UNIVERSIDADE DE AVEIRO
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MODULATION OF *HERMETIA ILLUCENS* LIPID PROFILE BY INCORPORATION OF AN INVASIVE MACROALGAE – A MULTIGENERATIONAL APPROACH

MODULAÇÃO DO PERFIL LIPÍDICO *DA HERMETIA ILLUCENS* ATRAVÉS DA INCORPORAÇÃO DE UMA ALGA INVASORA – UMA ABORDAGEM MULTIGERACIONAL

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica da Doutora Olga Maria Correia Chitas Ameixa, investigadora assistente do Departamento de Biologia & CESAM, Universidade de Aveiro.

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o júri

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palavras-chave

Hermetia illucens, Agarophyton vermiculophyllum, GC-MS, ácidos gordos polinsaturados, aquacultura, peixes, macroalga invasora, alimentação, insetos

resumo

Nos últimos anos, o aumento da procura por fontes de alimentação mais sustentáveis, a nível mundial, aumentou a atenção dada à aquacultura. De forma a ultrapassar a dependência do peixe de pesca e de outras opções de base vegetal, o uso de insetos já é atualmente usado como um ingrediente alternativo em rações. No entanto, a principal fonte de ácidos gordos polinsaturados (PUFA) nas dietas dos peixes ainda é o óleo de peixe, obtido através da pesca excessiva, e que compete diretamente com a cadeia de consumo humano, aumentando o preço destes ingredientes e, consequentemente, a necessidade de encontrar soluções alternativas para eles. A *Hermetia illucens*, vulgarmente conhecida como mosca soldado negro, é rica em proteína, no seu estado larval, e o seu conteúdo em ácidos gordos pode ser modulado de acordo com o substrato fornecido. Neste trabalho, fez-se a incorporação da macroalga *Agarophyton vermiculophyllum*, uma espécie invasiva abundante na Ria de Aveiro, nas dietas da *H. illucens*. O objetivo foi avaliar a capacidade de bioconversão da macroalga por parte da *H. illucens* e estudar a possível incorporação dos PUFA presentes na macroalga nos tecidos larvares, quando dietas contendo 25% e 50% de *A. vermiculophyllum* foram fornecidas durante quatro gerações. Paralelamente, foram calculados parâmetros para avaliar o crescimento larval e os índices de conversão, e o perfil lipídico foi analisado por cromatografia gasosa acoplada a espetrometria de massa (GC-MS). Os resultados obtidos mostram que houve aceitabilidade da dieta, uma vez que o conteúdo proteico das larvas foi constante durante a experiência (38 a 46% DM [matéria seca]), independentemente do substrato ou geração. Leves efeitos negativos foram observados para 50% de incorporação, nomeadamente um ciclo de vida mais lento nas primeiras duas gerações, e o alcance do estado de prepupa mais cedo no caso das duas últimas gerações. Na formulação da dieta, a incorporação de 50% de *A. vermiculophyllum* resultou na ocorrência de ácido araquidónico (C20:4) e docosa-hexaenóico (C22:6). Contudo, a presença de ácido araquidónico nas larvas apenas foi observada na terceira geração. Uma diminuição transgeracional foi verificada no conteúdo de ácidos gordos saturados, atribuída maioritariamente às alterações na abundância do ácido láurico. É reportada uma melhoria no conteúdo em PUFA, devida à abundância do ácido linoleico.

Em síntese, a incorporação de *A. Vermiculophyllum* nas dietas de larvas de *H. illucens* induziu mudanças no perfil lipídico, com um aumento no conteúdo de ácidos gordos polinsaturados e redução dos ácidos saturados. As larvas mostraram também capacidade transgeracional para se adaptar a dietas inusitadas. Embora não sejam adequadas para substituir fontes convencionais de n-3 PUFA na alimentação de peixes, as larvas da mosca soldado negro podem ser usadas como fonte de proteínas e de outros ácidos gordos importantes, aproveitando uma macroalga com comportamento invasivo como alimento.

keywords

Hermetia illucens, Agarophyton vermiculophyllum, GC-MS, polyunsaturated acids, aquaculture, fish, aquafeeds, invasive macroalgae, insect meals

abstract

The world's increasing demand for more sustainable food sources has brought more and more attention to aquaculture production in recent years. To overlap the dependence on pelagic fish and other plant-based options, insect meals are already being incorporated as alternative feed ingredient. However, the main source of polyunsaturated fatty acids (PUFA) in fish's diets are still fish oil, obtained from overharvesting, mostly of pelagic fish, which competes directly with human consumption, and increasing the price of these ingredients and the need to find alternative solutions. *Hermetia illucens*, commonly known as black soldier fly, has a high protein content in the larval stage and the fatty acid content can be improved and modulated according to the substrate provided. In this work, it was incorporated the invasive macroalgae species *Agarophyton vermiculophyllum*, found widely in Ria de Aveiro, in the diet of *H. illucens*. The aim was to evaluate the bioconversion capacity of *H. illucens* and its ability to incorporate macroalgae PUFA in larval tissue when fed with diets with replacement levels of 25% and 50% of *A. vermiculophyllum* over four generations. Alongside, larvae grown parameters and conversion indexes were calculated and fatty acid profile was analysed by gas chromatography coupled to mass spectrometry (GC–MS). The results show the acceptability of the diet, since the larvae protein content was constant throughout the experience (38 to 46% of DM [dry matter]), independently of the substrate or generation. Mild deleterious effects were observed for 50% inclusion rate. The life cycle was slower in the first two generations, and the prepupal stage was reached earlier in the last two generations. In the diet formulation, the incorporation of 50% *A. vermiculophyllum* led to the occurrence of arachidonic (20:4) and docosahexaenoic acids (22:6). However, arachidonic acid was present in the larvae only in the third generation. A transgenerational decreased was observed in the saturated fatty acids content, defined mainly by the lauric acid content. An improvement in the PUFA content was reported, mostly due to linoleic acid abundance.

Overall, the incorporation of *A. vermiculophyllum* in the diets of *H. illucens* larvae led to changes in its lipidic profile with increase in PUFA and reduction in saturated fatty acids. Larvae also demonstrated the ability to adapt to less suitable diets over several generations. Despite not being able to completely replace conventional n-3 PUFA sources in fish feed, black soldier fly larvae can be used as a protein source as well as other important fatty acids using an invasive macroalgae species as feeding substrate.

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List of abbreviations

(n-3) – omega-3

(n-6) – omega-6

ALA – α-linolenic acid

ARA – arachidonic acid

AV – *Agarophyton vermiculophyllum*

BCR – bioconversion ratio

BSF – black soldier fly

BW – body weight

CP – crude protein

DHA – docosahaenoic acid

DW – dry weight

EC – European Comission

EFA – essential fatty acids

EPA – eicosapentaenoic acid

FA – fatty acids

FAMEs – fatty acid methyl esters

FAO – Agriculture Organization of the United Nations

FCR – feed conversion ratio

G – generation

GC-MS - gas chromatography-mass spectrometry

GR – growth rate

LA – linoleic acid

MUFA – monounsaturated fatty acids

PAP – processed animal proteins

PUFA- polyunsaturated fatty acids

R – residue

SFA – saturated fatty acids

SR – substrate reduction

TAGs – triacylglycerols

TSE – transmissible spongiform encephalopathies

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1. Introduction

1.1. The role of aquaculture

The global population has rapidly increased over the last years due to several improves on life standers, such as advanced maternity and healthcare. This growth brings some concerns, and the major one is the assurance of food supply (FAO, 2020b). With an anticipated population of 10 billion people expected to inhabit the planet by 2050, the demand for animal protein will increase by 50 to 60 percent (FAO, 2016), setting off new challenges in agriculture, as the growing demand for animal protein has already overlapped the availability of cereal-based feed ingredients, mainly in low- and middle- income countries.

