 2014), its palatability (PAPATRYPHON & SOARES, 2001) and potential inflammatory factor of the digestive tract (MERRIFIELD et al., 2011) are the main negative reasons, since they can cause problems of digestibility and decrease in the absorption of nutrients.

The Edible insect's program from the Food and Agriculture Organization of the United Nations (FAO, 2013) suggests the use of processed insects in the flour form for the production of alternative animal feed. The reason for using insect meal as a partial substitute for fish meal or soybean meal comes from the easy way of production of these invertebrate animals, the short life cycle (CARVALHO et al., 2016) and the nutritional characteristics, such as the high protein content of large biological value (figure 1) ranging from 42-63% (MAKKAR et al., 2014). In addition of being rich in proteins, insects are sources of fiber and minerals, lipid contents up to 36% (MAKKAR et al., 2014) and are already part of the natural diet of freshwater and marine fishes (Howe et al. , 2014, WHITLEY & BOLLENS, 2014).

Figure 1. Differences of percentage of essential amino acids between insect orders (using fishmeal and soybean meal as reference)



Source: Barroso et al. (2014)

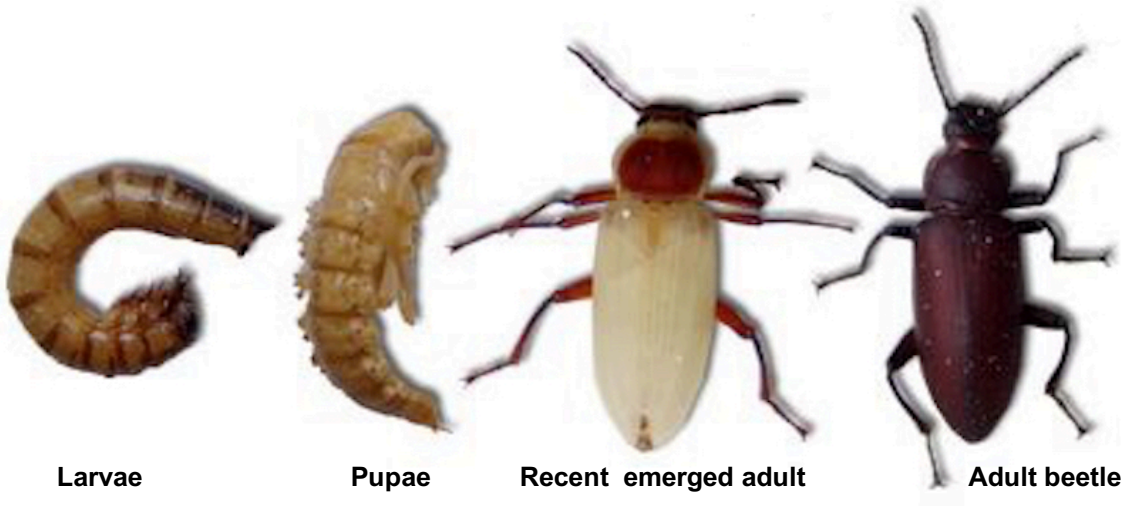
According to a study by Lock et al. (2016) with 100% of substitution of fish meal for black soldier fly (*H. Illucens*) larvae in salmon diets (*Salmo salar*), no significant differences were observed in the performance and weight gain of the animals. Another study carried out with Dourada (*Sparus aurata*) replacing 25% of the fishmeal by *Tenebrio molitor* larvae meal in the diets, found improvement in feed conversion and protein efficiency without negative effects on animal weight gain (Piccolo et al. 2017).

Chitin is a linear polysaccharide consisting of β(1-4) linked of N-acetyl-D- glucosamine, one of the most abundant polysaccharides in nature, and a common constituent of insect exoskeletons and fungal cell walls. Olsen et al. (2006) in a study with *Salmo salar* pointed out that chitin has beneficial effects on fish, acting as a prebiotic, acting on the pathogenic bacteria of the gastrointestinal tract of fish.Although there is a general literature on nutritional parameters of many teleosts, little is known about the influence of alternative feeds such as insect meal and its chitin content on modulating the immune system of commercial value species. Thus, the knowledge about its immunological variables can provide relevant information of changes in the physiological state of the cultivated species to the producer.

2.3 Super worm meal and its potential incorporation in fish diets

The super worm (*Zophobas morio*) (figure 2) is an insect belonging to the Coleoptera Order and Tenebrionidae Family (Table 1). The insects of this order occupy almost all the ecosystems, being about 30,000 species cataloged in Brazil (COSTA LIMA, 1952; FERREIRA, 2010). This extensive distribution is due to the morphophysiological adaptations of these insects in terms of the alteration of their natural habitats and the growing food trade since more than 500 species of this family are found associated to the degradation of stored grains (MOUND, 1989; HAINES, 1991; FERREIRA, 2010).

