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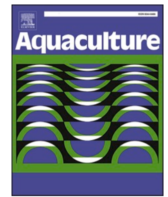

Efficiency of fatty acid-enriched dipteran-based meal on husbandry, digestive activity and immunological responses of Nile tilapia *Oreochromis niloticus* juveniles

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ABSTRACT

This study aimed to compare the enrichment capacity of polyunsaturated fatty acids (PUFA) and the long chain polyunsaturated fatty acids (LC-PUFA) of two dipteran species, *Hermetia illucens* - black soldier fly (BSF) - and a blowfly, *Chrysomya putoria* (CP), and to test its influence on growth, digestive activities and immune responses of Nile tilapia. Two types of enriched insect larval meal were produced using larvae cultured either on vegetable substrates (VGS) to formulate two diets rich in linolenic acid (ALA) (BSF/VGD and CP/VGD), or on fish offal substrates (FOS), in order to produce two diets rich in ALA and eicosapentaenoic acid (EPA) (BSF/FOD) or in ALA + EPA and docosahexaenoic acid (DHA) (CP/FOD). These four insect-based diets containing only palm oil as a lipid source were compared to a control diet based on fish meal (FM) and fish oil (FO). After 60 days of feeding, ALA or DHA muscle content of fish fed BSF/VGD or CP/FOD diet was comparable to that of the FMFO diet, and all insect diets increased the EPA muscle levels, except for a reduction by the BSF/VGD one. The CP/FOD diet induced similar fish growth, feed efficiency and protein efficiency ratio compared to the control FMFO diet, while a decrease was observed in fish fed other insect diets. Only BSF/VGD led to a decrease in protein and lipid digestibility. CP or BSF larval meal significantly increased alkaline phosphatase activity regardless of fatty acid (FA) enrichment. The expression level of *fads2*, *fads6* and *elovl5* was significantly higher in fish fed the BSF/VGD diet compared to fish fed the FMFO diet. FA-enriched insect diets increased some immune variables such as lysozyme, peroxidase and ACH50 values of fish fed CP/FOD, CP/VGD or BSF/VGD diets. Moreover, the expression level of *β-defensin-1* and *mhcII* genes were significantly higher in fish fed the BSF/FOD diet than the FMFO diet. Also, the expression of the pro-inflammatory gene *il-1-β* was significantly higher in fish fed FMFO diet than in those fed CP/FOD diet, but comparable to fish fed all other diets. No significant effects were observed for the other tested genes. The results showed a better efficiency in LC-PUFA enrichment of the CP larvae compared to BSF ones, resulting in a higher stimulation of the fish nutrient utilization processes and therefore, a higher growth capacity. Nonetheless, all dipteran larval meal stimulated the immune status whatever the insect species or dietary essential fatty acids.

1. Introduction

Global aquaculture fish production reached 82.1 million tons in 2018, representing 46% of total global fish production. Around 22 million tons of fish from global fish production were destined for non-food uses, mainly to produce fishmeal (FM) and fish oil (FO) (Stankus, 2021). FM is used as a protein source in compound feeds for many fish species, but its quantity and access are limited (Olsen and Hasan, 2012; Nguyen et al., 2020). In order to preserve natural resources and render aquaculture sustainable, it is imperative to reduce the use of ingredients

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derived from capture fisheries. The search for nutritionally appropriate and sustainable alternatives to FM and FO is an area of intense research, with possible alternative ingredients sourcing from terrestrial plants, animal by-products, micro- and macroalgae or insects (Gatlin et al., 2007; Olsen and Hasan, 2012; Barroso et al., 2014; Boyd and McNevin, 2015; Wan et al., 2019).

Larval fly meal has been identified as a high protein source with a well-balanced profile in essential amino acids (AAs), which is almost comparable to that of FM (Barroso et al., 2014; Henry et al., 2015; Müller et al., 2017). However, the available results on the inclusion of some types of larval fly meal in fish feeding remain contradictory, depending on fish species, dietary inclusion level tested, substrate used for fly larval production and experimental conditions. It has been shown that total or partial replacement of FM by black soldier fly (BSF, *Hermetia illucens* (L. 1758), Stratiomyidae)) larval meal reduced the growth of juveniles of African catfish (*Clarias gariepinus* (Burchell, 1822)), turbot (*Psetta maxima* (L. 1758)) and zebrafish (*Danio rerio* (Buchanan-Hamilton, 1822)), while it did not affect the food intake and growth performance of juvenile Atlantic salmon (*Salmo salar* L. 1758) and Jian carp (*Cyprinus carpio* L. 1758) (Kroeckel et al., 2012; Li et al., 2017a, 2017b; Belghit et al., 2018; Zarantoniello et al., 2019; Adeoye et al., 2020). It has been reported that a high inclusion of BSF in the range of 40–60%, could lead to a marked reduction in the apparent digestibility of crude protein in rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) or Atlantic salmon without affecting feed intake and feed conversion rate (Renna et al., 2017; Belghit et al., 2018; Nogales-Mérida et al., 2019). In a previous study, we have also shown that the total substitution of FM by BSF meal in a vegetable-based oil diet reduced the protein digestibility and the activity of several digestive enzymes in Nile tilapia juveniles (Agbohessou et al., 2021). In case of long-term feeding, this could negatively affect other physiological functions. Some studies have proven that replacement of 50% of FM by BSF larval meal associated with FO induced an imbalance of oxidative homeostasis mainly in kidneys but also in the liver of rainbow trout, whereas the replacement of 75% of FM by BSF larval meal in a vegetable oil-based diet did not affect blood parameters and differential leucocyte counts in African catfish (Elia et al., 2018; Fawole et al., 2020). The complete replacement of FM with housefly larval meal (*Musca domestica* L. 1758) in a vegetable oil-based diet also caused a significant decrease in survival rate, specific growth, feed conversion rate, while reducing the innate immunity in Nile tilapia (Wang et al., 2017).

Table 1
Analyzed composition of the leaves and seeds of *Euphorbia heterophylla* (EH), marine fish offal (FO), vegetable culture substrate (VGS) and fish offal culture substrate (FOS) used for the production and enrichment of *Hermetia illucens*-black soldier fly (BSF) and *Chrysomya putoria* (CP) larvae.

Ingredients (g/Kg of DM)	Leaves + seeds of EH	Marine FO	VGS	FOS
Soybean meal			777.4	320.1
Rapeseed oil			72.6	00
Leaves and seeds of EH.			150	00
Marine fish offal			00	679.9
Crude protein (g/Kg)	251.9	473.8	455.8	518.6
Fat (g/Kg)	31.8	97.2	100.3	100.1
FA composition (% of total identified FA)				
C4:0	28.31	10.87	4.97	15.46
C14:0	n.d.	4.39	n.d.	3.53
C16:0	17.06	28.45	9.89	25.05
C17:0	0.12	n.d.	0.05	n.d.
C18:0	4.59	9.33	2.47	7.80
C20:0	1.29	0.34	0.43	0.36
C22:0	n.d.	0.22	n.d.	0.13
C24:0	2.48	1.56	0.45	1.43
Total satured fatty acids	53.78	55.17	18.26	53.77
C16:1n-7	0.62	9.15	0.20	7.32
C17:1	0.26	n.d.	n.d.	n.d.
C18:1n-9	9.60	16.95	40.41	15.95
C20:1n-9	n.d.	0.83	0.84	0.90
C22:1n-9	n.d.	0.35	n.d.	0.18
Total monoenes	10.47	27.27	41.45	24.36
C18:2n-6 (LA)	14.23	1.67	32.85	10.02
C18:3n-6	n.d.	0.41	n.d.	0.32
C20:2	n.d.	0.18	n.d.	0.14
C20:3n-6	0.05	0.20	n.d.	0.13
C20:4n-6 (ARA)	n.d.	0.20	n.d.	0.04
Total n-6 PUFA	14.28	2.50	32.85	10.66
C18:3n-3 (ALA)	21.14	0.38	7.43	1.24
C20:3n-3	n.d.	2.54	n.d.	1.67
C20:5n-3 (EPA)	n.d.	3.88	n.d.	2.69
C22:6n-3 (DHA)	n.d.	8.13	n.d.	5.38
Total n-3 PUFA	21.14	14.94	7.44	10.97
n-3 / n-6 ratio	1.48	5.98	0.23	1.03

n.d. = not detected.

Terrestrial insects can accumulate fats, especially during their immature stages, but they are not able to bioconvert short-chains of polyunsaturated fatty acids (PUFA) into long-chain polyunsaturated fatty acids (LC-PUFA) (Barroso et al., 2017). As a result, insect meal are generally low in LC-PUFA (Barroso et al., 2014; Cardinaletti et al., 2019; Zarantoniello et al., 2018, 2020), which limits their combination with vegetable oils in fish feeds. The PUFA deficiencies in fish diet can affect fish health status, cause poor growth, low dietary intake, anemia and high mortality (Tocher, 2010; Olivotto et al., 2011; Piccinetti et al., 2012; Dumas et al., 2018). LC-PUFA deficiencies in plant-derived oil diets might induce fish health problems such as deformation of the digestive tract (Ribeiro et al., 2015), problems of gut morphology (Torrecillas et al., 2017), low bacterial resistance (Montero et al., 2010; Ferreira et al., 2015) or a reduction of some immune parameters (Montero et al., 2003; Conde-Sieira et al., 2018). The LC-PUFA are released from phospholipid membranes to participate in eicosanoid production by phospholipase (Rowley et al., 1995). This process is involved in the organism's immune defense system. The n-3 LC-PUFA have been demonstrated to play a role as anti-inflammatory factors in the immune system (Mullen et al., 2010; Wall et al., 2010; Calder, 2010, 2017; Stella Buoite et al., 2018).

It has been shown that some insect species are able to modulate their fatty acid composition according to the type of the culture substrate (Liland et al., 2017), but information on the enrichment of insect meal with fatty acids (FA) is still limited. Substrates containing *Schizochytrium* microalgae were shown to enrich BSF larvae in ARA, EPA and DHA (Belghit et al., 2018; El-dakar et al., 2020; Zarantoniello et al., 2020). Fisheries' waste is of great interest for the enrichment of insect larvae since it has been estimated that about a quarter of these waste materials is discarded, leading not only to a significant environmental impact but also losing the potential value of these products. Fish waste could be an effective substrate to enrich BSF larvae with EPA and DHA (St-Hilaire et al., 2007a, 2007b), depending on the quality of the waste used. We have previously shown that BSF larvae produced using a fish waste substrate contained a substantial amount of EPA, but little DHA, despite the high content of this FA in the production substrate (Agbohessou et al., 2021).

Insects could be produced on low quality organic wastes, require minimal water and cultivable land, and emit few greenhouse gases (van Huis, 2013). BSF is currently the main species widely studied for bioconversion and feed ingredient (Makkar et al., 2014). They are particularly interesting since adult insects with their atrophied mouthparts are not harmful to humans, food or buildings. Moreover, their larvae are saprophagous and are currently produced on an industrial scale in Europe and Africa for their suitability and their nutritional value (Wang and Shelomi, 2017; Devic et al., 2018; Li et al., 2020). BSF larvae have been successfully reared on a wide range of organic wastes, with particular success on plant wastes and manures (Wang et al., 2013; Tschirner and Simon, 2015). However, BSF larvae do not develop well on waste materials containing high levels of meat products or animal fat (Nguyen et al., 2013). In addition, their development time from eggs to adults of more than one month (Tomberlin et al., 2009) is relatively long

Table 2
Analyzed chemical composition of *Hermetia illucens*-black soldier fly (BSF) prepupal and *Chrysomya putoria* (CP) larval meal produced on different substrates: vegetable culture substrate (VGS) and fish offal substrate (FOS) (mean ± SD., n = 3). Means in a row without a common superscript letter differ ($p < 0.05$) as analyzed by two-way ANOVA and the TUKEY test. A protein-to-nitrogen conversion factor of 4.76 was used.