The Food and Agriculture Organization of the United Nations (FAO) assessed that in 2017, 34,2% of fish stocks were overfished at biological unsustainable (FAO, 2020a). Since capture fisheries have been static since the late 1980s (FAO, 2016), aquaculture has the potential to be a sustainable alternative to assure the world food supply, and it is responsible for the continuing impressive growth of the fish supply for human consumption. Under this scenario, following the need to feed a growing world population and an increased demand for food production, reducing the environmental footprint of aquaculture has become a high priority to achieve more sustainability in the food supply chain in modern society, as supported by a recent communication in science targeting blue farming - “Strategic guidelines for a more sustainable and competitive EU aquaculture for the period 2021-2030”.

Fish and livestock are the main sources of protein in most countries. Livestock production accounts for 70 % of all agricultural land use and it is expected to more than duplicate until 2050. (Ameixa, Duarte, & Rodrigues, 2019; FAO, 2020a) The rise in the consumption of animal products will require enormous resources and animal feed is the most challenging, mainly due to the associated costs incurred from the limited availability of natural resources, the current climatic changes and the food-feed-fuel competition (Makkar, Tran, Heuzé, & Ankers, 2014a).

1.2. Sustainability of aquaculture feed ingredients

The main challenge in reducing the environmental impact of aquaculture is the large dependence on the supply of terrestrial and, mostly, aquatic feed ingredients. For some types of aquaculture, mainly of carnivorous fish, which naturally have a diet rich in protein and oil from wild fish sources, it is a challenge to meet the nutritional requirements. Thus, the main ingredients used in aquafeeds are fish meal, fish oil and soybean meal (Tschirner, Fisheries, Kloas, & Fisheries, 2017). Unfortunately, fish feed represents over 50% of the operating costs in intensive aquaculture (Garcia-Vaquero & Hayes, 2016). The majority of feed ingredients originates from industry by-products, encompassing 25 to 35% of the total volume of fish meal and fish oil produced (FAO, 2020a). However, these fish by-products present significant environmental and technical challenges due to their susceptibility to fast degradation. Also, processing fish residues such as tails, bones and heads changes the quality of the fish meal, which becomes less protein-rich, and can negatively impact the inclusion rates in feed formulations (Tschirner et al., 2017). Still, part of fish meal and fish oil is originated from wild fish, mainly from pelagic forage fish (*e.g.,* mackerel, herring, sardine and anchovy), which creates a dependence on wild fisheries. The use of pelagic fish to produce feed ingredients is increasingly controversial due to several reasons, for instance, environmental concerns about the effects of these small fishes in the marine ecosystems; the perturbation of these stocks (Tacon & Metian, 2009), and a complex of ethical, food security and poverty issues (Alder, Campbell, Karpouzi, Kaschner, & Pauly, 2008). Also, when reduced to fish feed, pelagic forage fish is being taken out of the direct human food chain (either for direct human consumption or for nutrition supplements)(FAO, 2020a).

The limited availability and high prices of aquafeed raw materials, adding to the increasing production pressure on aquaculture, have determined the search for new and more compensatory sources of feed. The sustainable growth of the aquaculture sector will depend largely on its feed supply. The last years, fish meal and fish oil inclusion rates in aquaculture's feed had suffered a downward trend. Substitutes are mostly plant-based protein-rich feedstuffs (e.g., oilseed meal, seaweed and plant meal). However, the advantages overlap, mainly the low

protein content, unbalance essential amino acid profile, low palatability, and the presence of anti-nutrients, *i.e*., the absorption of other nutrients becomes blocked. In the last years, plant-protein for fish feed has also faced problems related to increasing prices and competition with other industries, such as human consumption, animal husbandry sector or biodiesel production (Moutinho et al., 2017). Nevertheless, fish meal and fish oil still are the most nutritious and the major source of omega-3 polyunsaturated fatty acids, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Despite this, terrestrial plant-based protein, mostly soybean meal, have been used as aquafeed ingredients. Other plant-based ingredients such as, corn, wheat and rapeseed were used as replacement of fish meal, with no significant consequences observed with a replacement of up to 98%, showing high acceptability, similar growth performances and nitrogen utilization by fish (Kaushik, Covès, Dutto, & Blanc, 2004). However, in these diets, high percentages of fish oils were supplemented, which may justify the overall positive results. For instance, in a different study, when the replacement of fish meal with soybean was higher (>50%), it was shown to reduce the voluntary daily feed intake, growth and welfare of fish at some point, in a study where only plant oils were fed to carnivorous fish (Olsen, 2011). Bottom line, the supplementation with fish oil in plant-based meals is crucial, since the oils of higher plants do not contain omega-3 (n-3) polyunsaturated fatty acids (PUFA), a dietary requirement for fish (Sargent et al., 1999). The most frequently used oils of terrestrial plants are rapeseed oil, linseed oil, and palm oil, that can be rich in C18 essential fatty acids (EFA), namely α-linolenic acid (ALA), and most of them are rich in linoleic acid (LA), but unable to meet the fatty acid requirements of fish (Olsen, 2011).

Another option recurrently used are terrestrial processed animal proteins (PAP) such as blood meal, bone meal, feather meal, and poultry by-product meal (Gasco et al., 2018), but the inclusion levels of these ingredients are limited by fish species poor digestibility, deficiency of some essential amino acids, variability in quality and high ash content (Hardy, 1996). Other options are being studied, such as grain legumes, single cell protein (microalgae, yeasts, fungi and bacteria), duckweed and insects (insect meal, insect oil or whole insect). Providing adequate feedstuff is crucial to achieve a greater fish performance and

success in the aquaculture system. To this end, in the last years, more attention has been given o insects.

1.3. Insects as alternative feed sources

Insect biomass is pointed as a sustainable solution to both livestock and aquaculture feed with potential to be part of a circular economy strategy (Borrello, Lombardi, Pascucci, & Cembalo, 2016). Insects are nutritious and can provide ample bioavailable proteins, fats, vitamins, minerals, and fibres. The nutritional composition is dependent on the taxonomic group but, in general, insects have a very high protein content, a balanced profile of amino acids (Barroso et al., 2014), and are rich in other beneficial nutrients such as vitamins and minerals (van Huis, 2013).

Insects present several advantages when compared to conventional sources of feed. They present higher reproduction rates and food conversion efficiencies than cows, pigs or poultry (Halloran, Roos, Eilenberg, Cerutti, & Bruun, 2016), since they are poikilothermic and do not require feed to maintain body temperature (van Huis & Oonincx, 2017). Another benefit of using insects is that they can be reared in a sustainable way using discarded organic by-products with low water input or biowaste. Moreover, rearing insects has a small ecological footprint, because there is no need for arable land, and they produce low concentrations of greenhouse gases (*e.g.,* CO_2, CH_4) and ammonia (NH_3) (Akhtar & Isman, 2000). The intensive animal production is a starting place for many health issues that can trigger the emergence of antibiotic-resistant pathogens, which may cause large-scale losses of animals. Regarding health safety, because of their lack of similarity with humans, insects present a lower risk of transmitting zoonotic infections (van Huis, 2013).

All these characteristics reinforce the potential of insects as a cheap ingredient to be produced in captivity. However, the expansion of insect rearing, at least in the European Union, is conditioned by the insect species and feed sources legally authorized to be used as rearing substrates. The legislation varies worldwide and encompasses mainly food safety, marketing, animal welfare and sustainability. In

the European Union, the use of animal-based sources in feed formulations is regulated by the Commission Regulation (EC) No 999/2001, created to prevent transmissible spongiform encephalopathies (TSE) spreading from poor control in meat and bone meal in animal feed, and therefore it is also known as the “TSE regulation”. With this regulation, alternative solutions to conventional animal-based diets were even more urgent. In 2013, a new regulation was released (Commission Regulation [EU] No 56/2013), enabling processed animal proteins derived from non-ruminant animals to be used as ingredients in aquaculture feed. In 2017, an amending to the TSE Regulation was adopted (Regulation [EU] No 2017/893), allowing, with restricted rules, the utilization of insect processed animal proteins for aquaculture animals. More recently, in 2021, a publication in the official journal pf the European Union of Commission Regulation 2021/1372 published that the use of PAP derived from insects in poultry and pig feed is now authorized.