Figure 2. Super worm (*Zophobas morio*) life cycle



Source: copyright 2005 the chameleon's dish

The *Z. morio* is widely used in animal feeding, especially for insectivores such as fish and birds, due to its ease of management and nutritional value in its larval phase (Schulte, 1996). The crude protein composition of the super worm meal varies from 50-80% and it is very similar to the 73% Fishmeal and the 50% soybean meal. However, this high protein value found in insect meal is largely due to the presence of chitin content in the exoskeleton of the insects, since it has nitrogen in its composition, which is accounted for and considered as a crude protein when it is measured by the Kjedahal method. In the dry matter basis, the insect meal has a high lipid content ranging from 39-43% (BARKER et al., 1998; JABIR et al., 2012; RUMPOLD and SCHLÜTER, 2013), but poor in minerals such as calcium and phosphorus (GHALY and ALKOAIK, 2009). These contents found by different authors can be influenced according to the composition of the food provided to the insects, as well as the microclimate, environment and sex (OONINCX & VAN DER POEL, 2011).

These small arthropods is minimally space intensive and do not need large areas, especially when compared to other crops such as soybeans. The use of insects contributes to the natural recycling of nutrients and could be a source of high quality animal protein derived from environmentally sustainable technology.

Table 1. Super worm (*Zophobas morio*) general review

Scientific name:	*Zophobas morio* (COLEOPTERA: Tenebrionidae)
Distribution:	Central and South America, grown in the USA and Europe.
Size:	Adult beetle: 30 to 34 mm in length, black. Pupae: 28 to 30 mm in length, white. Eggs: 1.2 to 1.4 mm in length, vitreous white. Larvae: Phase 1: 2 to 2.5 mm birth, yellow Phase 2: 60 mm last larval stage, approximate weight 1.5 g, 5 to 6 mm wide, yellow.
Sexual dimorfism:	The female has a larger length than the male, however the male head is slightly larger
Reproduction	The female spawns about 60 eggs per reproductive event.
Life time:	Adult beetle: 1 year
Life style (adults):	Night, daytime in dark places.

Source: FRIEDERICH and VOLLAND,1981 cited in SCHULTE 2006

Jabir et al. (2012) fed juvenile tilapia (*O. niloticus*) with diets containing 0, 25, 50, 75 and 100% of Zophobas morio meal in replacement of fish meal and observed that total replacement of fish meal by insect meal However, the diet containing 25% did not compromise the growth and body composition of the animals. Another study by Su et al. (2017) evaluating the effect of the inclusion of flour of *Tenebrio molitor* - another insect of the family Tenebrionidae, in diets for Catfish (*Pelteobagrus fulvidraco*) challenged by *E. ictaluri* demonstrated that the partial replacement of fishmeal by insect meal increases the plasma activity of the enzyme superoxide dismutase, lysozyme and immunoglobulin M, which shows the correlated action between insect meal and the immune system of animals.

In the literature, as presented above, it is possible to find work using *Zophobas morio* meal as a partial substitute for fishmeal in the feeding of some species of commercial value without compromising animal growth (JABIR et al., 2012; HENRY et al. al., 2015). However, to date, no studies have been found evaluating the immunomodulatory capacity of this insect's meal against an immunological challenge for fish.

2.4 General aspects of fish immunity

The knowledge of the nutritional needs that promote growth performance in teleosts is already well elucidated. However, more research needs to be done in order to define alternative sources and levels of inclusion of nutrients capable of promoting resistance and immunocompetence in face of the various health challenges. The immune system of fish is physiologically similar to most vertebrates, it is divided into innate immunity and adaptive immunity (URIBE et al., 2011). In both divisions, fish use physical, cellular and humoral mechanisms to confer protection against foreign substances such as microorganisms and toxins, responding to any exogenous or endogenous factor that stimulates the cells that make up this system (BAYNE and GERWICK, 2001).

The defense system includes the scales, gills, mucus and epithelial tissue (SHEPARD 1994, ELLIS, 2001). In addition, the immune system relies on several enzymes, plasma proteins and phagocytic cells present in tissues, such as macrophages, monocytes, and neutrophils that are recruited at the site of infection mainly by inflammatory cytokines (AOKI, 2008). The innate immune system is a fundamental mechanism for fish survival since it comprises the general defense system of the organism.

Among lytic enzymes triggered during the inflammatory process, lysozyme plays an important role in protecting against microbial invasion as a defense molecule of the innate immune system (SAURABH and SAHOO 2008). This enzyme is found in the bodily fluids of teleosts and has an antibacterial action acting mainly in the elimination of Gram-negative bacteria (MAGNADOTTIR, 2006). The activity of lysozyme is directly linked to the individual's conditions and it may vary according to age, water temperature, nutrition and their measurement may be an indicator of the animals' immunological condition (SAHOO, 2008). According to Møyner et al. (1993) Atlantic salmon (*Salmo salar*) when infected with *Aeromonas salmonicida* has a relevant lysozyme response prior to activation of the acquired response. It demonstrates the importance of the innate immune system in fish and the need to evaluate this response qualitatively and quantitatively.