Chemical composition	BSF		CP		*P*-value			
	BSF/VGS	BSF/FOS	CP/VGS	CP/FOS	Insects	Sub[1]	I × S[2]	F
Dry matter (DM (g/kg))	360.76 ± 5.60^{a}	358.08 ± 0.70^{a}	327.22 ± 0.66^{c}	344.65 ± 0.01^{b}	<0.001	0.022	0.008	$F_{3,8} = 2690$
Ash (g/kg DM)	68.90 ± 0.49^{b}	76.25 ± 1.41^{a}	60.56 ± 2.71^{c}	58.40 ± 0.22^{c}	<0.001	0.075	0.012	$F_{3,8} = 55.56$
Crude protein (g/kg DM)	416.00 ± 15.40 ^{b}	450.80 ± 8.10^{ab}	444.50 ± 9.0^{ab}	473.2 ± 3.60^{a}	0.022	0.011	0.689	$F_{3,8} = 12.87$
Crude fat (g/kg DM)	329.20 ± 22.10^{a}	267.10 ± 20.30^{b}	268.60 ± 15.60^{b}	269.60 ± 6.30^{b}	0.315	0.271	0.019	$F_{3,8} = 5.85$
Neutral detergent Fiber (NDF) (g/kg DM)[3]	218.50	218.50	238.10	238.10				
Fatty acid composition (% of total identified fatty acids)								
C10:0	1.04 ± 0.10^{a}	0.84 ± 0.02^{a}	00 ± 00^{b}	00 ± 00^{b}	<0.001	0.056	0.056	$F_{3,8} = 113.2$
C12:0	38.94 ± 1.49^{a}	32.00 ± 0.50^{b}	2.42 ± 0.02^{c}	0.00 ± 0.00^{c}	<0.001	0.001	0.015	$F_{3,8} = 1295$
C14:0	5.93 ± 0.12^{b}	6.37 ± 0.01^{a}	3.02 ± 0.01^{d}	3.69 ± 0.10^{c}	<0.001	0.001	0.159	$F_{3,8} = 1475$
C15:0	0.20 ± 0.00^{d}	0.58 ± 0.02^{c}	0.69 ± 0.01^{b}	1.16 ± 0.01^{a}	<0.001	<0.001	0.014	$F_{3,8} = 3163$
C16:0	7.90 ± 0.45^{d}	16.85 ± 0.44^{c}	29.41 ± 0.21^{b}	33.52 ± 0.35^{a}	<0.001	<0.001	0.001	$F_{3,8} = 1021.5$
C17:0	0.20 ± 0.02^{c}	0.71 ± 0.04^{b}	0.75 ± 0.00^{b}	1.26 ± 0.02^{a}	<0.001	<0.001	0.752	$F_{3,8} = 465.6$
C18:0	1.40 ± 0.36^{d}	3.48 ± 0.23^{c}	4.94 ± 0.01^{b}	7.10 ± 0.01^{a}	<0.001	<0.001	0.803	$F_{3,8} = 208.48$
C24:0	00 ± 00^{c}	00 ± 00^{c}	0.37 ± 0.03^{b}	1.25 ± 0.01^{a}	<0.001	<0.001	<0.001	$F_{3,8} = 3664$
Total satured fatty acids	55.60 ± 2.31^{a}	60.82 ± 1.24^{a}	41.60 ± 0.21^{c}	47.98 ± 0.48^{b}	<0.001	0.004	0.585	$\mathbf{F_{3,8} = 167.7}$
C14:1	0.19 ± 00^{b}	0.26 ± 0.01^{a}	0.00 ± 00^{c}	0.00 ± 00^{c}	<0.001	0.701	0.701	$F_{3,8} = 4.05$
C15:1	0.16 ± 0.01^{b}	0.31 ± 0.03^{a}	0.00 ± 00^{c}	0.00 ± 00^{c}	<0.001	0.923	0.923	$F_{3,8} = 4.10$
C16:1	2.60 ± 0.03^{a}	0.59 ± 00^{b}	2.37 ± 0.21^{a}	1.12 ± 0.01^{b}	0.229	<0.001	0.024	$F_{3,8} = 105.2$
C17:1	0.27 ± 0.01	0.88 ± 0.01	0.63 ± 0.001	2.16 ± 0.85	0.11	0.057	0.318	$F_{3,8} = 1355$
C18:1n9	22.13 ± 0.32^{c}	17.67 ± 0.88^{d}	32.83 ± 0.15^{a}	26.82 ± 0.28^{b}	<0.001	<0.001	0.104	$F_{3,8} = 157.7$
C20:1n9	0.64 ± 0.007^{a}	00 ± 00^{b}	00 ± 00^{b}	00 ± 00^{b}	<0.001	<0.001	<0.001	$F_{3,8} = 36{,}481$
Total monoenes	25.56 ± 0.04^{c}	19.42 ± 0.47^{d}	35.83 ± 0.36^{a}	30.09 ± 0.50^{b}	<0.001	<0.001	0.333	$F_{3,8} = 123.54$
C18:2n6 (LA)	10.08 ± 0.28^{b}	6.19 ± 0.52^{c}	14.43 ± 0.03^{a}	3.46 ± 0.08^{d}	0.019	<0.001	<0.001	$F_{3,8} = 397.9$
C18:3n6	00 ± 00^{c}	00 ± 00^{c}	0.35 ± 0.01^{b}	0.66 ± 0.05^{a}	<0.001	0.003	0.003	$F_{3,8} = 135.1$
C20:4n6 (ARA)	00 ± 00^{b}	00 ± 00^{b}	0.00 ± 00^{b}	0.09 ± 0.01^{a}	0.001	0.001	0.001	$F_{3,8} = 34$
C22:2	0.26 ± 0.05^{b}	0.00 ± 00^{c}	0.74 ± 0.03^{a}	00 ± 00^{c}	0.002	<0.001	0.001	$F_{3,8} = 100.3$
Total n-6 PUFA	10.34 ± 0.23^{b}	6.22 ± 0.57^{c}	15.59 ± 0.03^{a}	4.22 ± 0.02^{d}	0.002	<0.001	<0.001	$F_{3,8} = 403.8$
C18:3n3 (ALA)	2.30 ± 0.05^{b}	0.52 ± 0.04^{c}	3.38 ± 0.15^{a}	0.62 ± 0.03^{c}	0.002	<0.001	0.004	$F_{3,8} = 294.8$
C20:3n3	00 ± 00^{d}	0.91 ± 0.03^{c}	1.59 ± 0.03^{b}	2.58 ± 0.06^{a}	<0.001	<0.001	0.292	$F_{3,8} = 1188$
C20:5n3 (EPA)	00 ± 00^{c}	2.20 ± 0.06^{b}	00 ± 00^{c}	5.13 ± 0.18^{a}	<0.001	<0.001	<0.001	$F_{3,8} = 1151$
C22:6n3 (DHA)	00 ± 00^{b}	0.39 ± 0.06^{b}	00 ± 00^{b}	5.37 ± 0.20^{a}	<0.001	<0.001	<0.001	$F_{3,8} = 238$
Total_ n-3PUFA	2.30 ± 0.05^{d}	4.02 ± 0.18^{c}	4.97 ± 0.12^{b}	13.71 ± 0.01^{a}	<0.001	<0.001	<0.001	$F_{3,8} = 1855$
n-3:n-6	0.22 ± 0.003^{d}	0.64 ± 0.03^{b}	0.37 ± 0.01^{c}	3.88 ± 0.02^{a}	<0.001	<0.001	<0.001	$F_{3,8} = 12{,}631$

[1] Sub = Substrates; [2]I × S = Insects × Substrates interaction effect.
[3] Neutral detergent fiber in BSF prepupal meal (Renna et al., 2017) and larval meal of *Chrysomya megacephala* a species closely related to *C. putoria* according to Barroso et al. (2014) and Haasbroek (2016).

compared to other fly species such as *Chrysomya putoria* (Wiedemann, 1818) or CP. But, BSF larvae are much bigger than CP ones. Thus, CP larvae have been proposed as a candidate for animal feed (Barroso et al., 2014; Charlton et al., 2015; Parry et al., 2020), but its nutritional quality is not well known in fish nutrition and feeding. The CP larvae are considered as relatively polyvalent fly organisms that efficiently convert meat processing waste into proteins and lipids, but can also feed on vegetal waste (Parry et al., 2020). *C. putoria* is a very abundant species in urban centers of some countries, including Benin. It has a high reproductive capacity with high egg production. Moreover, scavenging larvae possess a very quick development rate with body mass increasing hundreds of times per day due to the rapid decomposition of their feeding substrate (Oliveira et al., 2007; Ferraz et al., 2011; Ferraz et al., 2012; Li et al., 2012; Čičková et al., 2015; Yakovlev et al., 2019).

The present study aims (1) to determine the specific effects of insect diets enriched with different levels of ALA, ALA + EPA or ALA + EPA + DHA on growth performance, flesh quality, fatty acid metabolism, digestive activities and immune system of Nile tilapia, (2) to compare the two types of dipteran larval meal (*H. illucens vs C. putoria*) in terms of exogenous larval FA accumulation using different types of culture substrates and their impact on fish physiological functions.

2. Materials and methods

2.1. Enrichment of insect larval meals

The production of BSF prepupal or CP larval meal and the *in vivo* fish feeding trial were conducted in the facilities of the research unit of Hydrobiology and Aquaculture (LHA) of the Faculty of Agricultural Sciences at the University of Abomey-Calavi (FSA/UAC) in Benin. Procedures for animal care and management were conducted according to the guidelines of the European (European directive 2010/63/UE).

The two species of larvae were reared separately in different insectariums in triplicate. In order to modulate their PUFA composition, two types of substrates with different biochemical compositions were used (Table 1). A vegetable culture substrate (VGS) was composed of soybean meal (777.4 g/Kg of dry matter), leaves and seeds of *Euphorbia heterophylla* L. (EH) (150 g/Kg) and rapeseed oil (72.6 g/Kg), containing high concentrations of LA and ALA. A fish offal culture substrate (FOS) was composed of soybean meal (320.1 g/Kg) and marine fish offal (679.9 g/Kg) rich in LC-PUFA (EPA and DHA) (Table 1). The leaves and seeds of EH were dried in the shade for three days. Fish waste was dried in an electric oven (Heraeus, Lille, France) at 70 °C for 3 days. In order to obtain homogeneous substrates and to facilitate the intake by the larvae, the different ingredients were crushed in an electric mill (David 4 V, Italy) and reduced to powder. The flours were then mixed according to the proportions of each diet and moistened up to 70%.

The production of the BSF prepupae was done in two stages: the breeding of the black soldier flies in nylon breeding cages (75 × 75 × 115 cm, Bugdorm, Taichung, Taiwan) and the growing of the larvae in white-colored, rectangular and transparent plastic containers (42 cm × 29 cm × 25 cm). BSF larvae were reared for 14 days at a temperature of 26.83 ± 0.48 °C during the experiment. The rearing containers have a downward sloping system to facilitate the removal of the prepupae from

the substrates and are equipped with a channel made of PVC tubing to allow automatic collection of the prepupae (Hoc et al., 2019).

For the production of CP larvae, a total of 100 g of fresh fish viscera were tied in plastic bags and introduced into nylon breeding cages (75 × 75 × 115 cm, Bugdorm, Taichung, Taiwan) to attract and trap flies. Once in the cage, the flies were identified according to Irish et al. (2014) and Lutz et al. (2018), the plastic bags containing the viscera were removed and the compound substrates were then introduced into the rearing cages. 24 h after the introduction of the substrates into the cages, egg laying and hatching were observed. CP larvae obtained were grown for 4–5 days and part of them was harvested. A second part was left in the substrates in order to constitute a new stock of breeding flies. After harvesting, larvae of each species were rinsed with sterilized water and then dried in an oven at 60 °C for 72 h until their weight stabilized. After drying, the larvae were ground to produce insect larval meal. These larval meals were stored at −20 °C and their biochemical composition was determined (Table 2) prior to incorporation in the formulation of experimental fish diets.

2.2. Facilities and fish

2.2.1. Experimental diets

Five isoproteic (330–347.1 g/kg dry matter) and isolipidic (88.4–93.4 g/kg dry matter) diets were formulated. The control diet (FMFO) contained fish meal (FM) and fish oil (FO) as sources of protein and fat, respectively. In the four insect diets, FM was completely replaced by the BSF prepupal or CP larval meal produced on different substrates, while FO was replaced by palm oil. The BSF/VGD and CP/VGD diet formulated using BSF prepupal or CP larval meal produced from larvae cultured on VGD substrates were both rich in ALA, with CP/VGD showing higher values. Concerning the insect diets formulated from larval meal produced from larvae cultured on the FOD substrate, the BSF/FOD diet was rich in ALA and EPA, while the CP/FOD one contained higher ALA, EPA and DHA levels. The formulation and chemical composition of these diets are shown in Tables 3A, B**.** All feed ingredients were homogenized and water was gradually added (35–40% of the dry ingredients) to the premixed ingredients and mixed for an additional 10 min. The homogenized mixture was passed through a pellet machine (BD-GP70, Henan BEDO Machinery Equipment, China) at a running speed of 700RMP and temperature was adjusted at 90–95 °C. The machine was equipped to adjust the size of the pellets (1.2–2.2 mm, diameter) in relation to the fish size. Pellets were then dried under a hood at room temperature for 12 h and finally stored at −4 °C.

2.2.2. Fish rearing and feeding

The feeding experiment was conducted in an aquaculture recirculating system (RAS) including mechanical and biological water filtration systems. A set of 450 juveniles (initial body weight = 6.57 ± 0.07 g) was randomly distributed into 15 tanks with a density of 30 fish per tank, and three tank replicates per dietary treatment. Water was equally aerated and exchanged at a flow rate of 3–4 L/min. Water temperature (27.90 ± 1.38 °C) and dissolved oxygen (6.03 ± 0.97 mg/L) were checked daily. Nitrite and ammonia were monitored weekly and averaged 0.09 ± 0.03 mg/L and 0.08 ± 0.004 mg/L, respectively.

Male sex-reversed Nile tilapia at early juvenile stage were acclimated to the experimental conditions for 3 weeks in fiberglass tanks. During this period, all fish were fed with commercial tilapia diet (BIOMAR, INICIO Plus, 2 mm) for the first 2 weeks and then with a mixture of the five experimental diets during the last week. Fish were hand fed to apparent satiation three times per day at 09.00, 13.00 and 17.00 h.