1.3.1. *The use of insects in fish meal formulations*

Since 2017, seven insect species are authorized to be reared in the EU for aquafeed use: Black Soldier Fly (*Hermetia illucens*), Common Housefly (*Musca domestica*), Yellow Mealworm (*Tenebrio molitor*), Lesser Mealworm (*Alphitobius diap perinus*), House cricket (*Acheta domesticus*), Banded cricket (*Gryllodes sigillatus*) and Field Cricket (*Gryllus assimilis*).

Several scientific evidence were gathered in the last years concerning the nutritional value of insect meal for fish feed. Results vary, depending on the species under study, insect life stage, feed substitution rate, among other factors (Barroso et al., 2014; Makkar, Tran, Heuzé, & Ankers, 2014b). In a study conducted by Barroso et al. (2014), where the potential of various insect groups (Coleoptera, Diptera and Orthoptera orders) as feed ingredients for fish was evaluated, it was shown that insect species have a high proportion of protein, between 40 and 60%, similar to soy meal levels (50% crude protein [CP]) but lower than fishmeal (73% CP). The Diptera order showed a balanced amino acid profile, indicating that this group of insects could be a suitable protein source for

aquaculture (Barroso et al., 2014). This study also referred that insects contained higher quantities of omega 6 (n-6) PUFAs than fish meal, but lower than soy meal. However, as in soy meal, lower levels of n-3 PUFA were observed in insect meals, when compared with fish meal. The previous acknowledgment that terrestrial insects do not contain EPA or DHA was supported by this article, and the lack of these essential fatty acids is one of the biggest limitations to its use in marine aquafeeds. Insect larvae are poor in highly unsaturated FA. Normally, increasing the levels of insect meal lead to a considerable change of the fatty acid profile in fish, with a decrease in EPA, DHA and in the (n-3)/(n-6) ratio (Gasco et al., 2018).

The most promising species for industrial feed production are the black soldier fly, common housefly, silkworms, yellow mealworms and grasshopper species. Among these species, the black soldier fly (BSF) stands out because of its capacity to convert several substrates into body mass, containing 42% protein and 35% fat on a dry matter basis (Craig Sheppard, Larry Newton, Thompson, & Savage, 1994).

1.3.2. *Potential of the Black soldier fly*

Hermetia illucens (Linnaeus, 1758) (Diptera: Stratiomyidae), the black soldier fly (BSF), is supposedly native to the Neotropics (Wang & Shelomi, 2017), even though this hypothesis was recently cast doubt on, brought forward by a possible Palearctic origin (Benelli, Canale, Raspi, & Fornaciari, 2014), and it is distributed in the tropics and warmer climates (Kim et al., 2011). BSF is a scavenger species and because of this feeding habit it can be used to accelerate the bioconversion of low-quality biomass, e.g., organic waste and by-products of the agri-food transformation chain such as coffee bean pulp, vegetables, distillers' waste and fish offal (fish processing by-products), into sustainable and nutritionally valuable protein and lipids for the production of animal feed, for both livestock and fish feed (Barragan-Fonseca, Dicke, & Loon, 2017; Diener et al., 2011; Newton, Lacy, Kozánek, & Helena, 2015; van Huis, 2013; van Huis et al., 2013; Wang & Shelomi, 2017). BSF adults do not bite or sting, and according to several reports, do not need to feed (Craig Sheppard et al., 1994; Sheppard, Tomberlin, Joyce,

Kiser, & Sumner, 2002; Tomberlin & Sheppard, 2002). The adult fly will survive on the fat stored during the larval stage (Liu et al., 2017; Sprangers et al., 2017). However, it is known that the adult fly can drink nectar from plants in the wild (Bertinetti, Samayoa, & Hwang, 2019; McAlpine et al., 1981). BSF was not described as vector of any diseases (Furman, Young, & Catts, 1959; Wang & Shelomi, 2017). However it was formally considered a medical-veterinary pest due to its potential to produce accidental myiasis or contamination of poultry wastes (Bosch et al., 2019),

The life cycle of *H. illucens* , represented in **Figure 1**, has four phases: egg, larva, pupa, and adult stage. Mating takes place two days after emergence, and the oviposition occurs two days after fertilization. The females lay their eggs in dry cracks and crevices near larval habitat (Salomone et al., 2017). The larvae pass through six instars and its development can last 2-4 weeks, depending on temperature, humidity and food availability (Diener, Zurbrügg, & Tockner, 2009). In the last larval stage, the last larval instar, often called prepupa, uses its mouthparts to pull their body along in search for a safe, dry and suitable site where it will pupate. From the pupa it can take from 10 days to even months to hatch into an adult fly (Surendra et al., 2020) .

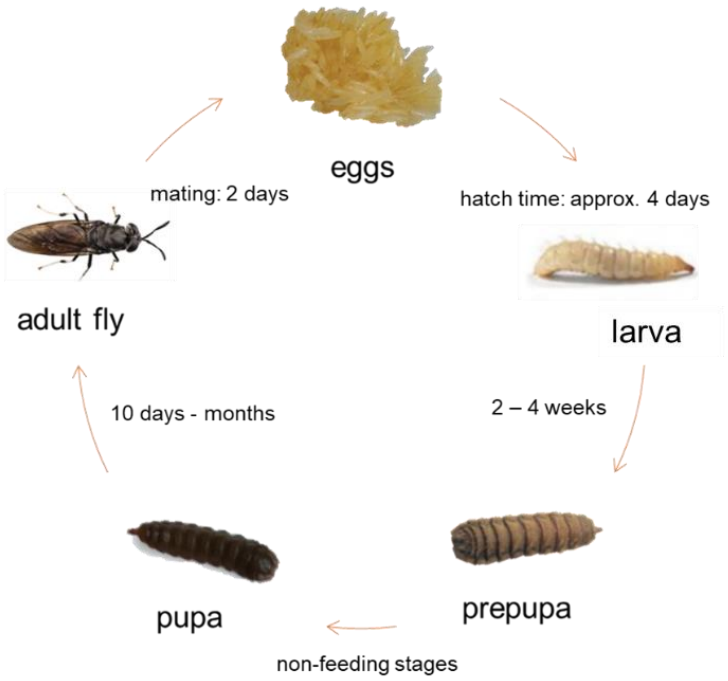

Figure 1. Life cycle of *Hermetia illucens*.

Several studies acknowledge the wide range of current and potential uses of the BSF. Initially, this species interest was triggered due to its value for the forensic sciences. The presence of BSF larvae on human remains allows to estimate a time of colonization and, therefore, is an indicator of the post-mortem interval (Lord, Goff, Adkins, & Haskell, 1994). Another important use of BSF larvae has been its use as a tool to manage waste, mainly of bioorganic waste (Newton et al., 2015; Zheng, Li, Zhang, & Yu, 2012). In the last years, the interest to study the BSF has increased exponentially mainly due to its capacity of convert low-value residual organic streams into high-value protein products that have the potential to be used as feed for farmed animals, for example, poultry and swine (Craig Sheppard et al., 1994), fish (Barroso et al., 2014; Cummins et al., 2017; Magalhães et al., 2017) and pets (reptiles, amphibians, and insects). BSF can also be used for biodiesel production, as new biomass feedstock, increasing the overall biodiesel yield from restaurant wastes (Zheng et al., 2012). Recent studies prove the value of BSF as a new source of antimicrobial peptides (AMPs) (Vogel, Müller, Heckel, Gutzeit, & Vilcinskas, 2018), with the potential to be an alternative to conventional antibiotics or feed preservatives (Buchon, Silverman, & Cherry, 2014). Also, the propensity of the larvae to grow on organic matter makes this insect a source of cellulose-, chitin-, and lignin-degrading enzymes with various potential industrial applications (Müller, Wolf, & Gutzeit, 2017).