The complement system is an important component of the innate immune system, being the main humoral mediator of the inflammatory process with the antibodies (ITURRY-YAMAMOTO and PORTINHO, 2001; LIU et al., 2016). It is composed of cytoplasmic and membrane proteins responsible for the biological processes of elimination of pathogens, pro-inflammatory functions, leukocyte chemotaxis, opsonization, histamine release from mast cells, basophils and active oxygen species by leukocytes (ADLER et al., 1986).

The activation of proteins from the complement system can occur through three pathways: the alternative pathway (more developed in teleosts, activated by a large number of molecules), the classical pathway (mediated by antibodies) and the lectin pathway (triggered by mannose-binding lectin) (NAKAO and YANO, 1998). Wang et al. (2009), analyzed the expression of the genes involved in the alternative pathway of the complement system in *Amphioxus Branchiostoma belcheri* challenged and re-challenged by three bacteria *Vibrio anguillarum*, *E. coli* and *Staphylococcus aureus*. Through the real-time PCR assays of the key genes (Bf, C3 and C6) involved in the alternative pathway, they found that the re-exposed animals had the expressions of these genes significantly high and reached the peak of gene expression in comparison to the exposure to the same bacteria. These results give rise to a probable link between the components of the alternative pathway and the immunological memory of this species.

The lymphoid tissues are usually associated with organs that may have an immuno-endocrine function, acting in the production of antibodies and catecholamines due to the absence of lymph nodes and bone marrow in teleost fish (URIBE et al., 2011). The major lymphoid organs of teleosts are the thymus, head kidney, spleen and liver (TORT et al., 2003; ZAPATA et al., 2006; URIBE et al., 2011). The spleen is an organ rich in leukocytes, which makes it a major site of substance phagocytosis and an important role in hematopoiesis (COSTA 2007). The thymus in teleosts is the site of differentiation and maturation of T lymphocytes (RAZQUIN et al., 1990; FALKON, 2007). The head kidney, in turn, plays the role of bone marrow, dealing with hematopoiesis in its cephalic portion and filtration of metabolites in its posterior portion (KARDONG, 2011). In addition to these organs, fish have gut-associated lymphoid tissues (GALT) that are formed during larval development and play a key structural role for innate and defense cells such as Natural-Killer cells, dendritic cells, thrombocytes, macrophages, lymphocytes among others (BILLER-TAKAHASHI and URBINATI, 2014).

The liver is responsible for producing the proteins of the complement system, the proteins of the acute phase and the homeostatic control of glucose, being a relevant organ in the production of innate humoral defense cells of fish (DALMO, 2005; COSTA, 2007). As in mammals, the liver acts on the metabolism of nutrients produces bile and it is responsible for metabolic homeostasis from the processing of nutrients such as lipids and proteins (CARDOSO et al., 2015). In addition, it is responsible for the synthesis of reactive proteins that is a fundamental part of the cascade of events of the complement system acting on the synthesis of plasma proteins (FLETCHER, 1981; CARDOSO et al., 2015).

Henry et al. (2018) fed juveniles of European Sea Bass (*Dicentrarchus labrax*) for six weeks with larvae of *Tenebrio molitor* and after the feeding period, the fish were challenged with gram-positive bacteria (*Micrococcus luteus*). The animals presented significant anti-inflammatory responses in the ceruloplasmin, myeloperoxidase and nitric oxide analyzes. The antibacterial activity of lysozyme and the inhibition of serum trypsin, generally linked to the antiparasitic activity of fish, were significantly increased. This may have occurred due to the similarities in the composition of the exoskeleton of parasites and insects that may, according to the author, act as immunostimulatory, potentially increasing antiparasitic activity.

A study with Yellow Catfish (*Pelteobagrus fulvidraco*) was carried out using 4 diets containing 0%, 25%, 50% and 75% of flour of *Tenebrio Molitor* in substitution of fishmeal. In this study, Su et al. (2017) verified through the real-time PCR technique performed in the kidney, spleen and liver that genes related to the immunological system Class II Histocompatibility Complex (MHC II), interleukin-2 (IL- 2), Cyclophilin A (CypA), Immunoglobulin M (IgM) and Hepcidin (HE) were found to be higher when compared to the control diet before and after the bacterial challenge (*Edwardsiella ictaluri*). The authors further report that the results indicate that IgM, MHC II, and IL-2 were involved in altering the immune status of the yellow catfish and could at least in part promote the innate immune response of the animals that received the insect-containing diets.