2.2.3. Growth performance and feed utilization

In order to evaluate the growth performance and feed utilization, all fish were individually weighed at the beginning and at the end of the experiment. Mortality was recorded daily. Growth performance and feed utilization were determined using the following formulas:

$$\text{Relative weight gain (RWG, \%)} = 100 \times (\text{Wf} - \text{Wi})/\text{Wi}.$$

$$\text{Specific growth rate (SGR, \%per day)} = 100 \times [\mathit{ln}\,(\text{Wf}) - \mathit{ln}(\text{Wi})\,]/\Delta\text{t}.$$

$$\text{Feed intake (FI)} = \text{FC}/(\text{N x } \Delta\text{t})$$

$$\text{Feed efficiency (FE)} = (\text{FB} - \text{IB})/\text{TFI}.$$

$$\text{Protein efficiency ratio (PER)} = \text{weight gain (g)}/\text{protein intake (g)}$$

$$\text{Retention of FA (\%)} = 100 \times ((\text{Wf x FAf}) - (\text{Wi x FAi}))/((\text{Wf} - \text{Wi}) \times \text{FCR x Fad})),$$

$$\text{Survival (\%)} = 100 \times (\text{number of final survivors per tank}/\text{initial number per tank})$$

where Wi and Wf are the initial and final body mass (g), Δt is the duration of the experiment, FC is the feed consumption per tank (g), N is the number of fish per tank, IB and FB are the initial and final biomass per tank (g), TFI is the total feed intake (g), FAi, FAf and Fad are the initial and final FA content in mg/g in fish and diet.

2.2.4. Sample collection

At the beginning of the experiment, 10 fish were randomly euthanized by immersion in ethyl-aminobenzoic acid (MS222: 240 mg/L) solution for analysis of initial whole-body composition. At the end of the feeding trial (day 60), the total number of fish and their body weight were recorded to determine the survival rate (SR) and specific growth rate (SGR). At the end of the growth trial, fish were starved for 24 h prior to the final sampling. Seven fish were randomly sampled in each tank and anesthetized with MS222 (120 mg/L, Sigma). Of these, three fish were frozen at −20 °C for the analysis of whole-body composition. Using heparinized syringes, blood was collected for plasma from the caudal vein of 4 fish in each replicate tank (12 fish per treatment). Plasma samples were obtained by centrifugation at 4000 ×*g* for 15 min using a high-speed refrigerated microcentrifuge and kept at −80 °C until subsequent analysis of innate immune parameters (lysozyme activity, alternative complement activity, Immunoglobulin total and total peroxidase activity). Muscle, stomach, intestine, whole kidney and liver were removed and frozen on dry ice after dissection and then stored at −80 °C until analysis in order to determine the fatty acid composition of the fish, the activity of digestive enzymes and the expression of lipid metabolism and immune genes in the liver and kidney.

2.2.5. Proximate analysis

Crude protein content in the feed and whole-body fish was estimated according to the Kjeldahl distillation method after digestion of the samples with sulfuric acid (Kirk, 1950). A protein-to-nitrogen conversion factor of 6.25 was used for the feed and whole body fish samples (Mariotti et al., 2008). For the BSF prepupal meals, a protein-to-nitrogen conversion factor of 4.76 was used, as suggested by Janssen et al. (2017), to avoid overestimating the protein content due to the presence of non-protein nitrogen from chitin in insects. Dry matter and ash were identified from three fish per tank pooled and homogenized with a mixer. Dry matter in fish homogenate was calculated from weight loss after drying in an oven at 105 °C for 24 h, while ash content was calculated from weight loss after combustion of samples in a muffle furnace at 550 °C for 12 h.

2.2.6. Fatty acid analysis

The fat content of the total homogenate and the dorsal muscles of the fish (3 fish per tank) were determined according to Folch et al. (1957). The fatty acid composition of the total homogenates and the dorsal muscles of 3 fish per tank (9 fish per treatment) were estimated by gas chromatography (GC). Ten mg of lipids were converted into fatty acid

methyl esters by reaction with boron trifluoride (Sigma-Aldrich, Overijse, Belgium) and methanol (VWR, Oud-Heverlee, Belgium) (Beccaria et al., 2016; Hoc et al., 2020). Fatty acid methyl esters were diluted in 8 mL of hexane (VWR) and analyzed with a Trace GC Ultra gas chromatogram (Thermo Fisher Scientific, Asse, Belgium), equipped with a split/splitless injector (240.0 °C) in splitless mode (splitless time: 0.85 min) and a flame ionization detector (250.0 °C). A Stabliwax DA column (Restek Corp., Bellefonte, PA, USA) (30.00 m × 0.25 μm × 0.25 mm in length × thickness × diameter) was used for the analysis. The temperature program was as follows: 50.0 °C (hold 1 min), increase to 150.0 °C at 30.0 °C/min, increase to 240.0 °C at 5.0 °C/min (hold 10 min). Fatty acid methyl esters were identified based on their retention data compared to a reference mixture of 37 fatty acid methyl esters (Supelco® 37 component FAME mix, Sigma-Aldrich, Overijse, Belgium) analyzed under the same conditions. The quantification of compounds was realized by comparing their areas with the internal standard using a response factor of 1. Data were elaborated using Chemstation software (Agilent Technologies, Palo Alto, CA, USA).

2.2.7. Digestive enzyme activities

Stomach and intestine samples from four fish per tank (12 fish per treatment) were homogenized in 10 volumes (*v/w*) of PBS (NaH_2PO_4: 20 mM, NaCl: 6 mM, pH: 6.9). Intestine alkaline phosphatase (AP) and intestine aminopeptidase (N) activities were assayed according to Bessey et al. (1946) using *p*-nitrophenyl phosphate (Sigma-Aldrich) and L-leucine p-nitroanalide (Sigma-Aldrich) as substrates, respectively. Intestine trypsin activity was assayed according to Holm et al. (1988) and Métais (1968), such as described by Gisbert et al. (2009). Stomach amylase activity of the crude extract was determined by the starch hydrolysis method, described by Bernfeld (1951). Stomach pepsin activity was assayed applying the method of Cuvier-Péres and Kestemont (2001). Protein was determined using the Bradford (1976) procedure. Enzyme activities are expressed as specific activities (U or mU mg protein^{-1}).

2.2.8. Digestibility assessment

A digestibility trial was conducted on the same fish used for the growth test but one month after the feeding with the different diets. Fish were fed a diet containing chromium oxide (Sigma, Adrich) serving as the inert marker at a level of 1% (Cr_2O_3, 10 g/kg) to the previous formulation, and fed to the fish under the same conditions as the growth experiment. One week after acclimation to the new diets, feces were collected with a siphon twice daily in the morning and afternoon during three weeks. Feces rapidly settled on the bottom of the tank and remained largely intact, so that nutrient and marker losses were minimized. The feed pellets rejected by the fish were carefully removed before feces collection. Sufficient amounts of feces were collected, freeze dried, and immediately kept at −20 °C until analysis. Concentration of chromium oxide in diets and feces was determined according to Furukawa and Tsukahara (1966).

$$ADC_{DM}: \text{Apparent dry matter digestibility} = 100\text{–}100\ (\%\text{marker in diet}/\%\text{marker in feces})$$

$$ADC_{protein}: \text{Apparent digestibility of protein}\ (\%) = 100\text{–}100\ ((\%\text{marker in diet}/\%\text{marker in feces})\ \text{x}\ (\%\text{protein in feces}/\%\text{protein in diet}))$$

$$ADC_{lipid}: \text{Apparent digestibility of lipid}\ (\%) = 100\text{–}100\ ((\%\text{marker in diet}/\%\text{marker in feces})\ \text{x}\ (\%\text{lipid in feces}/\%\text{lipid in diet}))$$

2.2.9. Evaluation of immune parameters

Serum lysozyme activity (LA) was determined according to the protocol of Ellis (1990) adapted for Nile tilapia. Thirty microliters of plasma were put into wells of microplates, and 100 μL of substrate of freshly prepared *Micrococcus luteus* (Schroeter, 1872) Cohn, 1872 (Sigma-Aldrich, Saint-Louis, USA) solution (0.6 mg/mL of Na2HPO4 0.05 M, pH 6.2) was added in triplicate. Absorbance corresponding to *Micrococcus luteus* lysis was measured at 450 nm during 30 min at regular intervals (5 min). One unit (U) of lysozyme was determined as an absorbance decrease of 0.001 per min.

The total peroxidase activity (PA) in the serum was measured according to Hossain and Koshio (2017), with some modifications. Briefly, 5 μL of serum was diluted with 70 μL of Hank ‘s balanced salt solution (HBSS) without Ca + 2 or Mg + 2 in flat-bottomed 96-well plates. Then, 25 μL of peroxidase substrate (3,3′,5,5′ tetramethyl benzidine hydrochloride) (TMB; ThermoScientific Inc., USA) was added. The serum mixture was incubated for 2 min. The color-developing reaction in the serum samples was stopped by adding 25 μL of 4 M sulfuric acid, and the OD (450 nm) was measured in a plate reader. PBS was used as a blank instead of serum.

Plasma hemolytic alternative complement activity (ACH50) was assayed following Sunyer and Tort (1995), later modified by Milla et al. (2010). Briefly, 10 μL of rabbit red blood cells suspension (RRBC, Biomerieux, Mary-l'Etoile, France) suspended at 3% in veronal buffer was mixed with serial dilutions of plasma (50 μL of total volume). Hemolysis 100% was obtained by adding 60 μL of distillate water to 10 μL of RRBC. Negative control (fresh water) was obtained by adding 60 μL of veronal buffer to 10 μL of RRBC. Samples were incubated 100 min at 27 °C and centrifuged (3000 ×*g*, 5 min, 4 °C). Then, 35 μL of supernatant was transferred to a new microplate in order to measure the absorbance at 405 nm. The ACH50 value was defined as the reciprocal of the plasma dilution which induced the hemolysis of 50% RRBC.

The total plasma immunoglobulin was separated from the plasma by precipitation with polyethylene glycol as described by Anderson and Siwicki (1995). Plasma (0.1 mL) was placed in plastic serum vials and 0.1 mL of 12% polyethylene glycol was added and incubated at room temperature for 2 h under constant mixing. After incubation, the solutions were centrifuged at 7000 *g* for 10 min. The protein content in the supernatant was determined using a protein assay kit. The total immunoglobulin content was determined by subtracting the protein content in the supernatant from the total protein content in the plasma.

2.2.10. Gene expression analyses

The total RNA of liver and whole kidney was individually extracted from a batch of three fish per tank (9 fish per treatment) using 1 mL trizol (Extract-all®, Eurobio, Courtaboeuf, France). The quality of extracted RNA was checked using a Nanodrop 2000 spectrophotometer (Thermo-Fischer Scientific, Waltham, MA, USA) and electrophoresis on a 1% agarose gel. Each individual RNA sample was then treated using a RTS DNAse™ kit (MO BIO Laboratories, Carlsbad, CA, USA) to avoid DNA contamination. Then, 1 μg of total RNA was reverse transcribed to cDNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). The cDNA was then diluted 15 times with ultrapure water (Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water, ThermoFisher scientific) and used for real-time qPCR to determine gene expression levels. Two housekeeping genes (*β-actin* and *18S*) were tested and the most stable gene in all studied conditions for each organ was used as the reference gene (*18S* for liver, *β-actin* for kidney). The expression of the lipid metabolism genes *elovl5* (very long elongase delta 5), *fads2* (FA desaturase 2) and *fads6* (FA desaturase delta 6) were analyzed in liver tissue. The expression of Toll like receptor (*tlr-2*, *tlr5* and *tlr7*), *β-defensin-1*, a humoral component *hepcidin*, Major histocompatibility complex class II (*mhcII*), complement *c4* and neutrophil (*mpo*), pro-inflammatory (*il-1- β*, *il-6*, *tnf-α*, *ifn-y*), anti-inflammatory genes (*il-10* and *tgf- β*) were determined in the kidney tissue using specific primers that were designed on Primer3 software and re-checked for quality on Amplifix software against sequences of the tilapia published on Genbank (Table 4). The efficiency of each gene was confirmed before analysis.

The amplification of cDNA was conducted in duplicate using Sso Advanced™ Universal SYBR® Green Supermix (BioRad Laboratories, Hercules, CA, USA). Thermal cycles and fluorescence detection were carried out using a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) under the following conditions: 10 min of initial denaturation at 95 °C, followed by 40 cycles at 95 °C for 30 s and 60 °C for 30 s. For analysis, a standard curve of a pool of the cDNA of all samples was included to calculate the PCR efficiency and normalize the transcript levels. The relative levels of RNA were quantified for each gene by densitometry, which was performed by measuring the photo stimulated luminescence values using StepOne Software v2.1. The relative target or reference gene expression of each sample was obtained using the standard curve method. Then, values for each sample were expressed as normalized relative expression (NRE), calculated with the formula NRE = relative concentration of target gene/relative concentration of reference gene. The results were expressed as average of values.

2.3. Statistical analyses

Data on the chemical composition of insect larvae and fish fed on larval meal-based diets are presented as mean values ± standard deviation of the mean (SD, n = 3). The normality and homogeneity of the variances in the data were assessed using the Shapiro-Wilk and Bartlett tests, respectively. When the data were heterogeneous or not normally distributed, they were log-transformed. Percentage values were also log-transformed before calculations. A bi-directional analysis of variance (ANOVA 2, with larval rearing substrate and insect species as factors) was performed on the data collected on insect larval composition and fish and organ samples, followed by a post-hoc LSD test using the tank as the statistical unit (n = 3). In all statistical analysis tests used, $p < 0.05$ was considered statistically significant. Statistical analyses were performed using R 3.03 software.