Bondari and Sheppard (1981) released a paper based on a 10-week feeding trial, where *H. illucens* larvae grown on chicken manure were fed to channel catfish and tilapia. The authors therefore mentioned that the black soldier fly "may be valuable" feedstuff in commercial fish production. Since this first study, the interest in study the BSF larvae has increased exponentially, and several studies were published in the last years concerning its use as fish meal. In a study by Magalhães et al. (2017), BSF prepupae meal was used as a fish meal replacement in the diets of European seabass (*Dicentrarchus labrax*) at 6,5%, 13%, and 19,5% replacement levels, equivalent to 15%, 30% and 45% of fish meal (FM), respectively. The study concluded that fish promptly accepted the experimental diets and during the trial no mortality occurred. The growth performance, feed intake and feed efficacy were not affected by the diet composition. Other studies were conducted for different fish species and similar

results were obtained: for Atlantic Salmon (*Salmo solar*) (Belghit et al., 2019) and for freshwater fish, the climbing perch (*Anabas testudineus)* (VonAVichith et al., 2019) and zebrafish (*Danio rerio*) (Zarantoniello et al., 2019).

The suitability of BSF as aquafeed ingredient is not limited to a protein source, in fact, it can be expanded with the modulation of its lipid profile through the rearing substrate. For the understanding of the fatty acids profile that is desirable to find in BSF larvae, the next chapter will clarify the nutritional requirements of fish, focusing on fatty acids.

1.4. Nutritional requirements of fish

In aquaculture, the farmed fishes are exposed to increased stress, due to environmental and health conditions. Possible negative impacts on fish well-being, immunity, growth, and performance can lead to economic losses. Besides that, fishes become more susceptible to infectious diseases, and deficient signs can appear (altered behaviour and pathological changes) (Trichet, 2010). For these reasons, it is important to adequate the supplied diet with the nutrient requirements. This understanding can avoid an unbalanced diet, which could induce negative interactions or antagonism between the nutrients, and can trigger toxicity (Oliva-Teles, 2012). Researchers have identified dietary constituents that affect both human and animal immune responses, namely amino acids, fatty acids, minerals, and vitamins.

Even with the differences in nutrient digestion between fish' species, metabolic utilization, and interactions among the nutrients, it is possible to indicate the general nutritional requirements. Nutrients are dietary constituents and can be obtained by digestion and absorbed from the digestive tract, but can also be obtained as products of metabolism of these dietary constituents (Lall & Dumas, 2015). A diet rich in protein is one of the major factors that influences the productivity of farmed fish. Fishes, as monogastric animals, do not have specific proteins requirements, but instead require amino acids. The 10 essential amino acids for fish, also referred to as indispensable, are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine

(Wilson, 2002). These cannot be synthesized by fish and must be obtained through the diet. There is not an absolute value for protein requirements, once it depends on the availability of the source, its amino acid profile, and the dietary energy level (Lall & Dumas, 2015).

1.4.1. *Fatty acids classification and importance*

Dietary lipids are the main source of available energy in fish diets, since carbohydrate utilization is not very efficient. Lipids are the suppliers of essential fatty acids, crucial for normal growth, development, reproduction, health and flesh quality of fish. Lipids also act as carriers of certain non-fat ingredients, among them the lipid-soluble vitamins A, D and K (Watanabe, 1982). Lipids are also sources of structural components of biomembranes, precursors of eicosanoids and hormones, and enzymes co-factors (Higgs & Dong, 2000). The fat content and fatty acids (FA) composition of fish lipids can vary largely, even within species, depending on abiotic and biotic factors: season, type and amount of feed available, water temperature, pH, salinity and reproductive cycle. The lipid content of farmed fish can also be different from wild-caught fish, due to the types of diet of each one.

Lipids are a chemically diverse group of compounds whose defining feature is their water-insolubility, which is the result of its hydrophobic hydrocarbon chain. In contrast, lipids are known to be soluble in non-polar organic solvents such as chloroform, benzene, ethers, and alcohols. They are responsible for several biological functions on living beings. Lipids include triacylglycerols, wax esters, sterols and phospholipids (Turchini, Torstensen, & Ng, 2009). Although biological lipids are not large macromolecular polymers, many are formed by several small constituent molecules chemically linked. Many of these molecular building blocks are similar or homologous in structure. Therefore, lipids can be classified in few major groups: fatty acids, fatty acid derivatives, cholesterol and its derivatives, and lipoproteins.

Dietary lipids contain both saturated and unsaturated FA. They can be found in two types of FA-containing compounds: triacylglycerols (TAGs) and waxes. Triacylglycerols, also called triglycerides or fats, are FA esters of glycerol and are the greatest source of dietary FA, accounting for up to 97%. Fats are constituted by three FA, each one linked to one of the -OH groups of a glycerol molecule (Cox & Nelson, 2000). Fats are the main sources of FA in the diet and many animals store them to be used as long-term energy reserves.

Fatty acids are carboxylic acids, composed by a hydrocarbon chain, which can be 4-36 carbons long, with a carboxyl group terminal (-COOH). The chain can be unbranched (i.e., with no double bonds) – saturated fatty acids [SFA]; can contain pars of carbon linked by one double bond – monounsaturated fatty acids (MUFA) or more double bonds – polyunsaturated fatty acids (PUFA). Fatty acids can also contain three-carbon cyclopropane rings, hydroxyl groups or methyl group branches. Unbranched fatty acids are more abundant and are generally represented by the number of carbon atoms separated by a colon from the number of double bonds (e.g., 20:3, 20 carbons and 3 unsaturations). The exact position of the double bonds can be added in two different ways, either counting from the carboxyl or from the methyl group. Since the nutritional role of PUFAs is more associated to the position of the first double bond near the methyl end (the omega carbon), this nomenclature is the most used. Omega (n- or ω-) indicates the positions of the first double bond when counting from the methyl group. On that account, in omega-3 fatty acids there is an unsaturation located between the 3rd and 4th carbon, and in omega-6 FA, the first unsaturation is between the carbons 6 and 7 (Lee, Lee, Kang, & Park, 2016).

Animals cannot synthesize two important polyunsaturated fatty acids, the precursors of the eicosanoids, represented in **Figure 2.**, and so they must obtain them from their diets. For this reason, these precursors are called essential fatty acids. The two EFA are the 18:2 linoleic acid family, referred to as the n-6 fatty acids series, and the EFA of the 18:3 linolenic acid family, called n-3 fatty acids series.

Overall of the nutritional compounds that make up an organism's body, many are required as a nutritional supplement (Malcicka, Visser, & Ellers, 2018). Like all the vertebrate species, fish have a full dietary requirement for both n-3 and n-6 PUFA, since they cannot synthesize *de novo* polyunsaturated fatty acids (PUFA) of the n-3 and n-6 series. These FA play an important role in cell physiology, signalling and reproduction, besides being vital for body functions, such as the formation and functioning of cell membranes and immune system (Malcicka et al., 2018). The biological active forms of the EFA required in fish diets are the C20 and C22 metabolites of linoleic acid (LA, 18:2 n-6) and α-linolenic acid (ALA, 18:3 n-3): arachidonic acid (ARA, 20:4 n-6), eicosapentaenoic acid (EPA 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3). The biosynthesis of PUFA can be observed in **Figure 2.**