In addition to nutrition, several factors, both external and internal, can influence the immune response of fish, such as stress, age, water quality, storage density, handling, and temperature changes (DAVIDSON et al., 2009; BARROS et al., 2014) . These limitations may contribute to the suppressive effects of this type of response, however, the use of food additives and immunostimulants may increase their efficiency (MAGNADOTTI, 2010). In recent years the use of immunostimulants has been widely used, however, the understanding of the mechanisms involved in the immune response in fish is limited (ROMBOUT et al., 2010). The evaluation of the immune system of the fish linked to its nutrition can be a good parameter of the health of the animals. Also, this information opens new possibilities and strategies of immunomodulation of the organism by feeding.

3. GENERAL CONCLUSION

Research about nutrition linked to immunological defense are one of the most helpful tools for a better understanding of how feed, besides the quality of the nutrients, may also improve growth and healthiness. Although many studies carried out in the past decades improving considerably the knowledge about fish nutrition, more research needs to be done in

order to elucidate how some nutrients would help during a bacterial challenge. Legitimate concerns are being raised about aquaculture future and its actual and finite protein sources. This context stimulates the search for alternative protein sources which would promote the ideal development of the animals, improve healthiness, besides favoring the sustainability of the aquaculture production in economic and environmental level.

Hemato-immunological studies as a differential leukocyte counts, lysozyme and hemolytic activity as well as the parameters presented in the manuscript below have indicated that insect meals act modulating the immune system promoting host resistance to infections. Since the nutritional conditions of the animals are directly linked to their immune response and, it plays a fundamental role in the defense of the body, it is necessary to understand the immunomodulatory activity of the substances present in the insect meal against a stimulus of the immune system. For this reason, how feeding can influence the immune system is the target of interest on this study.

Due to the great diversity of fish being cultured along with a lack of understanding regarding its immunology, nutrition, and its relations to growth performance and health, additional research is warranted in this field.

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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 h post-challenge. The results revealed that dietary inclusion of SWM had no negative effects on growth performance of tilapia ($p < 0.05$). The moisture and lipid content increased ($p < 0.05$) 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 ($p < 0.05$) in those fed SWM diets mainly 6 hours after challenge. Increased lysozyme activity in serum and liver was registered in both groups fed SWM ($p < 0.05$). Fish fed SWM diets showed an increase in HACS in which higher hemolysis was registered at 0 h and 3 h ($p < 0.05$). 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: Insect meal; *Zophobas morio*; Nile tilapia; Growth performance; Immune response; Cellular counts.

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 (Makkar, Tran, Heuzé, & Ankers, 2014; van Huis, Dicke, & van Loon, 2015). 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, Razak, & Vikineswary, 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 (Jabir et al., 2012; Henry, Gasco, Piccolo, & Fountoulaki, 2015). Many studies have focused on a nutritional perspective, searching for ingredients which would promote the ideal development of fish, besides favoring 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, have been shown to activate the immune response in mammals and fish (Gopalakannan & Arul, 2006; Henry, Gasco, Chatzifotis, & Piccolo, 2018; Jiang, Bao, Merzendorfer, & Yang, 2019; Kumar, Murthy, Patil, Doddamani, & Patil, 2015; Kumar, Kaur, & Kamilya, 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 defense against pathogens. Nutraceutical substances can increase resistance to infectious diseases by enhancing non-specific defense mechanisms (Zhang, Gong, Yu, & Yuan, 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, Lunde,

Engstad, & Robertsen, 2003), including several studies in Nile tilapia (Ha, Gonçalves, Sousa, Biller-Takahashi, & Takahashi 2017; Lazado, Skov, & Pedersen, 2016; Liu et al., 2016). It can trigger fierce immune response in animals that leads to a signaling cascade including humoral and cellular components e.g., 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 h), and stored -5°C. Ingredients composition and proximate analyses of the diets and SWM are shown in Table 1.

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 fiber 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 5kg/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).

2.2 Fatty acids determination

For fatty acid (FA) analysis of the diets, lipid extraction and fatty acid (FA) profile were analyzed according to Araújo et al. (2017). For each sample, total lipid was extracted with chloroform and methanol using the Folch method (1957) with minor modifications. FA profile was analyzed 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^{-1}). 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 fiberglass 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 h.

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 h of fasting. Three fish per tank (n = 12/treatment) were randomly sampled after being euthanized with an overdose of 250 mg L^{-1} 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^{-1} of body weight; L2880, Sigma, USA) at swim bladder as previously described by Matushima & 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 hours, injected with saline solution) and then, 3, 6, and 9 hours 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 hematological procedures. The second blood set was added to the EDTA-free tube and centrifuged at 4800g 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 analyzed according to AOAC methods (2012). The samples were dried to a constant weight at 105°C for 24 h 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, Vincken, Van Den Broek, Fogliano, & Lakemond (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, Lawrence, & Swakon (1993). Ash content was determined by incineration in a muffle furnace at 550°C for 12 h.