3. Results

3.1. Chemical composition of BSF prepupal and CP larval meal and efficiency of FA enrichment

Chemical composition of BSF and CP larval meal varied depending on the culture substrate (Table 2). The highest values ($p < 0.001$) of dry matter (DM) and crude ash were obtained in BSF larval meal and the lowest values in CP larval meal. Crude protein levels were higher ($p < 0.05$) in the CP/FOS larval meal compared to the BSF/VGS ones, but comparable to those of the BSF/FOS and CP/VGS larval meal. Total lipid level was significantly higher ($p < 0.05$) in BSF/VGD meal than the other insect meals.

Regarding the influence of culture substrates on FA profiles of the larval meal (Table 2), the highest levels of saturated fatty acids (SFA; $p < 0.001$) were found in the BSF larval meal and the lowest levels were found in CP larval ones. Monoene's concentrations were higher ($p < 0.001$) in the BSF or CP larval meal produced from the VGS substrate than in those produced from the FOS substrate, with higher values ($p < 0.001$) in CP larval meal than in BSF. LA contents were higher ($p < 0.001$) in the BSF and CP larval meal produced from VGS substrate than in those produced from FOS substrate. The relative LA accumulation by the larvae was estimated to be about 31% or 62% for the BSF larvae, and 44% or 35% for the CP ones compared to the amounts supplied in the VGS or FOS substrates. ALA content was also higher ($p < 0.001$) in larval meal produced from VGS substrates compared to those from FOS ones whatever the fly species, but with the highest values for CP/VGS larval meals. The relative ALA accumulation by the larvae was estimated to be about 31% or 42% for BSF larvae, and 45% or 50% for CP ones relative to the amounts provided in VGS or FOS substrates.

The BSF larval meal did not contain ARA regardless of the production substrate, only the CP/FOS larval meal contained low levels of ARA. As expected, the FOS substrate significantly enriched ($p < 0.001$) the larval meal in EPA whatever the fly species compared to the VGS substrate, with the highest accumulation in the CP/FOS larval meal ($p < 0.001$). The relative EPA accumulation by the larvae was estimated to be about 82% for BSF larvae and 191% for CP larvae compared to the amounts supplied in FOS substrates. A differential enrichment was observed for the DHA level with a very significant higher ($p < 0.001$) accumulation in the CP/FOS meal and low amounts in the BSF/FOS one, which nevertheless were higher ($p < 0.001$) than in the BSF or CP/VGS meals. The relative accumulation of DHA by larvae was estimated to be about 7% for BSF larvae and 100% for CP larvae compared to the amounts supplied in FOS substrates.

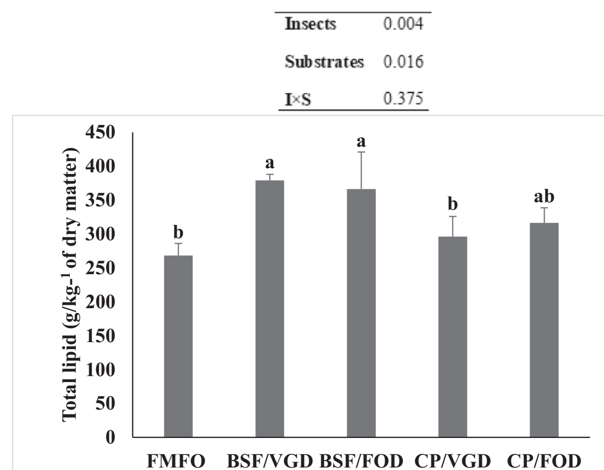

Fig. 1. Total lipid content in whole body of juvenile Nile tilapia fed with fish meal (FM) and fish oil (FO) or *Hermetia illucens*-black soldier fly (BSF) prepupal or *Chrysomya putoria* (CP) larval meal enriched with (LC)-PUFA for 60 days (mean ± S.D., $n = 3$). The different letters indicate significant differences ($p < 0.05$) analyzed by two-way ANOVA and the TUKEY test.

3.2. Growth performance, feed utilization and nutrient digestibility

Data on growth, food utilization and survival are presented in Table 5. The CP/FOD diet induced similar growth performance (final body weight, RWG and SGR) as the control FMFO diet, while a significant ($p < 0.05$) decrease was observed for other insect-based diets in relation to lower ($p < 0.01$) feed efficiency (FE) and protein efficiency ratio (PER). However, values for FE and PER did not differ between fish fed the CP/FOD diet and the FMFO control fish. No significant differences in growth parameters were observed between fish fed the BSF/VGD and BSF/FOD diets, while values were higher ($p < 0.05$) in fish fed the CP/FOD diet than those that received the CP/VGD diets. Survival rate and feed intake (FI) were not significantly affected by the experimental diet.

Mean ADC_{DM} values were the highest for fish receiving the FMFO or CP/FOD diet, but no significant differences were observed between treatments due to high intra-variability (Table 6). As for growth and feed utilization, fish fed the CP/FOD diet showed comparable $ADC_{protein}$ and ADC_{lipid} to those receiving the control FMFO diet, while a trend of decrease was observed in fish fed other diets, especially in BSF/VGD ones ($p < 0.05$).

3.3. Activities of digestive enzymes

Data on digestive enzyme activity levels are presented in Table 7. Amylase activity was not affected by any insect-based diet. Both CP-based diets increased ($p < 0.01$) the level of pepsin activity compared to the control FMFO diet, and induced higher ($p < 0.01$) values than both BSF-based diets. Trypsin activity was significantly higher ($p <$

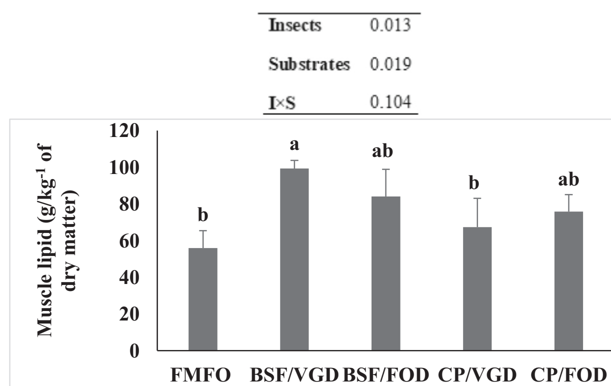

Fig. 2. Muscle lipid content of juvenile Nile tilapia fed with fish meal (FM) and fish oil (FO) or *Hermetia illucens*-black soldier fly (BSF) prepupal or *Chrysomya putoria* (CP) larval meal enriched with (LC)-PUFA for 60 days (mean ± S.D., n = 3). The different letters indicate significant differences ($p < 0.05$) analyzed by two-way ANOVA and the TUKEY test.

0.05) in fish fed the CP/VGD diet compared to the other insect-based diets and values were comparable to those of the FMFO control fish. Aminopeptidase activity was significantly higher ($p < 0.05$) in fish fed the CP/FOD diet than in all other fish groups including the FMFO controls. Alkaline phosphatase activity increased in fish fed all the insect-based diets ($p < 0.05$), with comparable values among all insect-diets.

3.4. Whole body and fish muscle lipid content

Both BSF diets, but not CP diets increased ($p < 0.05$) total body lipid content compared to the FMFO control diet (Fig. 1). No significant differences were observed in the total body lipid content of fish fed the CP/FOD diet and all other diets. The muscle total lipid content of fish fed the BSF/VGD diet was significantly higher ($p < 0.05$) than those fed the CP/VGD diet and the FMFO control, but was comparable to those fed the BSF/FOD and CP/FOD diets (Fig. 2).

3.5. Fatty acid metabolism

3.5.1. FA profiles in dorsal fish muscles and retention coefficients of LA and ALA

The total saturated fatty acid (SFA) content in dorsal muscle was significantly higher in fish fed the BSF/FOD diet compared to those fed the FMFO control, while the level of SFA was comparable between fish fed the BSF or CP larval meal diets (Fig. 3A). Regarding PUFA contents, fish fed the BSF/FOD and CP/FOD diets had significantly lower ($p < 0.01$) concentrations of ALA compared to those fed the BSF/VGD, CP/VGD and FMFO diets (Fig. 3B). The LA level was significantly lower ($p < 0.05$) in dorsal muscle of fish fed the BSF/FOD or CP/FOD diets compared to fish fed the FMFO control diet, but comparable between fish fed the VGD insect-based diets (Fig. 3B). For LC-PUFA, no significant difference was observed in the concentrations of ARA in the dorsal muscles between fish receiving the different diets (Fig. 3C), despite the fact that the BSF/VGD, BSF/FOD and CP/VGD diets did not contain ARA. Regarding EPA, values were comparable between fish fed the insect-based diets and control diet, except for lower values for those fed the BSF/VGD diet ($p < 0.05$). In contrast, only the CP/FOD diet induced a DHA muscle level comparable to that of the control FMFO diet. Moreover, DHA values of fish fed the CP/FOD were significantly higher than those of fish fed the BSF/FOD, CP/VGD and BSF/VGD diets ($p < 0.001$), indicating differences in both, the species and substrate of larval meal production. Therefore, n3/n6 values of fish fed the CP/FOD diet were significantly higher than those of fish fed other diets ($p < 0.05$), and even significantly higher for BSF/VGD fish ($p < 0.001$).

Interestingly, the retention coefficients of OA (Fig. 4A) and ALA

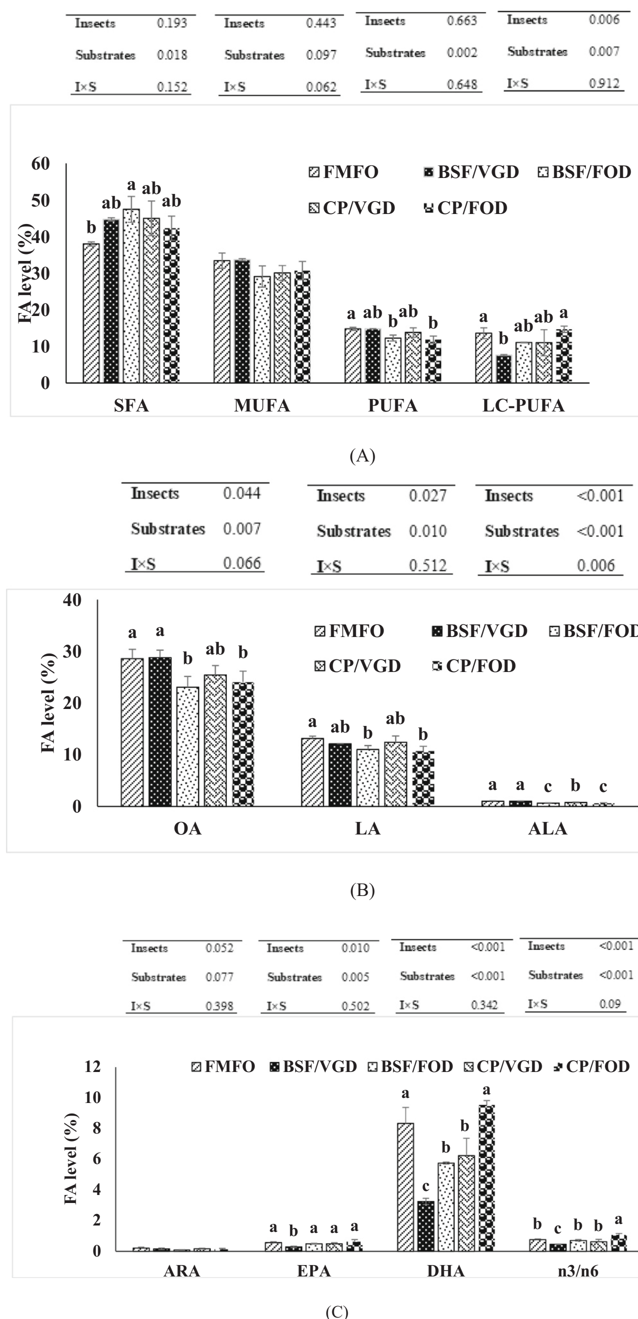

Fig. 3. Muscle fatty acid composition (% of total fatty acids identified) for (A) all fatty acid groups, (B) C18 unsaturated fatty acids and (C) LC-PUFAs in juvenile Nile tilapia fed with fish meal (FM) and fish oil (FO) or *Hermetia illucens*-black soldier fly (BSF) prepupal or *Chrysomya putoria* (CP) larval meal enriched with (LC)-PUFA for 60 days (mean ± S.D., n = 3). SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: C18 polyunsaturated fatty acids, LC-PUFA: long chain polyunsaturated fatty acids, OA: oleic acid, LA: linoleic acid, ALA: α-linolenic acid, ARA: arachidonic acid, EPA: eicosapentanoic acid, DHA: docosahexanoic acid. The different letters indicate significant differences ($p < 0.05$) analyzed by two-way ANOVA and the TUKEY test.

(Fig. 4C) of fish fed the CP/FOD diet were comparable to those in the control FMFO fish, and were significantly higher ($p < 0.05$) than values in fish fed other insect larval meal (Fig. 4A and C). In addition, LA retention coefficients in the CP/FOD fish were significantly ($p < 0.05$) higher than in other insect-fed fish or control (Fig. 4B).