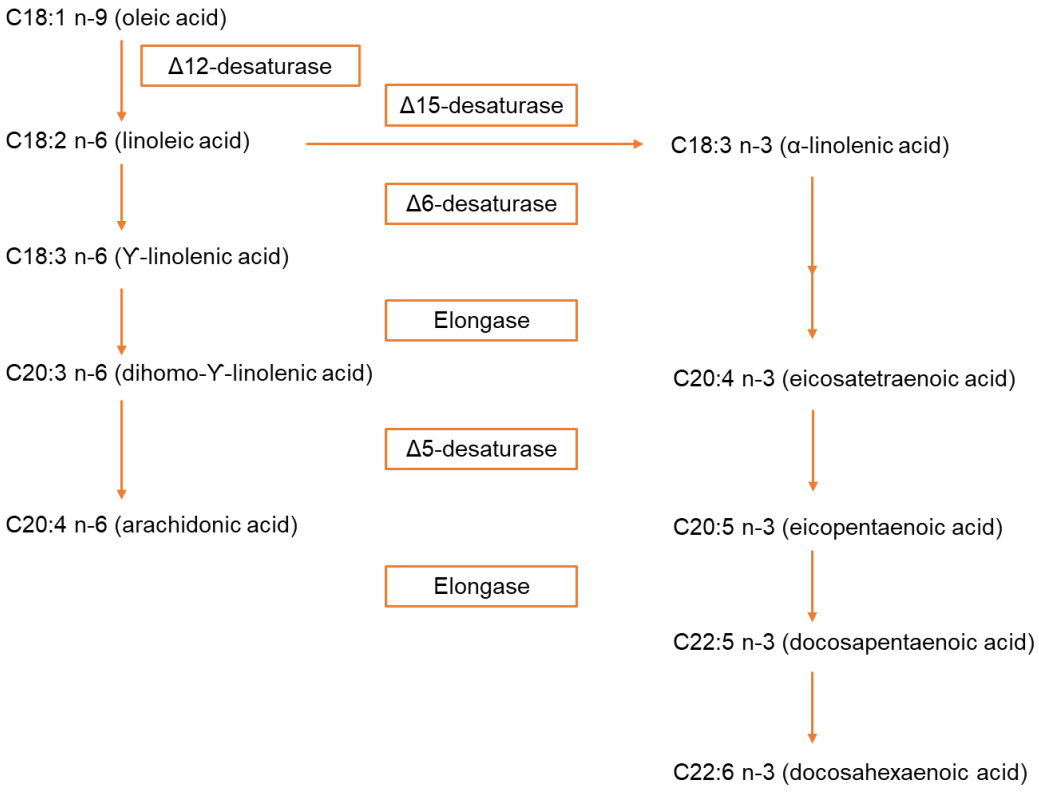

Figure 2. Biosynthesis of fatty acids, adapted from *(P. Calder, 2003)*.

The lipid content depends largely on the diet. Marine fish, especially carnivore ones, have a natural diet rich in n-3 PUFA and it is understandable that long-chain n-3 PUFA occur in higher concentration in marine fish muscle. Because of that, most farmed fish are usually fed with high-lipid diets to maximize the growth

rates. An EFA deficient diet can result in nutritional deficiency signs, for instance, fin rot, myocarditis, reduced growth rate and feed efficiency, shock syndrome, and mortality (Lall & Dumas, 2015; Oliva-Teles, 2012).

Fish oil has been the most important and significant resource for the health promoting marine PUFA in farmed fish feed, being the major source of EPA and DHA. Specific requirements are different in freshwater and marine species. On one side, freshwater fish can convert C18 PUFA to C20 or C22 by a series of chain elongation and desaturation reactions, therefore their requirements are met by linolenic acid (ALA, 18:3 n-3) and linoleic acid (LA, 18:2 n-6). Marine fish, on the other side, are not able to perform such conversion, because of their reduced expression or even lack of delta-15 desaturase enzyme (Gladyshev, Arts, & Sushchik, 2009). Emphasis to the fact that is not only the n-3 PUFA quantity that can vary in fish and fish oils, but also the relative proportions of the individual PUFA (EPA and DHA), and consequently the n-3/n-6 ratio (P. C. Calder & Yaqoob, 2009).

Regarding human consumption, fish and fish oil are the sources for EPA and DHA intake. Both n-3 and n-6 fatty acids are vital for good health and must be obtained from the diet. However, in western society, an unbalanced consumption of n-6 fatty acids, provided by vegetable oils, leads to an atypical higher ratio of n-6/n-3 FA. The n-3 PUFA perform a wide range of critical roles in physiologic functions in the human body, for example, enhancing oxidation and as potent cellular modulators. Therefore, they play an important role in the healthy function of the cardiovascular and nervous system, decreasing the risk of many diseases due to their anti-inflammatory properties (Bentsen, 2017; Ristić & Ristić, 2003; Zárate, el Jaber-Vazdekis, Tejera, Pérez, & Rodríguez, 2017). Excessive amounts of n-6 PUFAs can have deleterious effects such as proinflammatory (Simopoulos & DiNicolantonio, 2016). Therefore, it is recommended for humans to increase the consumption of food rich in n-3 fatty acids.

1.5. Modulation of Black soldier fly nutrient composition

The inclusion of BSF larvae into animal feed is still limited, mainly due to its high lipid content (approximately 40%) and unbalanced fatty acid profile (Hoc et al., 2020). A major problem in the inclusion of BSF in fish feed diets, is the resulting low content of α- linolenic acid (ALA; C18:3), eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) in fish fillet, which could be a problem for both producers and consumers, and nowadays makes the supplementation of insect meal with fish oil mandatory.

The nutrient composition on insects is strongly dependent on their diets and stage of development (Stanley-Samuelson, Jurenka, Cripps, Blomquist, & de Renobales, 1988) and, more specifically, the fatty acid profile of BSF is known to be strongly dependent on the quality and quantity of the diet. By analysing the fatty acid profile in BSF fed with different diets, a high level of saturated FA that were not present in the diets were observed, such as lauric acid, which has antibacterial and antiviral activities (Hoc et al., 2020). Myristic, palmitic and stearic acids were also found in BSF, such as hexadecenoic and octadecenoic unsaturated acids. However, very low concentrations of polyunsaturated as well as branched FA were observed (Oonincx, Broekhoven, Huis, & Loon, 2015). When diets rich in mono- and polyunsaturated FA were fed to BSF larvae, a bioaccumulation of some of those FA was observed, namely oleic acid C18:1, linoleic acid (LA,C18:2) and α-linolenic acid (ALA,C18:3), assumed to be metabolized mainly into lauric acid (Giannetto et al., 2020). According to the EU Regulation of 2017, the rearing substrate of the feeding insects can contain non-animal origin products and some specific products of animal origin (e.g., fishmeal, blood products from non-ruminants, hydrolysed proteins from non-ruminants or from hides and skins of ruminants, egg and milk-based products, rendered fats).

One of the first reports concerning the improvement of BSF fatty acids was published by St.Hilaire et al. (2007). In this study, mixed diets of cow manure and fish offal (homogenized heads, viscera, and some bony structures from rainbow trout) were fed to larvae of BSF and compared with the control diet. Larvae fed fish offal were richer in PUFA, with substantial enrichment (2.5-3.8% of total lipid

content) of n-3 FA (EPA, DHA and ALA). The incorporation of fish meal after only 3h of feeding trial can represent an EPA and DHA enrichment of 2.7% of total FA, according to a study conducted by Barroso et al. (2017), resulting in a desired lower n-3/n-6 ratio. In this study, it was also observed that DHA does not accumulate over time, in contrast to EPA. A more recent study support this find, showing that larval retention values of BSF reared in diets based on organic waste sources (mussels, bread, fish and food waste) go up to 40% of the initial EPA but only 10% of DHA. In this experience, larvae fed on mussels presented the higher amounts of n-3 FA (8% EPA and 5% DHA) (Ewald et al., 2020). As plants are considered the main feed option for BSF production in the actual legislative, plant-based by-products can be explored as a source of desirable PUFA. Oilseed by-products can provide omega-6 and omega-3 rich cakes, which can be incorporated in BSF diets providing an increase of the ALA content which is favourable to rainbow trout needs.

Some other important discoveries have been made in the last years concerning BSF nutritional composition. For instance, a connection between the levels of FA available in the diet and their incorporation on larvae body mass: when higher levels of PUFA were fed to BSF (>10%), the majority of these FA remained in the residues (Ewald et al., 2020). Also, the larval stage appears to affect the FA composition (Liu et al., 2017).

An increase of 70% in the animal production will demand an extra 235% of animal feed needed to sustain the expected growth, and an increasing use of the global arable land, which is not sustainable in the future. Consequently, it is urgent to find new feed sources, even for insect rearing. Resources not currently being used directly as food can potentially be processed to be used as feed ingredients. One option is wild and cultured marine macroalgae, already considered as a feed alternative not only to aquaculture, but as well to ruminant and swine feed industries (Miranda, Lopez-Alonso, & Garcia-Vaquero, 2017).