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 100x in immersion oil on blood smears (two slides per fish, 5 to 10 µL blood drop), fixed in methanol, and colored 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^{-1}.

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, Sharp, Secombes, & Robertsen (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, Rosa, & Gatlin, 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: $[(\Delta_{\text{absorbance (4-1 min)}}/3)/0.001]$ x 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 Hemolytic activity of the alternative complement system

Hemolytic activity of the alternative complement system (HACS) was measured using sheep red blood cells as targets (Sutili, Gatlin, Rossi, Heinzmann, & Baldisserotto, 2016). Briefly, tilapia serum (10 µl) was incubated at room temperature for 1 hour with 2%-sheep blood (25 µL). After the incubation time, 100 µL of cold-PBS was added and centrifuged at 2500 x g for 5 minutes 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 hemolysis

of the saline solution for each sample was calculated using the absorbance of a total hemolysis control (distilled water + sheep blood) and spontaneous lysis (PBS 0.1 M + sheep blood) according to the following calculation: % hemolysis = $[(A_{405}\ sample - A_{405}\ no\text{-}hemolysis)/(A_{405}\ total\ hemolysis - A_{405}\ no\text{-}hemolysis)] \times 100$.

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).

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 h) and 3, 6, and 9 h 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.

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

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.

3.2 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) ($P > 0.05$). Also, the treatments did not interfere with fish mortality values before or after challenge ($P > 0.05$) (Table 3).

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 $^{-1}$)	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

Initial fish average weight was of 3.00 ± 0.20.
The values are means of four replicate tanks, n = 12 individuals/treatment. S.E.M. is pooled of standard error.

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 ($P < 0.05$) (Table 4). Ash and protein contents of fish fed 30% SWM were lower than in the other groups ($P < 0.05$).

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

The values are means of four replicate tanks, n = 12 individuals/treatment. S.E.M. is pooled of standard error. Means in the same row with letters indicate significant differences ($p < 0.05$).

3.4 Cellular counts

Total leukocyte and the numbers of lymphocytes, monocytes, and erythrocyte were not changed by the diet ($P > 0.05$) (Figure 1). However, fish fed 30% SWM diet showed a higher number of neutrophils at 6 hours after challenge compared to the control group ($P < 0.05$). Also, fish fed 15% and 30% SWM diet showed a higher number of thrombocytes mainly 6 hours after challenge compared to the control group ($P < 0.05$). Comparing the hours (0, 3, 6, 9 h), there was an increase in WBC in all treatments, where the highest values were observed between 3 and 6 hours, gradually returning to the baseline values after 9 hours in most counts ($P < 0.05$) (Figure 2). Differential leukocyte counts were characterized by the predominance of lymphocytes.