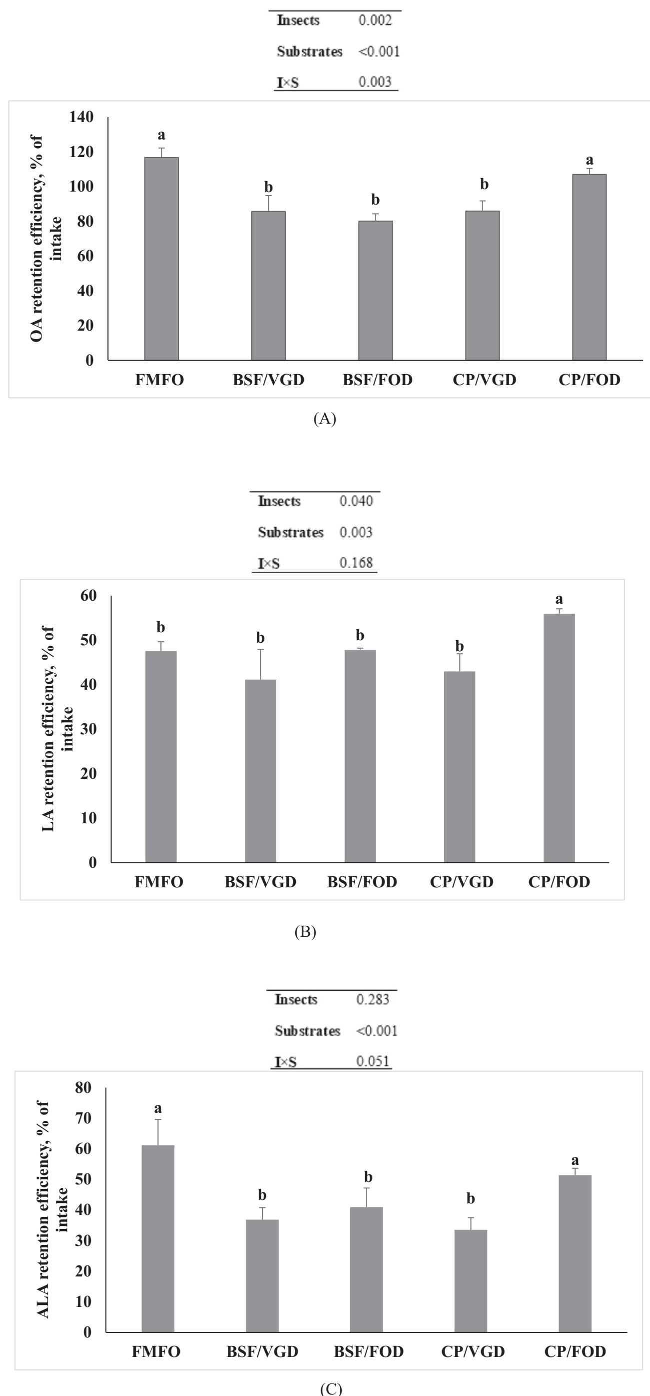

Fig. 4. Retention of fatty acids (A) OA, (B) LA and (C) ALA of juvenile Nile tilapia fed with fish meal (FM) and fish oil (FO) or *Hermetia illucens*-black soldier fly (BSF) prepupal or *Chrysomya putoria* (CP) larval meal enriched with (LC)-PUFA for 60 days (mean ± S.D., n = 3). The different letters indicate significant differences ($p < 0.05$) analyzed by two-way ANOVA and the TUKEY test.

3.5.2. Relative expression of genes related to FA biosynthesis processes in liver

The level of *fads2* expression (Fig. 5A) was higher ($p < 0.05$) in fish fed the BSF/VGD ($p < 0.05$) or BSF/FOD diets compared to fish fed the CP-based diets or the FMFO control. No significant differences were observed in the expression of *fads6* between fish fed the insect-based diets but values were lower ($p < 0.05$) in the FMFO control fish than in the BSF/VGD ones. The expression of *fads6* was comparable between fish fed the BSF/FOD, CP/FOD, CP/VGD diets and the FMFO control. The expression of *elovl5* was comparable between fish fed the diets BSF/VGD, BSF/FOD and CP/FOD, and lower ($p < 0.05$) in CP/VGD and FMFO control fish than BSF/VGD fish (Fig. 5C).

3.6. Influence of BSF prepupal or CP larval meal on the innate immune status in Nile tilapia

3.6.1. Humoral immune status

The humoral innate immune status was characterized by the levels of serum lysozyme and peroxidase activities as well as plasma hemolytic activity of complement (ACH50) (Fig. 6A, B and C) evaluated after 60 days of feeding. Lysozyme activity values of fish fed the CP/FOD and CP/VGD diets were significantly ($p < 0.05$) higher than those of fish fed the FMFO control diet, but comparable to those of fish fed both BSF-based diets (Fig. 6A). Lysozyme values of fish fed BSF diets were not significantly different from those of fish fed the FMFO control diet. Fish fed the CP/FOD diet had significantly higher peroxidase activity ($p < 0.05$) than fish fed the FMFO control diet (Fig. 6B). No significant differences in peroxidase activity were observed between fish fed the BSF or CP larval meal and between fish fed the BSF/VGD, BSF/FOD, CP/VGD and FMFO control diets. Only the BSF/VGD diet induced significantly higher ($p < 0.05$) ACH50 activity in fish than all other diets, but ACH50 activity was comparable between fish fed the other diets (Fig. 6C). Total serum immunoglobulin content was not affected by any insect diet (Fig. 6D).

3.6.2. Relative expression of immunity genes in whole kidney

Regarding the levels of gene expression related to the innate immunity functions, the expression of *β-defensin-1* was significantly higher ($p < 0.05$) in fish fed the BSF/FOD diet than those fed all other diets (Table 8). The expression of the *mhcII-α* in fish fed the BSF/FOD diet was also significantly higher ($p < 0.05$) than those receiving the FMFO control diet, but comparable between fish receiving all other diets (Table 8). No significant differences were observed for the *tlr2*, *tlr5*, *tlr7*, *hepcidin*, *mpo* and *c4 complement* values between all diets (Table 8). The expression of the pro-inflammatory gene *il-1-β* was significantly higher ($p < 0.05$) in fish fed the FMFO diet than in those fed CP/FOD but comparable to fish fed all other diets (Table 8). No significant differences were observed in the expression of pro-inflammatory *il-6*, *tnf-α* and *ifn-y* (Table 8) or anti-inflammatory *il-10* and *tgf-β* genes between all diets.

4. Discussion

4.1. Efficiency of PUFA enrichment of BSF prepupae and CP larvae and effects on fish fatty acid metabolism

Both BSF prepupal meal contained higher levels of SFAs than the CP ones, and lower levels of LC-PUFA (Table 2), which may limit their use as fish feed (Barroso et al., 2017; Liland et al., 2017). However, the PUFA contents (LA and ALA) were higher in the BSF or CP larval meal produced using the VGS substrate than those produced on the FOS substrate (Table 2) in relation to the lower content of PUFA in the latter substrate. In fact, the VGS substrate contained a mixture of leaves and seeds of *Euphorbia heterophylla* and rapeseed oil which were rich in LA and ALA. Despite this high level of PUFA in the VGS substrate, it appeared that the relative accumulation of these PUFA in BSF larvae-based vegetable substrate was lower (31%) than BSF larvae-based FOS substrate (58%). It has recently been reported that BSF larvae-based vegetable substrate may degrade a large LA or ALA intake to produce SFAs, and that they can metabolize about two-thirds of ALA intake to SFAs such as lauric or myristic acid (Hoc et al., 2020). Further differences in the FAs enrichment were observed between the two fly species concerning the larval EPA and DHA contents. CP larvae-based FOS substrate were rich in EPA and DHA, while only EPA was observed for BSF larvae-based FOS

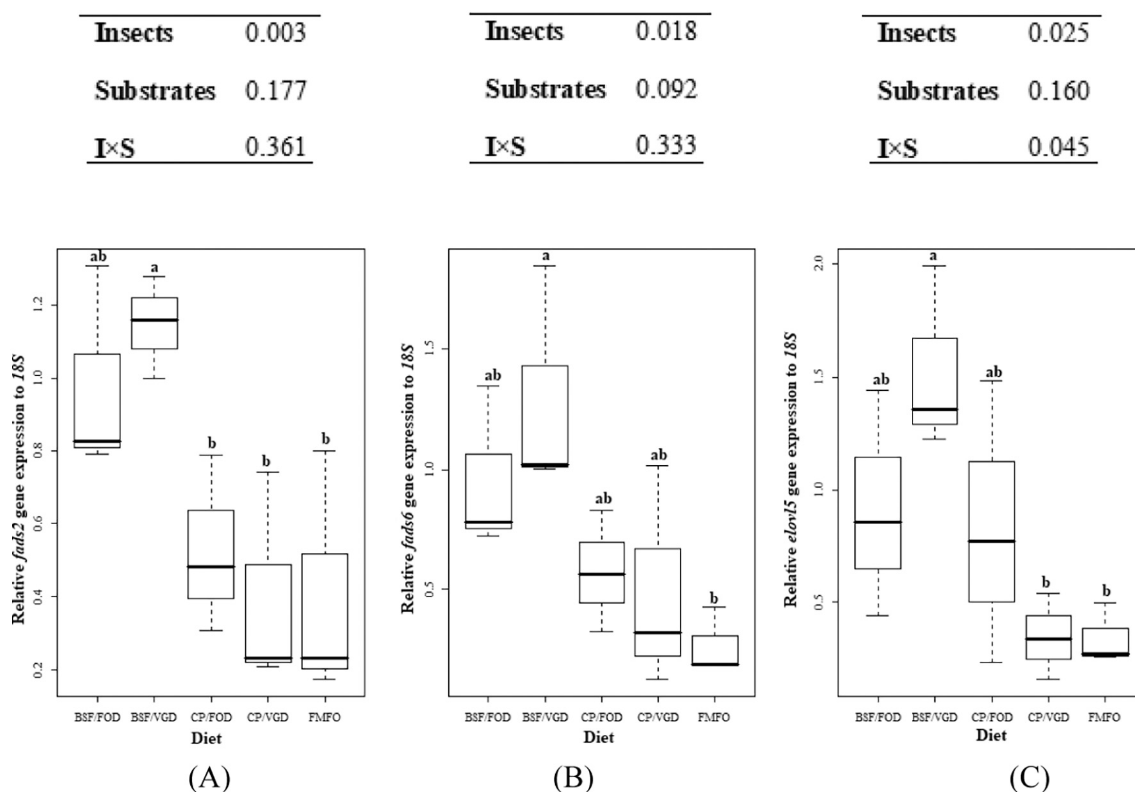

Fig. 5. Liver relative expression levels of (A) *fads2*, (B) *fads6*, and (C) *elovl5* genes involved in the FA biosynthesis in juvenile Nile tilapia fed with fish meal (FM) and fish oil (FO) or *Hermetia illucens*-black soldier fly (BSF) prepupal or *Chrysomya putoria* (CP) larval meal enriched with (LC)-PUFA for 60 days. Boxplots represent the quartile distribution of target gene expressions (n = 3). The different letters indicate significant differences ($p < 0.05$) analyzed by two-way ANOVA and the TUKEY test.

substrate. As reported for PUFA, a possible degradation in these LC-PUFA could explain the low level of DHA (0.39%) in the BSF/FOS larvae despite the high level of this fatty acid in the FOS substrate. The results of the current study confirm our previous experiment showing that the BSF larvae produced using a substrate containing 23% fish waste and 77% maize bran were low in DHA (0.37%) (Agbohessou et al., 2021). Other authors have observed that terrestrial insects are poor in EPA and DHA except *Chrysomya megacephala* (Fabricius 1794) and *Calliphora vicina* Robineau-Desvoidy 1830 (Finke, 2002, 2007; Barroso et al., 2014), which are fly species close to CP. This could also indicate that the processes of fatty acid accumulation in larvae is variable according to the insect species and would but would also depend on the quality of the rearing substrate. A study conducted on the differences in accumulation efficiency of FAs in three insects species, namely the domestic cricket (*Acheta domestica* (L. 1758); Orthoptera: Gryllidae), the lesser mealworm (*Alphitobius diaperinus* Panzer 1797; Coleoptera: Tenebrionidae), and the BSF had shown high accumulation of ALA in the domestic cricket and the mealworm compared to BSF when they were fed a diet enriched with 1%, 2% or 4% linseed oil during their larval stage (Oonincx et al., 2020).

The results of the current study also showed that the profiles of essential FAs in the fish dorsal muscles varied according to the fly species after two months of feeding. Indeed, the LA or ALA muscle levels in the fish fed the BSF/VGD and CP/VGD diets were higher than those fed the FOD-based ones, and comparable or close to those in the FMFO control fish (Fig. 3B) in relation to the differences reported above. But, EPA and DHA muscle levels of the CP/VGD fish were higher than those of the BSF/VGD fish (Fig. 3C) despite comparable dietary levels of LA and ALA, known as precursors of LC-PUFA in fish such Nile tilapia (Tocher et al., 2001; Tocher, 2015; Sourabié et al., 2018; Stoneham et al. (2018); Nguyen et al., 2020). The low valorization of the dietary PUFA of the BSF/VGD diet could be due to the higher levels of SFAs in this diet compared to the CP/VGD diet as mentioned above. The high inclusion of BSF in the rainbow trout diet increased the level of SFAs and consequently decreased the ALA, EPA and DHA content of fish fillets (St-Hilaire et al., 2007a, 2007b). The high content of EPA and DHA in the CP/FOD diet as well as the high retention of OA, LA and ALA in fish fed the CP/FOD diet (Fig. 4A, B and C) could also explain the high content of DHA in the muscle of the CP/FOD fish. As with PUFA, the fish muscle DHA level reflected those of the DHA-rich diets, particularly for the CP/FOD fish.