1.6. Macroalgae as feedstuff

The potential of macroalgae has been acknowledged to fulfil the biggest challenges of the animal feed industry: provide high quality products as a sustainable and environmentally friendly alternative to traditional feed ingredients. However, macroalgae still present some limitations as feed for some animals, mainly due to its high content of water, salt and complex carbohydrates (Makkar et al., 2016). Marine macroalgae harvested from natural resources have been used to produce marine biopolymers, additives for food and pharmacological and medical purposes. More recently, macroalgae are also used for direct consumption by humans, not only in Asian countries but at a world scale.

Marine macroalgae can adapt to extreme marine environmental conditions, such as temperature, salinity, nutrients, radiation, light and oxygen combination, by producing secondary metabolites such as proteins, polysaccharides, lipids, pigments and minerals (Collins, Fitzgerald, Stanton, & Ross, 2016). Macroalgae have a highly variable composition that varies with multiple factors namely the species, date of collection and the environmental conditions.

Protein is the most expensive nutrient in animal feed and new feed formulations have its major challenge on protein substitution. Macroalgae have high protein contents (Miranda et al., 2017), and red macroalgae have the highest protein content, from 35 to 47%, depending on the geographic location and harvesting season. These values are closed to protein-rich foods, such as soybean, cereals, eggs and fish (Garcia-Vaquero & Hayes, 2016), hence, macroalgae can be used as a protein-rich feedstuff for various animals, namely the *H. illucens* larvae, which have a requirement for high protein diets.

1.6.1. *Marine algae as a source of polyunsaturated fatty acids*

Aquatic ecosystems are the main source of n-3 PUFA for most animals. The ability to synthetize both n-6 and n-3 PUFA in fish and humans is suppressed, and direct consume is preferred. For most primary producers in freshwater and

marine habitats, such as macroalgae, this does not happen, and these fatty acids are synthesized. So, in contrast with higher plants mentioned previously, marine macroalgae can be a source of n-3 fatty acids (Pereira, Polo, Rešek, & Engelen, 2012). Like proteins, the lipid composition varies within the species and with the season. The total lipid content in macroalgae is usually small, up to 4.5% dry weight, and their contribution as a food energy source is low, but the proportion of PUFA can be high. The main sources of lipids from marine algae are glycolipids, neutral lipids, and phospholipids. Marine macroalgae PUFA include α-linolenic (18:3 n-3), octadecatetraenoic (18:4 n-3), arachidonic (20:4 n-6) and eicosapentaenoic acids (20:5 n- 3)(Imbs et al., 2012; Khotimchenko, Vaskovsky, & Titlyanova, 2002) .

Marine algae can be introduced in the diet of BSF, as larvae was shown to successfully grown on media containing up to 50% of brown algae *Ascophyllum nodosum* (Liland et al., 2017). Above that incorporation rate, the presence of *A. nodosum* had a deleterious effect, decreasing the growth rate, survival and nutrient utilization by BFS. In the same study, all larvae grown on algae were slightly enriched with EPA (1% of the total FA), which was present in the algae. Increasing the incorporation of *A. nodosum* was noticed to do not affect the FA composition of the larvae. Generally, the use of seaweed-fed BSF larvae does not present a risk of food borne pathogens such as E. coli 0157, *Salmonella* spp., *Listeria* spp. and *Vibrio* spp. (Swinscoe et al., 2018). In the sea, algae can suffer rapid colonization by human pathogens and therefore marine algae utilization may pose a risk if harvested and processed at poor conditions (e.g., high temperature, poor water quality at the harvesting place). In the foreseeable future, it is expected that algae handling should be managed as part of Good Agriculture Practice (GAP) which would help to reduce the probability of food contamination from the start of the production chain, as well as robust Hazard Analysis and Critical Control Point (HACCP) guidelines throughout the whole production chain (Swinscoe, Oliver, Ørnsrud, & Quilliam, 2020). It is projected that these regulatory improvements will raise the acceptability of algae-fed insects for feed.

In this work, the marine macroalgae *Agarophyton vermiculophyllum* was incorporated as feed for BSF larvae. The red algae *A. vermiculophyllum* was originally from Japan and widely distributed in East Asia. Nowadays, it is

recognized as an invasive species (Saunders, 2009), found in North America and along the European coasts, from Sweden to Southern Portugal (Rueness, 2005), as reported in “Decreto-Lei n.º 92/2019 de 10 de julho”. Invasive algae species can have ecologic impacts and real consequences for the local ecosystems, by modifying the habitat for both fishes and invertebrates and by competing with native algae, with strong impacts in the local economies. In Portugal, *A. vermiculophyllum* was often misidentified as *Gracilaria verrucosa* and *G. bursa-pastoris*. In 2008, the taxonomic classification of this algae largely found in Ria de Aveiro was confirmed by Saunders (2009). It is expected that *A. vermiculophyllum*, as like other species from *Gracilaria* genus, presents a high level of EPA and DHA.

1.7. Goals

The sustainability of aquaculture is largely dependent on aquafeed ingredients. The use of insect meal is already implemented worldwide, but the lack of n-3 polyunsaturated fatty acids sources, usually provided by fish meal and fish oil, are an obstacle to achieve a greater performance by non-conventional diets. The nutrient composition of *H. illucens* body mass can be modulated by the rearing substrate and therefore present a possible solution for this problematic.

The specific objective of this work was to investigate the multigenerational effects of the supplementation of the macroalgae *Agarophyton vermiculophyllum* in the diet of the black soldier fly, to explore the long-term effects of a diet rich in polyunsaturated acids on insect growth, reproduction and fatty acid profile.

2. Methods and Materials

2.1. Reagents/chemicals

Dichloromethane (CH_2Cl_2) and methanol (MeOH) were purchased from Fisher Scientific (Leicestershire, UK). Purified water (Synergy®, Millipore Corporation, Billerica, MA, USA) was used whenever necessary.

2.2. *Hermetia illucens* rearing

The larvae of these experiments were obtained from a continuous colony of *H. illucens* established since 2018. Larvae were fed with a control diet consisting of chick feed and tap water (1:1, v/v) and maintained at controlled conditions of temperature 27±3 °C, photoperiod 16:8 (L/D), 50% relative humidity and a constant ventilation flow. This colony is maintained at the rearing facilities in the Center for Extension and Environmental and Marine Research (CEPAM) at ECOMARE – Laboratory for Innovation and Sustainability of Marine Biological resources of University of Aveiro, in the frame of the SUShI project – SUStainable use of Insect protein in aquaculture feed. All the trials were carried at these facilities.

2.3. Macroalgae collection and processing

The algae biomass consisted of 100% *Agarophyton vermiculophyllum* (AV) **(Figure 3)** harvested in July 2020 in Ria de Aveiro. The whole thalli were collected to plastic buckets together with some water during the transport. At the facilities, in the same day, the algae were rinsed in cold freshwater to remove salt, adhering residues and other algae species or animals, and then were stored in small plastic bags at -20°C.

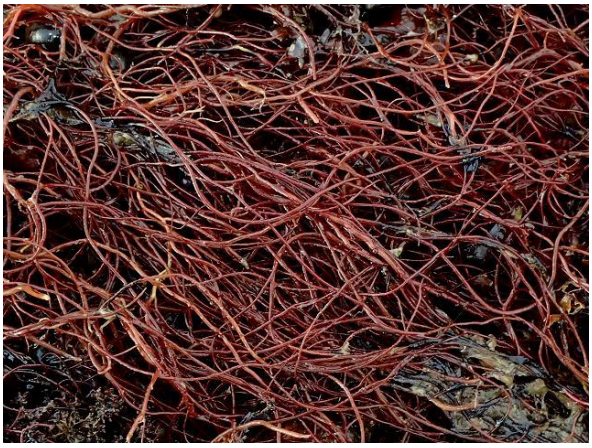

Figure 3. Macroalga *Agarophyton vermiculophyllum.*

2.4. Substrate preparation

The incorporation rates of *A. vermiculophyllum* under test were 0%AV (control diet), 25%AV and 50%AV. To avoid larvae from escape from the containers, or any potential oviposition by other flies, mosquito mesh was clamped on top of the container with a rubber band. The rearing substrate was elaborated right before being provided to the larvae. The algae were picked from the -20°C freezer and grounded to small particles using a knife. With the help of a paper towel, the excess of water was soak up. The respective proportion of algae and dry chicken feed were mixed, and ~63% of moisture was achieved by adding tap water.