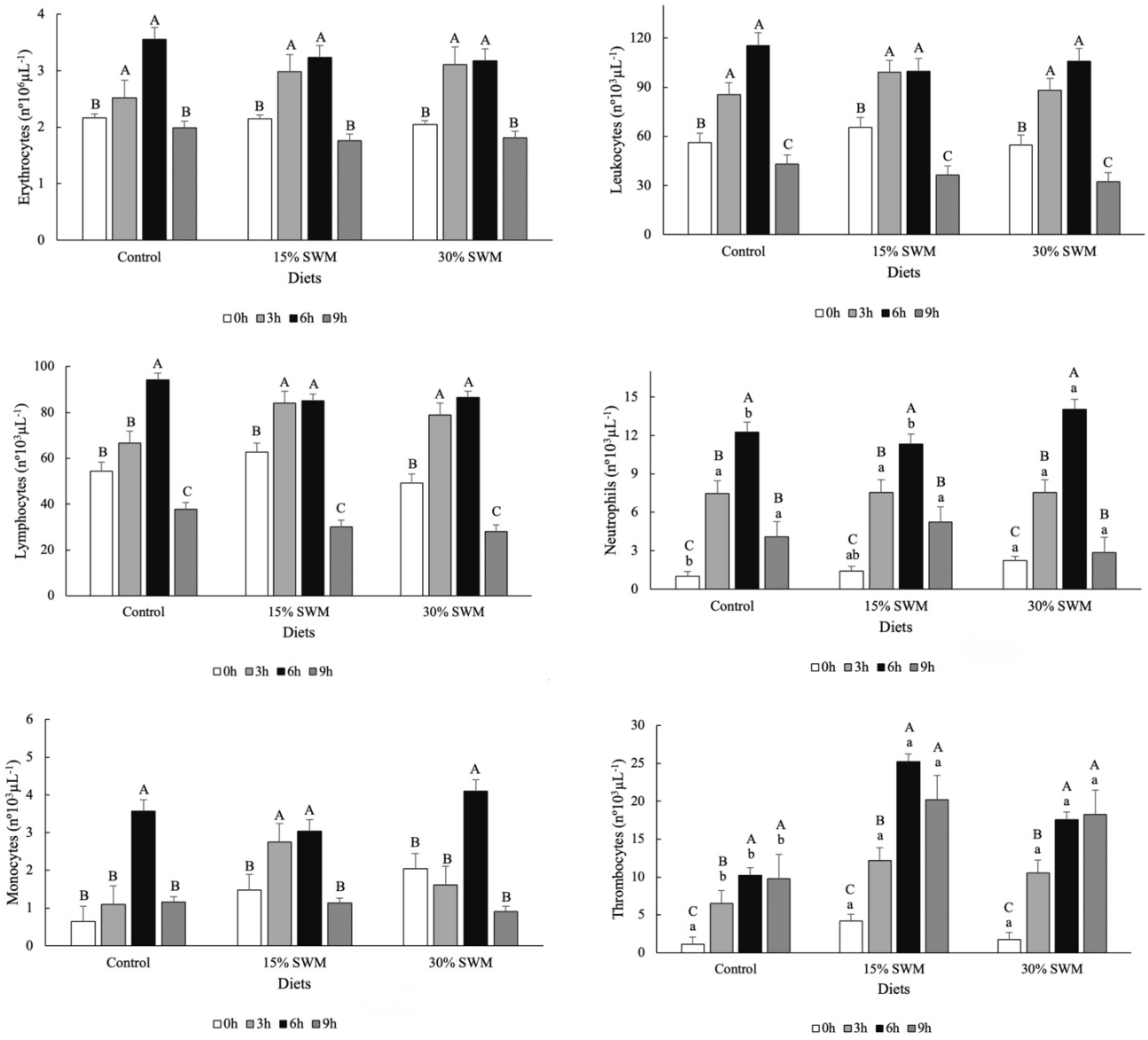

Figure 1. Number of circulating erythrocytes, leukocytes, lymphocytes, neutrophils, monocytes and thrombocytes in Nile tilapia at the end of the feeding trial (pre challenge – time 0) and 3, 6 or 9 h post challenge with *E. coli* LPS (n= 8/treatment). Data are reported as the mean ± SEM with their standard deviation represented by vertical bars. a, b, c: significant difference ($P < 0.05$) among different treatment within the same period. A, B, C: significant difference ($P < 0.05$) within the same treatment among different periods. Letter were omitted when there was no statistical difference.

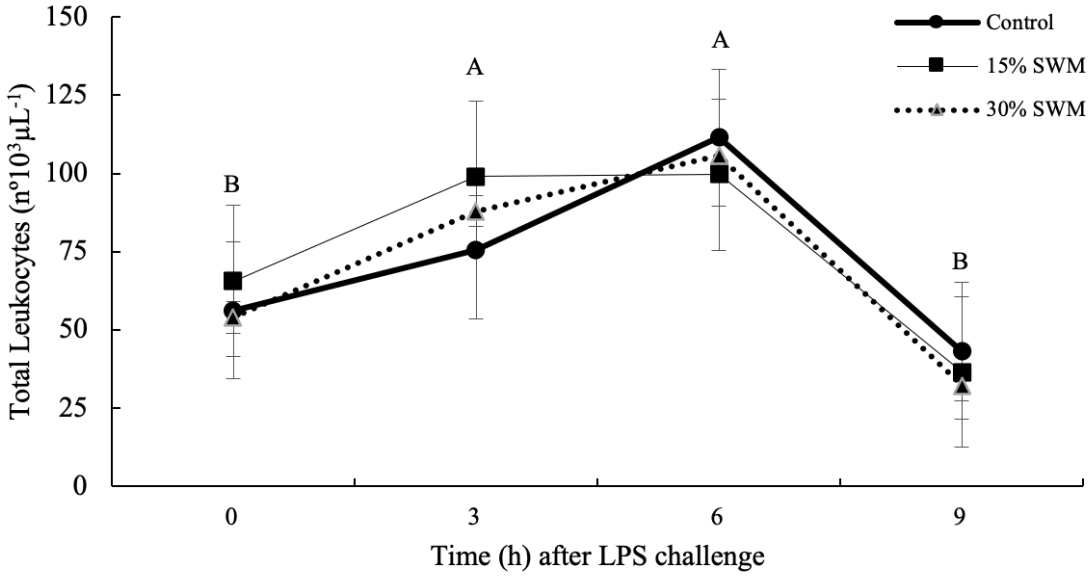

Figure 2. Total leukocytes in Nile tilapia at the end of the feeding trial (pre challenge – time 0) and 3, 6 and 9 h post challenge with *E. coli* LPS (n= 8/treatment). The peak was reached 3 to 6 h after LPS challenge gradually returning to the baseline values. Data are reported as the mean ± SEM with their standard deviation represented by vertical bars. A, B, C: significant difference ($P < 0.05$) within the same treatment among different periods.