In the current study, the results showed that the relative expressions of the *fads2*, *fads6 and elovl5* genes were stimulated in fish fed the BSF/VGD diet compared to those receiving the FMFO control diet, further confirming the ability of Nile tilapia to accumulate substantial amounts of EPA and DHA in the muscle from a diet only rich in LA and ALA. Previous studies have already shown an increase in the expression of the *fads2*, *elovl5* genes and the enzymatic activity of these genes in fish fed with olive, rapeseed, palm, flax, perilla, soybean and rice oils compared to fish oil for rainbow trout (Buzzi et al., 1996), Atlantic salmon (Zheng et al., 2005), European sea bass (Geay et al., 2010), Spotted Flag, *Scatophagus argus* (Xie et al., 2014), Eurasian perch (Geay et al., 2015), Asian sea bass (Glencross et al., 2016), African catfish (Sourabié et al., 2018) and common carp (Nguyen et al., 2019). The expressions of the genes *fads6* and *Elovl5* were comparable between fish fed the BSF/FOD, CP/FOD and FMFO diets containing EPA and DHA. Dietary fatty acids can regulate the transcription of *elovl5* in fish and, in general, the expression of elongase genes is suppressed by dietary n-3 LC-PUFA (Yamamoto et al., 2010; Ren et al., 2012; Kuah et al., 2015; Morais et al., 2015; Zuo et al., 2016; Li et al., 2016; Li et al., 2017a, 2017b).

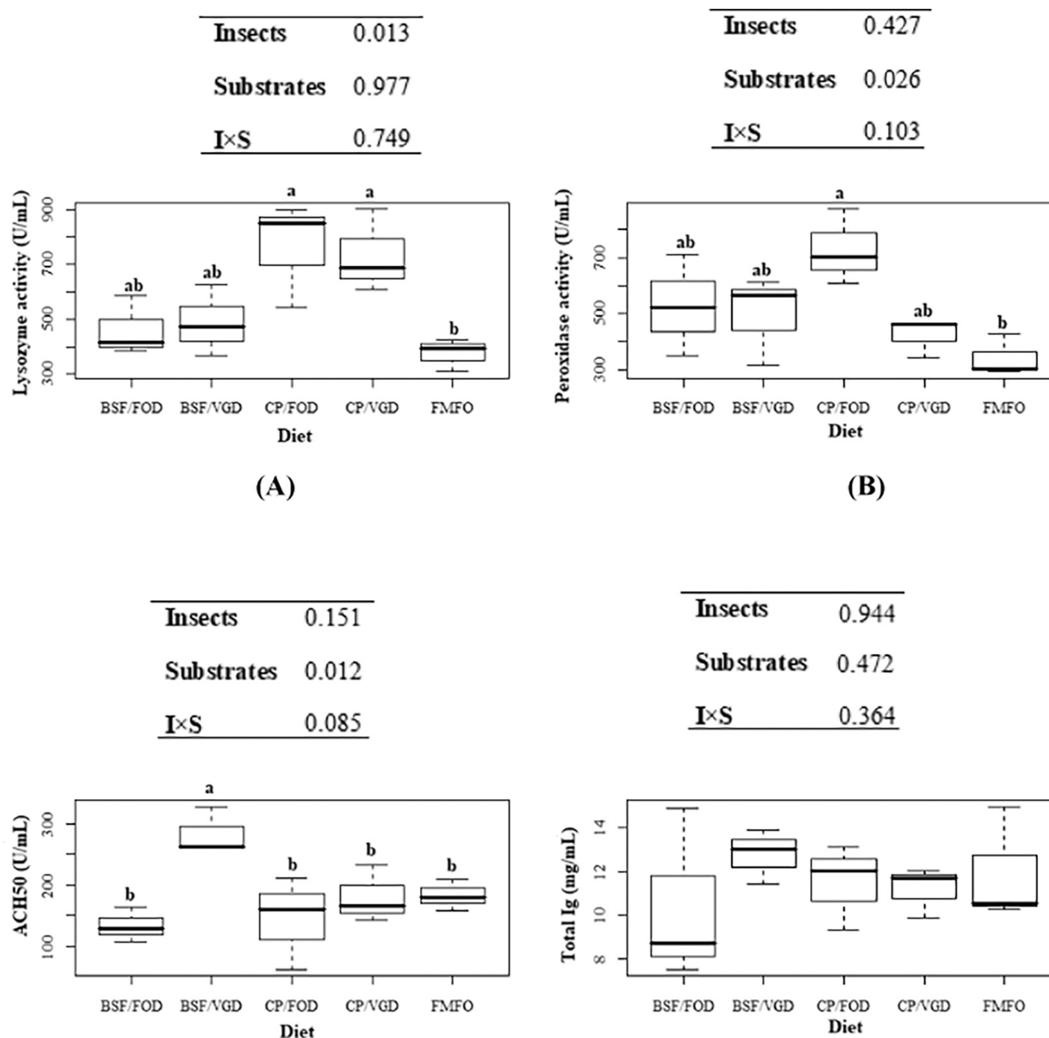

Fig. 6. Levels of (A) plasma lysozyme activity (U/mL), (B) plasma peroxidase activity (U/mL), (C) plasma alternative complement pathway activity (ACH50) (U/mL) and (D) Total Ig (mg/mL) of juvenile Nile tilapia fed with fish meal (FM) and fish oil (FO) or *Hermetia illucens*-black soldier fly (BSF) prepupal or *Chrysomya putoria* (CP) larval meal enriched with (LC)-PUFA for 60 days. Boxplots represent the quartile distribution of target gene expressions (n = 3). The different letters indicate significant differences ($p < 0.05$) analyzed by two-way ANOVA and the TUKEY test.

4.2. Effects of PUFA-enriched BSF prepupal or CP larval meal on growth, feed utilization, digestive features

Survival was high in all fish groups and not affected by the larval meal whatever the fly species or the substrate type. Fish fed the CP/FOD diet, rich in ALA, EPA and DHA, showed growth rates comparable to fish fed the control FMFO diet, with higher values than those of fish receiving other insect diets poor in this FA. FI did not seem to have any effect on growth, because values were similar in all the insect-based groups and comparable to the FMFO control fish. This result does not corroborate with the observation from our previous experiment where a reduction in FI was observed in Nile tilapia juveniles fed BSF meal compared to the control feed containing FM and FO (Agbohessou et al., 2021). Similar results have previously been found for the replacement of FM with BSF larval meal (Devic et al., 2018), housefly meal (Ogunji et al., 2007; Wang et al., 2017) or mealworm meal (Sánchez-Muros et al., 2016) in the diets of Nile tilapia juveniles.

The reduction in growth rate observed in the current study was associated with a significant decrease in FE and PER for fish fed the ALA-enriched diets (BSF/VGD or CP/VGD)) but also for fish fed the ALA and EPA-enriched diet (BSF/FOD). This confirms our previous results that the substitution of FM by BSF larval meal enriched with ALA or ALA and EPA did not allow a sufficient feed utilization, and thus, a good growth rate in Nile tilapia (Agbohessou et al., 2021). Other research on tilapia juveniles has also shown that complete replacement of FM with housefly (*Musca domestica*) meal in combination with vegetable oil resulted in significantly lower survival, weight gain, specific growth rate and feed conversion rate (Wang et al., 2017). However, the partial (70%) replacement of FM and FO with BSF larval meal did not affect tilapia juveniles reared in a lake cage system (Devic et al., 2018). Our results have shown that only an enrichment of fly larval meal with both, PUFA and LC-PUFA, provides a good substitute for fishmeal and fish oil in the diet of Nile tilapia. The physical properties, the length of the carbon chains and the degree of unsaturation of such PUFA associations, particularly the inclusion of DHA, could explain the positive effect on growth performance. In salmonids such as rainbow trout, it has been reported that a DHA-enriched diet improved the growth performance of juveniles compared to an LA-enriched diet (Cornet et al., 2018). Indeed, DHA plays only a minor role as an energy source but rather strongly influences membrane flexibility and fluidity while reducing membrane thickness (Hishikawa et al., 2017). It has been shown that the water permeability of membranes was improved when cell membranes were composed of PUFA bilayers compared to monounsaturated bilayers (Huster et al., 1997; Cornet et al., 2018). Thus, in our study, the membranes of tilapia fed with a DHA-enriched diet could be thinner, softer and more fluid than those of fish fed with an ALA- or ALA + EPA-

Table 3A
Analyzed composition of ingredients (g/Kg dry matter) and nutrient contents of experimental diets.

Diets	FMFO	BSF/ VGD	BSF/ FOD	CP/ VGD	CP/ FOD
BSF larval meal	0	225.60	225.60	0	0
Fish meal[a]	110	0	0	0	0
C. Putoria larval meal	0	0	0	225.60	225.60
Soybean meal[b]	335	359	359	330	330
Corn flour[c]	249.60	140	140	159	159
Wheat bran[d]	150	160	160	160	160
Betaine[e]	20	20	20	20	20
Menhaden fish oil[f]	30	0	0	0	0
Palm oil[g]	30	20	20	30	30
Blood meal[h]	30	30	30	30	30
Carboxymethyl cellulose[i]	20	20	20	20	20
Mineral Premix[j]	10	10	10	10	10
Vitamin premix[k]	10	10	10	10	10
BHT[l]	0.20	0.20	0.20	0.20	0.20
BHA[m]	0.20	0.20	0.20	0.20	0.20
L-Methionine[n]	5	5	5	5	5
TOTAL	1000	1000	1000	1000	1000
Dry matter (g/kg)	894.92	925.85	922.07	917.59	923.12
Ash (g/kg dry matter)	83.06	82.52	85.43	54.99	53.79
Protein (g/kg dry matter)	330.00	336.80	335.00	333.30	347.10
Lipid (g/kg dry matter)	88.40	89.50	88.10	92.90	93.40
NDF (g/kg DM)[o]	138.40	185.20	185.20	189.60	189.60

FMFO = control diet containing fish meal (FM) and fish oil (FO) as protein and lipid sources.
BSF/VGD / CP/VGD = diet formulated using *Hermetia illucens*-black soldier fly (BSF) prepupal or *Chrysomya putoria* (CP) larval meal produced from larvae cultured on vegetable culture substrate (VGD).
BSF/FOD / CP/FOD = diet formulated using BSF or CP meal produced from larvae cultured on fish offal substrate (FOS).
a, e, f, i, j, k, l, m, n Sigma-Aldrich, St Louis, MO, USA.
b, c, d, g, h Veto Service group, Cotonou, Benin.
BHA, butylated hydroxyanisole; BHT, butylated hydroxyl toluene.
jMineral premix (g/kg of mix) was prepared in the lab. From ($CaHPO_4$) $2H_2O$. 727.77; ($MgSO_4$) $7H_2O$. 127.50; NaCl. 60.00; KCl. 50.00; ($FeSO_4$) $7H_2O$. 25.00; ($ZnSO_4$) $7H_2O$. 5.50; ($MnSO_4$) $4H_2O$. 2.54; ($CuSO_4$) $5H_2O$. 0.78; ($CoSO_4$) $7H_2O$. 0.48; ($CaIO_3$) $6H_2O$. 0.29; ($CrCl_3$) $6H_2O$. 0.13.
kThe vitamin premix was formulated following (Abboudi et al., 2009), (to provide g/kg mixture, except as noted): retinyl acetate (1,500,000 IU/g), 0.67; ascorbic acid, 120; cholecalciferol (4,000,000 IU/g), 0.1; tocopheryl acetate (1000 IU/g), 34.2; menadione, 2.2; thiamin, 5.6; riboflavin, 12; pyridoxine, 4.5; calcium-panthotenate, 14.1; p-aminobenzoic acid, 40; vitamin B12, 0.03; niacin, 30; biotin, 0.1; choline chloride, 350; folic acid, 1.5; inositol, 50; canthaxanthin, 5; astaxanthin, 5; butylated hydroxytoluene, 1.5; butylated hydroxyanisole, 1.5; αcellulose, 325.
oNeutral detergent fiber (NDF) values were theoretically calculated.

enriched diet, resulting in better nutrient uptake. As a corollary, FE and PER were increased and thereby growth performance in fish fed the CP/FOD, rich in ALA + EPA and DHA as reported above. The lipid sparing effect of maximizing protein utilization for growth and improving PER has previously been reported in various fish species (Luo et al., 2005; Song et al., 2009; Chatzifotis et al., 2010; Giri et al., 2011; Han et al., 2014; Nayak et al., 2018). For the first time, we demonstrate here that a balanced diet containing specific PUFA and LC-PUFA improves the lipid sparing protein process and thereby the overall feed utilization in fish compared to diets containing only PUFA or PUFA + EPA. Such a strategy could be useful for other alternatives proposed as substitutes for fish-meal and fish oil.

Our results showed no clear influence on nutrient digestibility and FE or growth rate as only the BSF/DVG diet induced low protein and lipid ADCs unlike other insect diets. This diet had high levels of SFAs and was devoid of EPA and DHA with a very low n-3/n-6 ratio (Table 2), which could reduce nutrient digestibility. The limited digestibility of lipids in poor phospholipid oil sources has previously been reported (Caballero et al., 2003; Menoyo et al., 2003; Teoh et al., 2011). It has also been documented that marine microalgae *Schizochytrium* sp. with high lipid and unsaturated fatty acid content induce a high lipid ADC in Nile tilapia (Sarker et al., 2016). This relationship between LC-PUFA dietary content and nutrient digestibility was confirmed by our study as fish fed the CP/FOD diet displayed the highest levels of EPA and DHA in muscles associated with high protein and lipid ADCs compared to fish fed other insect-diets. The levels of palm oil containing palmitic acid added in the different diets may have modified the level of SFAs, and thus affected overall fat digestibility.