New freshly made medium was weighted and added to the plastic box initially and every other day, at a feeding rate of 33 mg/larva/day, chosen after a preliminary test.

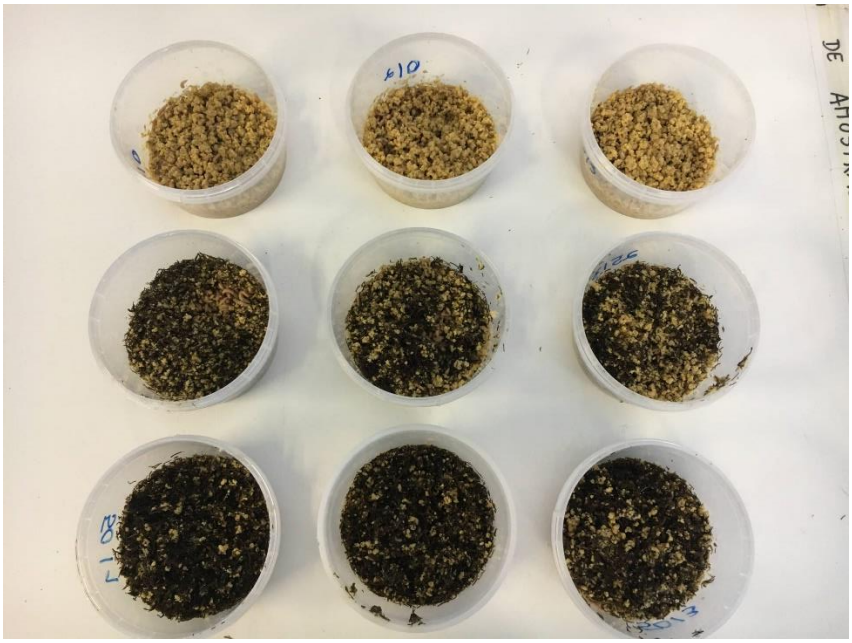

Figure 4. Preparation of the diets. From the top to the bottom: control diet (0%AV), 25% and 50 % inclusion rate of *A. vermiculophyllum*.

2.5. Experimental design

The study consisted of three types of diet, each one with a different inclusion rate of macroalgae *A. vermiculophyllum*, fed through four generations of black soldier fly (G0, G1, G2, G3).

A suitable medium for egg deposition (stacks of wooden rips with small gap in between), a source of water, and a source of food, were placed in each cage to stimulate the oviposition. Eggs with less than 24 hours were collected from the rearing colony and left on a container with the standard diet consisting of chicken feed and tap water (1:1, v/v). After 3 days the eggs hatched, and all the larvae were left to grow for 7 days in the control diet fed *ad-libitum*.

On the 7th day, the larvae were collected, cleaned up with a paper towel, and counted into groups of 1000 larvae. For each type of diet, three replicates were under study **(Figure 4)**. In each container, a group of 1000 larvae were placed together with the respective experimental diet.

Feeding was carried out until more or less 50% of the larvae have reach the non-feeding (prepupal) instar, distinguished by its dark brown colour – equivalent to 10 days. By that time, the larvae and prepupae were manually collected from the residues and counted separately. The residues were also weighted. The remaining larvae were placed in new containers and more food was provided until all reached the prepupal stage. With the majority of the BSF larva achieving the prepupal and pupal stage, the replicates were grouped by treatment (type of diet) and placed in a cage for the pupae emergence.

2.5.1. *Transgenerational experiment*

To observe the transgenerational effects of *A. vermiculophyllum* supplementation, in the end of the larval stage, the following precautions were taken, as represented in **Figure 5**.

The G0 pupae were separated in containers placed in different cages for each experimental diet under study, and the pupae were left to emerge. The adult flies

were placed in cages according to the experimental diet under study. The conditions in the cages to stimulate the oviposition were the same as described before, and the eggs were removed every two days until a quantity of 24-hour eggs was considered adequate to start the next generation of the experiment. The eggs were placed separately on different containers with the standard diet and the larvae was maintained there for 7 days, after what they were removed to containers with the tested diet treatments in which G0 completed their larval stages. This procedure was repeated for 4 generations.

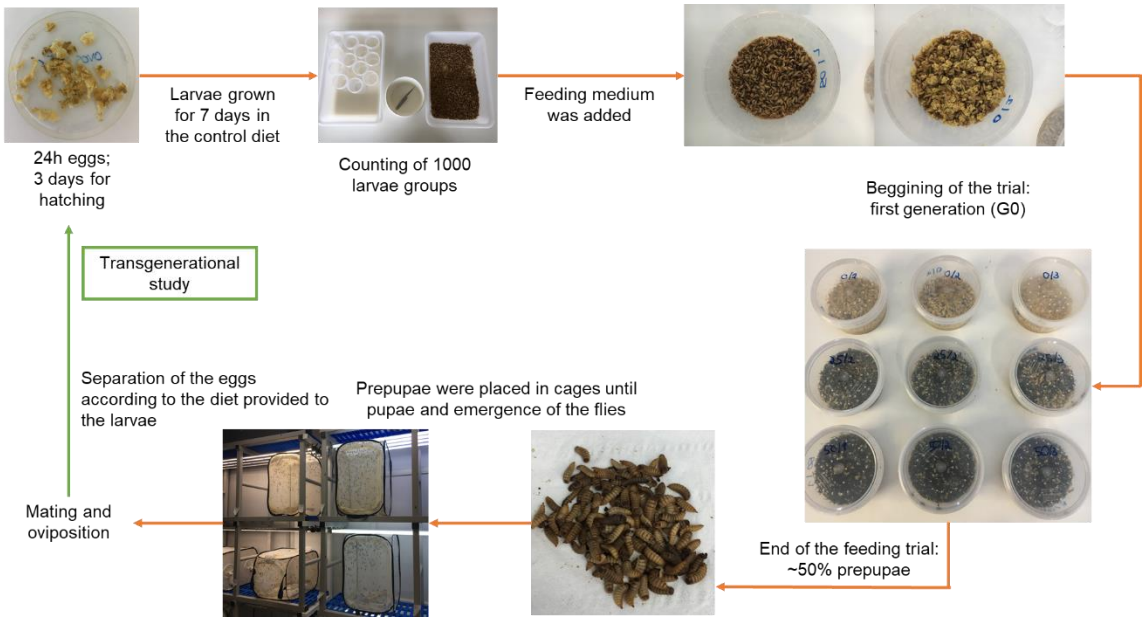

Figure 5. Scheme of the experimental design.

2.6. Larval growth

The basic parameter used to evaluate the performance of the larvae throughout the experiment was the body weight, obtained by weighting 50 larvae from each replicate.

The weights of the diet provided, larvae and residue allow the estimation of how efficiently the larvae converted the experimental diets into body mass (larval biomass) or how efficiently they reduced it. The residue (R) weight, constituted by diet, exuviae and excreta (Bosch et al., 2019), was obtained by the end of each generation when larvae were harvested from it.

The value of the body weight (BW) gained was used to calculate the Growth Rate (GR). The substrate reduction (SR) estimates the efficiency of the BSF to consume organic matter content in the fed substrate. The efficiency of the feed conversion into body mass was evaluated by the Feed Conversion Ratio (FCR) and the Bioconversion Ratio (BCR).

$$\%\ survival = \frac{Larvae_{in}}{Larvae_{out}} \times 100 \quad (1)$$

$$Growth\ rate\ (GR) = \frac{|final\ BW - initial\ BW|\ (g)}{trial\ duration\ (d)} \quad (2)$$

$$Substrate\ reduction\ (SR) = \frac{|total\ feed\ added - residue\ feed|}{total\ feed\ added} \times 100 \quad (3)$$

$$Bioconversion\ ratio\ (BCR) = \frac{final\ BW - initial\ BW}{Diet\ provided} \times 100 \quad (4)$$

$$Feed\ conversion\ rate\ (FDR) = \frac{total\ feed\ added}{|final\ BW - initial\ BW|} \times 100 \quad (5)$$

2.7. Fatty acid analysis

2.7.1. *Lipid extraction*

Total lipid extraction based on Bligh & Dyer method (Bligh & Dyer, 1959).