3.5 Lysozyme activity

Liver lysozyme activity (LYZ-L) increased at 3h in fish fed with 15% and 30% SWM ($P < 0.05$) (Figure 3). Head kidney (LYZ-HK) and spleen lysozyme activity (LYZ-SP) did not show differences among the treatments ($P > 0.05$). 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 ($P < 0.05$).

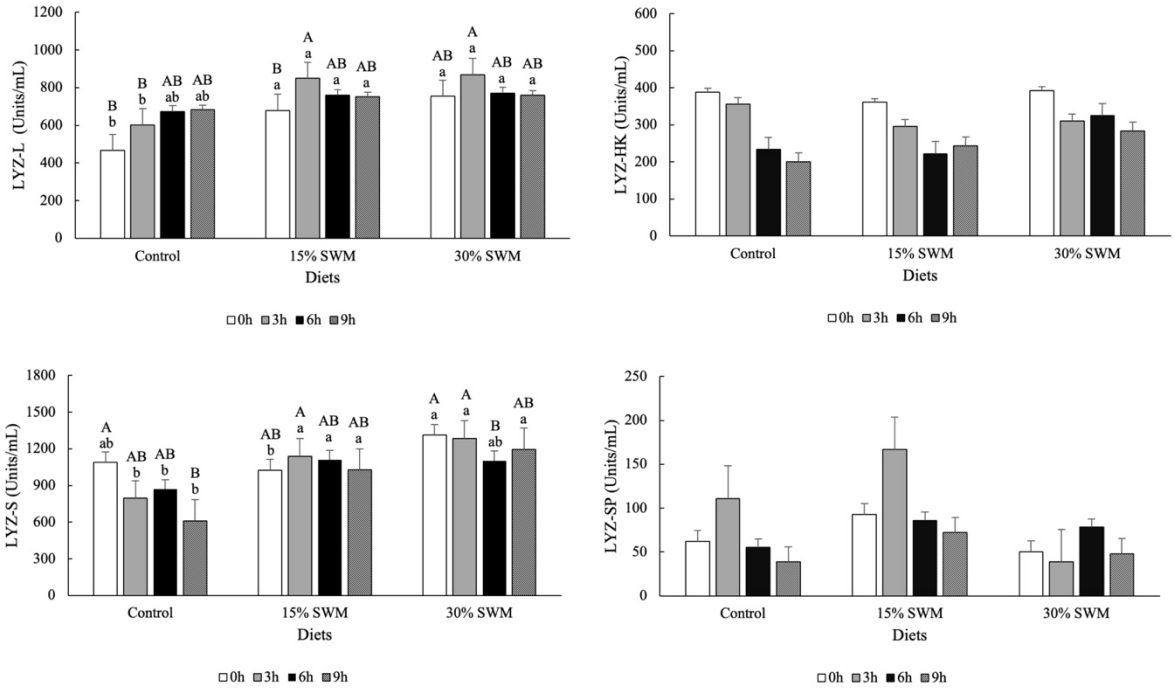

Figure 3. Lysozyme activity on head liver, kidney, serum and spleen in Nile tilapia at the end of the feeding trial (pre challenge – time 0) and 3, 6 or 9 h post challenge with *E. coli* LPS (n= 8/treatment). Data are reported as the mean ± SEM with their standard deviation represented by vertical bars. a, b, c: significant difference ($P < 0.05$) among different treatment within the same period. A, B, C: significant difference ($P < 0.05$) within the same treatment among different periods. Letter were omitted when there was no statistical difference.

3.6 Hemolytic activity of the alternative complement system

Fish fed diets containing 15% and 30% SWM showed an increase in hemolytic activity of the alternative complement system (HACS) ($P < 0.05$) (Figure 4). In addition, higher hemolysis percentages were registered at 0 and 3 h after challenge for all feeding trials ($P < 0.05$).