Table 4
Primer sequences used for the analysis of the expression of certain genes related to the immune response, pro-inflammatory, anti-inflammatory and fatty acid biosynthesis, in Nile tilapia.

Genes	Primer sequence (5′-3′)	Reference
Fatty acid biosynthesis genes		
Fads2	Fw:5'-CTGGTCATCGATCGAAAGGT-3'	KF268464.1
	Rv:5'-GCGGCTTCAGAAACTTATGC-3'	(Chen al., 2018)
Fads 6	Fw:5'-GCGGGAAAGCTAAAGGTGTT-3'	AB069727.1
	Rv:5'-TGGCATCCTCTCCAGCATAG-3'	
elovl5	Fw:5'-GATCACGTTCCTGCACCTCT-3'	NM_001279460.1
	Rv:5'-GCTGAGAGGCCATAGTACGA-3'	
immune genes		
tlr-2	Fw:5'-GGGGCTAAGTTCACAGCCAT-3'	XM_013264298.2
	Rv:5'-TGGGGAAGGATAGCCCAGAA-3'	(Ting et al., 2018)
tlr-5	Fw:5'-CCTGGTGTGGCTTTCAGAAC-3′	XM_019353524.1
	Rv:5'-AAAGCGCTGTTCCGTATGAC-3′	
tlr-7	Fw:5'-CAGCAGGGTGAGAGCATAC-3'	XM_005477981.1
	Rv:5'-ACATATCCCAGCCGTAGAGG-3'	(Ting et al., 2018)
β- defensing-1	Fw:5'-ATGTAGAAAGGTTTGCCTCCCA-3′	KJ577575.1
	Rv:5'-CAGCCCAGAGGTCCAAAGAAC-3′	(Abarike et al., 2018)
hepcidin	Fw:5'-GAGCTCTGCCATCCCATTTG-3′	XM_003450530.5
	Rv:5'-GTTGCGGGAATCCTCAGAA-3′	
mhcII-α	Fw:5'-ATCCACCATCAACGTTCCCT-3′	AF212856.1
	Rv:5'-TCAGACCCACTCCACAGAAC-3′	
mpo	Fw:5'-GGGGAGAAATGTACAAAGCAGA-3′	GBDC01000001.1
	Rv:5'-GAAACCGACGCTTCACCTTG-3′	
complement C4	Fw:5'-AAAAGCCTCCCAGTCCAGAA-3′	XM_005472473.4
	Rv:5'-CAGTCAGATGCACGGTGATG-3'	
il-1β	Fw:5'-ACCTGCTTTCCATCATGCTG-3'	DQ061114.1
	Rv:5'-CGACATGTTCAGGTGCACTAT-3'	
il-6	Fw:5'-AACCAGTCCCCTCCTCCTAT-3'	XM_019366098.2
	Rv:5'-GCCCCAATTCAACACGTTCT-3'	
tnf-α	Fw:5'-GTCGTCGTGGCTCTTTGTTT-3'	NM_001279533.1
	Rv:5'-AGTCGCTGCCTTCTAGATGG-3'	
ifn-y	Fw:5'-GAGCTGAAGAAACACCGCTACCA-3'	NM_001287402.1
	Rv:5'-GCGTCTCCTCTGGATCTTGA-3'	
il-10	Fw:5'-TTCATCCTGGCACTTTACGC-3'	XM_019352826.2
	Rv:5'-GTGGACAAGGAGGAGGTGAA-3'	
tgf-β	Fw:5'-GAGGAGGTGGCTGTGAAGAT-3'	XM_003457515.5
	Rv:5'-TTGATCATGCCCTCCACTGT-3'	
Housekeeping genes		
β-actin	Fw:5'-CCATGTACGTTGCCATCCAG-3'	EF206801.1
	Rv:5'-TGATGTCACGCACGATTTCC-3'	
18 S	Fw:5'-GGCAACCAACGGTAAAACAA-3'	DQ397879.1
	Rv:5'-AGCTAGCTGCGTTCTTCATTG-3'	

The activity of digestive enzymes in fish may be variable depending on the different food ingredients used (Zambonino Infante and Cahu, 2007; Lin and Luo, 2011). In the present study, aminopeptidase or trypsin values were the highest in fish fed the CP/FOD or CP/VGD diets. Only alkaline phosphatase was enhanced by all the insect-based diets. The better stimulation of the proteolytic digestive enzymes by the CP-based diets could explain the highest ADCs of proteins in the CP/FOD fish, and thereby their better feed utilization and growth performance. In an earlier study, pepsin activity in the stomach of Nile tilapia had significantly increased with increasing dietary protein levels (Durigon et al., 2019). A decrease in protease activities in the gut and in hepatopancreas has been observed with the increase of soybean meal as a

Table 5
Growth performance, feed utilization and survival of juvenile Nile tilapia fed with fish meal (FM) and fish oil (FO) or *Hermetia illucens*-black soldier fly (BSF) prepupal or *Chrysomya putoria* (CP) larval meal enriched with (LC)-PUFA for 60 days (mean ± S.D., n = 3). Means in a row without a common superscript letter differ ($p < 0.05$) as analyzed by two-way ANOVA and the TUKEY test.

		BSF		CP		*P*-value			
Parameters	FMFO	BSF/VGD	BSF/FOD	CP/VGD	CP/FOD	Insects	Sub[1]	I × S[2]	F
Initial body weight (g)	6.55 ± 0.05	6.55 ± 0.02	6.62 ± 0.07	6.50 ± 0.07	6.62 ± 0.04	0.423	0.051	0.518	$F_{4,10} = 2.24$
Final body weight (g)	47.84 ± 1.64^a	37.52 ± 1.98^b	36.69 ± 0.59^b	40.26 ± 2.60^b	47.23 ± 0.67^a	<0.001	0.013	0.004	$F_{4,10} = 46.53$
Relative weight gain (RWG, %)	630.40 ± 26.66^a	472.51 ± 29.07^{bc}	454.20 ± 7.88^c	519.45 ± 46.59^b	614.04 ± 26.66^a	<0.001	0.049	0.009	$F_{4,10} = 24.41$
Specific growth rate (SGR, % d^{-1})	3.31 ± 0.06^a	2.91 ± 0.08^{bc}	2.85 ± 0.02^c	3.04 ± 0.12^b	3.28 ± 0,03^a	<0.001	0.071	0.011	$F_{4,10} = 23.57$
Feed intake (FI, g/fish/day)	0.73 ± 0.01	0.73 ± 0.05	0.71 ± 0.004	0.74 ± 0.02	0.75 ± 0.01	0.24	0.901	0.414	$F_{4,10} = 0.61$
Feed efficiency (FE)	0.94 ± 0.03^a	0.71 ± 0.1^b	0.70 ± 0.02^b	0.76 ± 0.05^b	0.90 ± 0.002^a	0.004	0.077	0.05	$F_{4,10} = 13.45$
Protein efficiency ratio (PER)	2.85 ± 0.08^a	2.11 ± 0.29^b	2.10 ± 0.05^b	2.29 ± 0.16^b	2.61 ± 0.007^a	0.007	0.162	0.127	$F_{4,10} = 13.42$
Survival (%)	97.78 ± 1.92	96.67 ± 3.33	100 ± 00	96.67 ± 3.33	96.7 ± 3.33	0.347	0.347	0.347	$F_{4,10} = 0.85$

FMFO = control diet containing fish meal (FM) and fish oil (FO) as protein and lipid sources.
BSF/VGD / CP/VGD = diet formulated using *Hermetia illucens*-black soldier fly (BSF) prepupal or *Chrysomya putoria* (CP) larval meal produced from larvae cultured on vegetable culture substrate (VGD).
BSF/FOD / CP/FOD = diet formulated using BSF or CP meal produced from larvae cultured on fish offal substrate (FOS).
[1] Sub = Substrates; [2]I × S = Insects × Substrates interaction effect.

Table 6
Apparent digestibility coefficients (ADC) for nutrients in juvenile Nile tilapia fed fish meal (FM) and fish oil (FO) or *Hermetia illucens*-black soldier fly (BSF) prepupal or *Chrysomya putoria* (CP) larval meal enriched with (LC)-PUFA (mean ± S.D., n = 3). Means in a row without a common superscript letter differ ($p < 0.05$) as analyzed by two-way ANOVA and the TUKEY test.

		BSF		CP		P-value			
Parameters	FMFO	BSF/VGD	BSF/FOD	CP/VGD	CP/FOD	Insects	Sub[1]	I × S[2]	F
ADC_{DM} (%)	74.80 ± 2.63	62.91 ± 7.68	66.73 ± 5.11	64.42 ± 4.71	73.13 ± 8.01	0.107	0.099	0.493	$F_{4,10} = 2.36$
$ADC_{protein}$ (%)	89.54 ± 1.25^a	71.51 ± 8.87^b	81.10 ± 5.92^{ab}	82.02 ± 3.39^{ab}	88.73 ± 3.37^a	0.009	0.023	0.646	$F_{4,10} = 5.74$
ADC_{lipid} (%)	93.94 ± 1.00^a	87.48 ± 2.59^b	91.28 ± 0.99^{ab}	89.69 ± 2.31^{ab}	93.50 ± 1.26^a	0.013	0.004	0.996	$F_{4,10} = 6.92$

FMFO = control diet containing fish meal (FM) and fish oil (FO) as protein and lipid sources.
BSF/VGD / CP/VGD = diet formulated using *Hermetia illucens*-black soldier fly (BSF) prepupal or *Chrysomya putoria* (CP) larval meal produced from larvae cultured on vegetable culture substrate (VGD).
BSF/FOD / CP/FOD = diet formulated using BSF or CP meal produced from larvae cultured on fish offal substrate (FOS).
[1] Sub = Substrates; [2]I × S = Insects × Substrates interaction effect.

Table 7
Digestive enzyme activities of juvenile Nile tilapia fish meal (FM) and fish oil (FO) or *Hermetia illucens*-black soldier fly (BSF) prepupal or *Chrysomya putoria* (CP) larval meal enriched with (LC)-PUFA for 60 days (mean ± S.D., n = 3). Means in a row without a common superscript letter differ ($P < 0.05$) as analyzed by two-way ANOVA and the TUKEY test.

		BSF		CP		*P*-value			
Parameters	FMFO	BSF/VGD	BSF/FOD	CP/VGD	CP/FOD	Insects	Sub[1]	I × S[2]	F
Amylase (U mg. protein^{-1})	2.10 ± 0.71	2.45 ± 0.87	3.66 ± 0.57	2.33 ± 0.68	2.82 ± 0.79	0.291	0.08	0.427	$F_{4,10} = 2.10$
Pepsin (mU mg. protein^{-1})	219.14 ± 20.91^b	240.61 ± 23.37^b	249.73 ± 30.65^b	382.99 ± 80.82^a	397.20 ± 114.43^a	0.008	0.788	0.953	$F_{4,10} = 5.03$
Trypsin (mU mg. protein^{-1})	131.32 ± 14.82^{ab}	111.39 ± 5.92^b	110.77 ± 10.65^b	149.56 ± 7.87^a	115.70 ± 11.28^b	0.027	0.051	0.056	$F_{4,10} = 4.79$
Aminopeptidase (mU mg. protein-1)	45.37 ± 6.96^b	41.82 ± 7.74^b	43.98 ± 6.57^b	53.87 ± 19.84^b	83.75 ± 4.24^a	0.004	0.04	0.067	$F_{4,10} = 8.04$
Alkaline phosphatase (mU mg. protein^{-1})	203.20 ± 35.44^b	302.27 ± 40.84^a	286.84 ± 59.54^a	313.58 ± 12.78^a	293.93 ± 24.43^a	0.753	<0.001	0.614	$F_{4,10} = 4.03$

FMFO = control diet containing fish meal (FM) and fish oil (FO) as protein and lipid sources.
BSF/VGD / CP/VGD = diet formulated using *Hermetia illucens*-black soldier fly (BSF) prepupal or *Chrysomya putoria* (CP) larval meal produced from larvae cultured on vegetable culture substrate (VGD).
BSF/FOD / CP/FOD = diet formulated using BSF or CP meal produced from larvae cultured on fish offal substrate (FOS).
[1] Sub = Substrates; [2]I × S = Insects × Substrates interaction effect.

substitute for FM in Nile tilapia diet (Lin and Luo, 2011). Activity of some proteases such as alkaline phosphatase may vary depending on the type of proteins used. As an example, the partial replacement of FM by differently treated soybean meal in European sea bass (*D. labrax*) diets had an inhibitory effect on alkaline phosphatase activity (Tibaldi et al., 2006). In contrast, the incorporation of enzyme-treated soy protein (produced using different types of proteases to hydrolyze the protein) in the diet of juvenile carp increased the alkaline phosphatase activity

Table 8
Relative expression of innate immune genes in whole kidney in juvenile Nile tilapia fed with fish meal (FM) and fish oil (FO) or *Hermetia illucens*-black soldier fly (BSF) prepupal or *Chrysomya putoria* (CP) larval meal enriched with (LC)-PUFA for 60 days (mean ± S.D., n = 3). Means in a row without a common superscript letter differ ($p < 0.05$) as analyzed by two-way ANOVA and the TUKEY test.