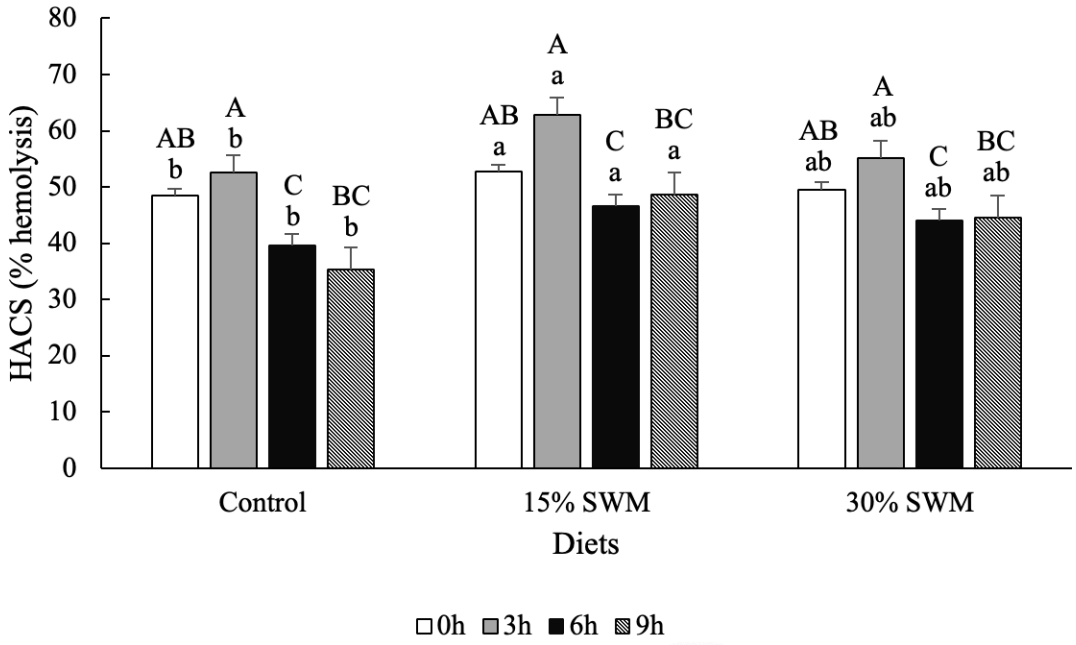

Figure 4. Serum hemolytic activity of alternative pathway of complement system in Nile tilapia at the end of the feeding trial (pre-challenge – time 0) and 3, 6 or 9 h post challenge with *E. coli* LPS (n= 8/treatment). Data are reported as the mean ± SEM with their standard deviation represented by vertical bars. a, b, c: significant difference ($P < 0.05$) among different treatment within the same period. A, B, C: significant difference ($P < 0.05$) within the same treatment among different periods.

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, Arsiwalla, & Waagbø, 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 (Piccolo et al., 2017;

Lin & Mui, 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.*) (Ng, 2001; Gasco et al., 2016). 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 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, Taufek, Kamarudin, & Razak (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, Latimer, & Burnley, 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, El-Ashram, AbdelHamid, & Gadalla, 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 defense of the body (Sahu, Das, Mishra, Pradhan, & Sarangi, 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*) (Taufek et al., 2018; Okore, Ekedo, Obeagu, & Christian, 2018).

Erythrocyte counts were not changed by the diet and the highest value, in all treatments, was registered 6 hours 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 hours 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, Fausto, & Picchietti, 2018), we observed a reduction in the number of lymphocytes in fish blood 9 h after challenge, suggesting the migration of these cells to the inflammation focus recruited by the defense mechanism as verified by Lamas, Santos, Bruno, Toranzo, & Anadón, (1994) in *Oncorhynchus mykiss* blood and Garcia, Moraes, & Martins, (2009) in *Piaractus mesopotamicus* blood.

Fish fed 15% and 30% SWM diet showed the highest number of thrombocytes mainly 6 and 9 hours after challenge. Thrombocyte is an important cell involved in fish defense, 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, Abessa, Santos, Silva, & Seriani (2017), studying *O. niloticus* reported thrombocytes as the main cells present in the inflammatory exudate of fish, suggesting that thrombocytes act as defense cells in fish, besides presenting hemostatic 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).

The assessment of neutrophils migration in the blood system is an important health status indicator of fish population. Neutrophils are defense 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, Mitroulis, Renesse, Hajishengallis, & Chavakis, 2017). According to Sebastião, Nomura, Sakabe, & Pilarski (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 h 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 h). 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.

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 h after challenge. In addition, the levels of LYZ-S remained between 120 – 150 U/mL, similar to those reported for Nile tilapia (Aly, Ahmed, Ghareeb, & Mohamed, 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).

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 defense, such as cellular activation, phagocytosis, inflammatory response, and lysis of bacterial cell (Biller-Takahashi, 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, Li, & Sunyer, 2006). In turn, we are the first to investigate the hemolytic 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 h after challenge for all feeding trials. Comparing the

hours, there is no difference between 0 h and 3 h, the highest percentage of hemolysis. It is therefore likely that the peak of hemolysis occurred between these times.

Complement-mediated hemolytic 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., 2011; Lee, Silva, Lee, Hartl, & Elias, 2008). 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, hemato-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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