		BSF		CP		P-value			
Parameters	FMFO	BSF/VGD	BSF/FOD	CP/VGD	CP/FOD	Insects	Sub[1]	I × S[2]	F
il-6	2.48 ± 1.18	1.28 ± 0.43	1.72 ± 1.06	1.03 ± 0.37	0.88 ± 0.71	0.071	0.765	0.541	$F_{4,10} = 1.87$
tnf-α	0.99 ± 0.49	1.37 ± 0.39	1.26 ± 0.42	0.93 ± 0.32	1.29 ± 0.39	0.493	0.6	0.337	$F_{4,10} = 0.70$
ifn-γ	1.83 ± 0.89	0.94 ± 0.12	0.89 ± 0.08	0.86 ± 0.13	1.29 ± 0.53	0.055	0.498	0.392	$F_{4,10} = 2.29$
il-10	1.20 ± 0.75	1.07 ± 0.31	1.70 ± 0.49	1.71 ± 0.43	2.14 ± 0.81	0.191	0.153	0.767	$F_{4,10} = 1.60$
tgf- β	0.44 ± 0.10	0.64 ± 0.26	0.77 ± 0.18	0.75 ± 0.15	0.74 ± 0.06	0.065	0.56	0.463	$F_{4,10} = 2.06$
hepcidin	1.51 ± 0.86	1.13 ± 0.57	1.08 ± 0.87	0.79 ± 0.34	0.48 ± 0.31	0.187	0.632	0.734	$F_{4,10} = 1.08$
mpo	2.91 ± 0.95	1.75 ± 0.08	2.66 ± 1.50	2.51 ± 1.13	2.17 ± 0.54	0.598	0.62	0.29	$F_{4,10} = 0.65$
c4 complement	0.44 ± 0.34	1.03 ± 0.34	0.96 ± 0.48	0.51 ± 0.10	0.66 ± 0.57	0.13	0.853	0.642	$F_{4,10} = 1.33$
tlr2	0.82 ± 0.23	1.01 ± 0.66	1.53 ± 0.80	0.67 ± 0.25	1.74 ± 0.89	0.595	0.054	0.473	$F_{4,10} = 1.60$
tlr5	1.40 ± 0.49	1.39 ± 0.39	1.43 ± 0.74	0.60 ± 0.17	0.93 ± 0.53	0.098	0.523	0.619	$F_{4,10} = 1.65$
tlr7	0.89 ± 0.05	1.38 ± 0.48	1.44 ± 0.54	0.61 ± 0.08	1.22 ± 0.42	0.082	0.149	0.243	$F_{4,10} = 2.62$
il-1β	1.16 ± 0.16^{a}	0.97 ± 0.08^{ab}	0.89 ± 0.57^{ab}	0.74 ± 0.05^{ab}	0.41 ± 0.04^{b}	0.027	0.225	0.434	$F_{4,10} = 3.23$
β- defensin-1	0.13 ± 0.19^{b}	0.10 ± 0.12^{b}	2.64 ± 0.90^{a}	0.34 ± 0.25^{b}	0.66 ± 0.96^{b}	0.029	0.002	0.010	$F_{4,10} = 9.12$
mhcII-α	0.94 ± 0.12^{b}	1.81 ± 0.81^{ab}	2.62 ± 0.28^{a}	2.03 ± 0.54^{ab}	1.79 ± 0.70^{ab}	0.026	0.399	0.131	$F_{4,10} = 3.54$

FMFO = control diet containing fish meal (FM) and fish oil (FO) as protein and lipid sources.
BSF/VGD / CP/VGD = diet formulated using *Hermetia illucens*-black soldier fly (BSF) prepupal or *Chrysomya putoria* (CP) larval meal produced from larvae cultured on vegetable culture substrate (VGD).
BSF/FOD / CP/FOD = diet formulated using BSF or CP meal produced from larvae cultured on fish offal substrate (FOS).
[1] Sub = Substrates; [2]I × S = Insects × Substrates interaction effect.

Table 3B
Fatty acid composition (% of total fatty acids identified) analyzed in the experimental diets.

Fatty acid	FMFO	BSF/VGD	BSF/FOD	CP/VGD	CP/FOD
C10:0	n.d.	0.41	0.37	n.d.	n.d.
C12:0	0.46	20.76	16.70	n.d.	n.d.
C13:0	0.62	0.33	0.12	1.19	1.20
C14:0	1.08	4.50	4.24	1.06	1.60
C15:0	n.d.	n.d.	0.37	n.d.	n.d.
C16:0	25.24	20.96	29.37	31.70	36.81
C17:0	n.d.	n.d.	0.45	n.d.	0.55
C18:0	3.54	2.59	3.46	4.69	4.92
C22:0	0.56	n.d.	n.d.	n.d.	n.d.
C24:0	n.d.	n.d.	n.d.	n.d.	0.60
Total satured fatty acids	31.50	49.55	55.08	38.64	45.68
C16:1	1.15	1.12	0.46	1.39	7.39
C18:1n9	29.23	26.30	24.28	32.41	32.60
C20:1n9	1.53	n.d.	0.38	n.d.	n.d.
Total monoenes	31.91	27.42	25.12	33.80	39.99
C18:2n6 (LA)	28.49	20.41	15.78	23.78	7.40
C18:3n6	0.79	n.d.	0.16	0.71	1.27
Total n-6 PUFA	29.36	20.41	15.94	24.49	8.72
C18:3n3 (ALA)	2.61	2.41	1.21	2.95	1.24
C20:3n3	n.d.	n.d.	0.49	n.d.	1.01
C20:4n6 (ARA)	0.08	n.d.	n.d.	n.d.	0.05
C20:5n3 (EPA)	1.65	n.d.	1.21	n.d.	1.97
C22:6n3 (DHA)	2.37	n.d.	0.10	n.d.	1.95
Total n-3 PUFA	6.63	2.41	3.01	2.95	4.93
n-3/n-6	0.23	0.12	0.19	0.12	0.57

FMFO = control diet containing fish meal (FM) and fish oil (FO) as protein and lipid sources.
BSF/VGD / CP/VGD = diet formulated using *Hermetia illucens*-black soldier fly (BSF) or *Chrysomya putoria* (CP) larval meal produced from larvae cultured on vegetable culture substrate (VGD).
BSF/FOD / CP/FOD = diet formulated using BSF or CP meal produced from larvae cultured on fish offal substrate (FOS).
n.d. = not detected.

(Xiao et al., 2017). Alkaline phosphatase is a major defense enzyme against pro-inflammatory compounds in the gut and its secretion maintains the gastrointestinal health of fish (Lallès, 2020). Therefore, the increase in alkaline phosphatase activity during the total replacement of FM with BSF or CP larval meal indicates the good gastrointestinal health of Nile tilapia. In the present study, amylase activity was not affected by any insect-based diet. The proportions of carbohydrate-containing ingredients were comparable between the BSF and CP diets (Table 3A), which may account for the absence of a significant difference in amylase activity in the fish. From the analysis of the results on digestive enzymes, it can be concluded that fly larval meal would not be a limiting factor for the nutrient utilization and would therefore not interfere with growth processes.

4.3. Influence of FA-enriched BSF prepupal or CP larval meal on immune status

The interaction between nutrition and the immune system in fish has been known for a long time, but the relationship is much more complex than originally thought (Siwicki et al., 2006). In the present study, some important immune functions were stimulated by the insect-based diets, particularly those containing the FOS larval meal regardless of the species. Fish fed the CP/FOD diet had higher peroxidase activity than those fed the FMFO control diet, but comparable to that of fish fed the other insect larvae meal diets. The CP/FOD diet contains the highest level of LC-PUFA with the highest n3/n6 level. These molecules can be oxidized due to their susceptibility to oxidative stress. The high peroxidase activity in fish fed the CP/FOD diet could be explained by activation of the antioxidant defense system in these fish. Fish fed both CP-based diets displayed higher lysozyme activity but comparable to that of fish fed BSF-based diets. Fish fed the BSF/VGD diet had a higher ACH50 activity than fish fed the FMFO control diet. The nutritional composition of BSF larvae depended in part on the composition of the feeding medium. Micronutrients, such as minerals and vitamins, can easily accumulate in BSF larvae (Liland et al., 2017). In the present study, leaves and seeds of EH were used in the VGS substrate to enrich BSF larvae with ALA. The leaves of EH contain quercetin which is an antioxidant (Falodun, 2004) and is used in medical practices as a laxative, anti gonorrhoea, migraine and worm remedies (James and Friday, 2013). The nutrients and pharmacological properties of EH leaves could have stimulated immunity in fish fed with BSF/VGS larval meal, which could explain the significant differences in plasma ACH50 activity between fish fed with BSF/VGD and BSF/FOD diets. These results showed that BSF or CP meal increased the innate immune system in Nile tilapia, particularly, when enriched with LC-PUFA. Previous studies have shown that dietary inclusion of the house fly increased the innate immune

system and resistance to diseases of the Red Sea bream (*Pagrus major* (Temminck and Schlegel, 1843)) (Ido et al., 2015). The present study confirms our previous results which have shown that BSF meal stimulated innate immunity by increasing the activity of lysozyme and ACH50 in Nile tilapia (Agbohessou et al., 2021). CP and BSF larvae live in very dirty environments that are often infested with many types of microorganisms (Choi et al., 2012; Ferraz et al., 2012). The defense strategies of insects living in these substrates result in the presence of numerous bioactive peptides with various functional properties, such as antimicrobial, antifungal and antiviral functions (Elhag et al., 2017; Józefiak and Engberg, 2017; Vogel et al., 2018). The antibacterial property of CP larvae is known for their potential uses in larval therapy (Dallavecchia et al., 2014).

The LC-PUFA contained in the BSF larval meal produced with the FOS substrate would have stimulated the expression of the gene *β-defensin-1* in fish fed with BSF/FOD. The *β-defensins* are small cationic amphiphilic peptides that are widely distributed in plants, insects and vertebrates and are important for their antimicrobial properties (Dong et al., 2015). The *β-defensins* also play a key role in boosting innate and acquired immunity. They are capable of killing a variety of pathogens, including bacteria, fungi, viruses and parasites. In addition, *β-defensins* are chemotactic attractants for immune cells and are involved in immune regulation (Ellis, 2001). These biological characteristics suggest that BSF may be rich in AMPs and other substances with activity against pathogenic bacteria (Choi et al., 2012; Park et al., 2014). The insect AMPs not only act against pathogenic bacteria, but may also enhance the innate immune system of certain species and induce certain immunomodulatory effects (Nogales-Mérida et al., 2019). Therefore, in veterinary production and animal husbandry, these peptides have been considered as an alternative to antibiotics (Nogales-Mérida et al., 2019). In the present study, a higher content of EPA in fish fed the BSF/FOD diet compared to those receiving the BSF/VGD one could explain the significant differences in the expression of the *β-defensin* gene between these fish groups. The LC-PUFA contained in the BSF larval meal produced with the FOS substrate would also have stimulated the expression of major histocompatibility complex class II molecules (*mhcII-α*) in fish fed the BSF/FOD diet. Proteins of the (*mhcII-α*) are surface glycoproteins of cell membranes. These proteins play a role in the organism's specific immune response. The molecules of this system are responsible for the binding, transport, and presentation of a foreign antigen to helper T lymphocytes (Th, CD4). They also stimulate the multiplication of specific B lymphocytes and determine the type of antibodies produced (Handunnetthi et al., 2010). They further participate in important selection processes that lead to the differentiation of immature into mature lymphocytes. In adults, *mhcII* molecules are expressed on the surface of cells identified as antigen-presenting cells (APCs), macrophages, B lymphocytes, microglial and dendritic cells (Keller et al., 1988; Wierzba-Bobrowicz et al., 2001). The higher expression of the *mhcII-α* gene in fish fed with BSF/FOD larval meal may indicate a better stimulation of the immune response compared to other fish groups.

The low expression of *il-1-β* in fish fed the CP/FOD diet (Table 8) showed that CP meal enriched with LC-PUFA did not increase inflammatory reactions in fish, indicating a good health status in these fish. Pro-inflammatory cytokines can be induced during an immune response to promote the migration of immune cells to an infection site by binding to and activating chemokine receptors (Fernandez and Lolis, 2002). All the results obtained for the innate immunity genes studied do not indicate any marked negative effect of BSF or CP meal enriched with LC-PUFA on various immune functions, namely bactericidal and pro-inflammatory processes. EPA, ARA and DHA are capable of promoting or suppressing the inflammatory and immune response by affecting the fluidity of the cell membrane (Stenberg et al., 2019).

5. Conclusion

The results of this study showed a higher efficiency of LC-PUFA enrichment for CP larvae compared to BSF ones, and a CP larval-based diet balanced in PUFA and LC-PUFA resulted in a greater stimulation of the processes of nutrient utilization, and thus a greater growth capacity. In addition, an improved DHA content was observed in the muscles of fish fed with the CP/FOD larval meal compared to those fed with all other insect larval meals. Nevertheless, all types of dipteran larval meal were palatable to Nile tilapia and enriched fish muscles with EPA except those fed the BSF/VGD diet. In addition, all types of dipteran larval meal boosted the immune status regardless of species or dietary essential fatty acid quality. Further studies are needed to elucidate the potential beneficial effects of BSF or CP larval meal on pathogen resistance in Nile tilapia.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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