Total lipid content was determined using representative samples of approximately 10 mg of BSF larvae powder, dry weight (DW). Grinded larvae were mixed with 1mL of Mili Q-water. Then, the solution of 1:2 (v/v) CH_2CL_2: MeOH (3.75 mL) was added, and the mixture was homogenized for 2 min. The glass tubes were placed on ice for 30 min and vortexed a few times for 30 s during this period. After this procedure 1.25 mL of CH_2CL_2 were added, vortexed for about 1 min after which 1.25 mL of Mili Q-water were added and vortexed for another minute. To separate organic and aqueous phases, samples were centrifuged (Selecta JP Mixtasel, Abrera, Barcelona, Spain) for 5 min at 2000 rpm and the organic phase was collected into another glass tube. This process was repeated a second time to extract any residual crude lipid in the glass tube by adding 1.88 mL CH_2Cl_2, vortexed this for 1 min, and centrifuged for 5 min at 2000 rpm, after which the organic phase was recovered. Lipid extracts were collected in the same tube and were dried under a nitrogen stream, resuspended in 0.400 mL of CH_2Cl_2, vortexed, and transferred to a glass vial, previously weighted. This

step was repeated twice to ensure the maximal transfer of the total lipid extract to the vial. The lipid extract was dried in the vials under a nitrogen stream and the extracted content was estimated by gravimetry. The vials were stored at -20 ˚C before gas chromatography-mass spectrometry (GC–MS) analysis.

2.7.2. *Analysis of Fatty Acid Methyl Esters*

Fatty acid methyl esters (FAMEs) were prepared from total lipid extracts using a methanolic solution of potassium hydroxide (2.0 M) according to the methodology previously described by Aued-Pimentel et al. (2004). Briefly, 2.0 µL of a solution of hexane containing the FAMEs and 1.175 µg mL^{-1} of methyl nonadecanoate (Sigma, St. Louis, MO, USA) as internal standard was analysed by GC–MS. GC–MS data were acquired using an Agilent Technologies 8860 GC System (USA) equipped with a DB–FFAP column with the following specifications: 30 m long, 0.32 mm internal diameter, and 0.25 µm film thickness (123-3232, J&W Scientific, Folsom, CA, USA). The oven temperature was programmed as follows: (1) the initial temperature was set up to 58 ºC for 2 min; (2) a linear increase to 160 ºC at 25 ºC min^{-1}; (3) a linear increase at 2 ºC min^{-1} to 210 ºC; and (4) a linear increase at 20 ºC min^{-1} to 225 ºC followed by 20 min at this temperature. Helium was used as carrier gas at a flow rate of 1.4 ml min^{-1}. A total of five replicates were run. The identification of each FA was performed by mass spectrum comparison with those available in Wiley 275 library. Quantitative analysis of fatty acids was achieved from calibration curves of each methyl ester of fatty acids from a FAME mixture (Supelco 37 Component FAME Mix, CRM47885, Sigma Aldrich, St. Louis, MO, USA), analysed by GC-MS under the same conditions of extracts and results expressed as mg kg^{-1} of dry biomass. The relative amounts of FAs were calculated by the ratio of the amount of each FAME and the sum of all identified FAMEs, results were expressed as means (%).

2.8. Statistical analysis

Beforehand, a normality test (Shapiro-Wilk test) was performed to evaluate if the dependent variables were normally distributed for each combination of group factors. The recorded and calculated parameters were subjected to a two-way ANOVA and significant differences were evaluated using post-hoc Tukey's multiple comparison test, at 5% probability level. The analysis was performed using Graphpad Prism v8.

A chemometric statistical method (MetaboAnalyst (v4.0)) was used to find potential patterns in lipid molecular species within groups of substrates and relative abundances of different fatty acids in *H. illucens* larvae tested for different diets and throughout the four generations. Data were log-transformed followed by auto-scaling to decrease the influence of more and less abundant molecular species. Variance in fatty acids' profile was assessed using a Partial Least Squares – Discriminant Analysis (PLS-DA).

2.8.1. *Partial Least Squares – Discriminant Analysis (PLS-DA)*

PLS-DA is a supervised classification technique that is often used for high-dimensional data and, in this work, it was used to visually understand the relationships between the inclusion of *A. vermiculophyllum* in the diet and the fatty acid profile on BSF prepupae. It performs a variable reduction on the data set by calculating new variables (called factors), combining the variables in the data set, to find the maximum correlation with the class variable and, thus, the maximum separation among two classes. This method uses multivariate regression techniques to extract from the original variables the information that can predict the class membership (type of diet). This statistical method, therefore, finds a linear regression model by projecting these variables to a new space.
This analysis reduces the dimensionality of the data set to new ones, the principal components (PC_s), as a way to summarize the information. In the current work,

PLS-DA was used to generate models for distinguishing the three incorporation rates of algae in the diet.

2.8.2. *Clustering heatmap*

A hierarchical cluster analysis was carried out using the Euclidean distance similarity measure to assemble a heatmap and Ward's linkage was used to construct the dendrogram for group averages. Significant differences were assumed at a critical p-value <0.05.

3. Results

3.1. Feeding trials – larval development and substrate bioconversion

In **Table 1** are reported the values of the crude protein and crude lipid for the substrate and *H. illucens* larvae over four generations. Transgenerational significant differences can be observed in the lipid content. Statistical analysis performed for this data are presented in **Table 6 to 8** (appendix).

Table 1. Crude protein and crude lipid content of the feeding diet and of BSF larvae (g/100 g total of dry weight) (mean ± SD; n = 3).

		Protein	Lipid
Diet	**0%**	18.65±2.40^{a}	2.16±0.32^{a}
	25%	22.22±6.03^{ab}	1.69±0.36^{a}
	50%	15.23±1.80^{ac}	1.65±0.63^{a}
G0	**0%**	41.23±0.36^{a}	38.19±0.38^{a1}
	25%	41.74±1.14^{a}	25.37±3.11^{b2}
	50%	43.27±0.18^{a}	24.57±1.82^{b3}
G1	**0%**	38.51±1.54^{a}	31.30±1.74^{ab4}
	25%	42.99±0.38^{a}	38.95±7.63^{a235}
	50%	43.30±0.08^{a}	26.13±6.37^{b156}
G2	**0%**	44.21±0.11^{a}	21.64±1.09^{ab145}
	25%	44.00±0.15^{a}	26.01±5.90^{a157}
	50%	46.03±4.74^{a}	18.61±2.28^{b1458}
G3	**0%**	41.59±0.45^{a}	16.50±2.11^{a14567}
	25%	41.89±0.55^{a}	29.24±1.59^{b158}
	50%	44.33±0.82^{a}	28.22±6.82^{b158}

[1] Within a generation, mean values with different letters in the same column are significantly different ($p<0.05$).
[2]Between generations, statistically different values are identified by the same number.

Individual prepupal weight, survival rate and prepupae rate per treatment are presented in **Table 2.** A two-way ANOVA was performed followed by post-choc Tukey's test ($p<0.05$). Significant differences provided by the diet modulation were evaluated for every parameter, within **(Table 9 to 16, appendix)** and between every generation **(Table 17, appendix)**.

Table 2. Individual prepupal weight (mg), survival rate (%), and prepupae ratio (%) of 4 generations of BSF fed on diets with different inclusion rates of *A. vermiculophyllum* (AV)[1,2]. Values are presented on a wet weight basis (mean ± SD; n=3).

Generation (Duration of the trial)	Inclusion rate	Individual prepupal weight (mg)	Survival rate (%)	Prepupae (%)
G0 (10 days)	**0%**	152.0±12.4^{a1}	99.0±4.2^{a}	72±1.2^{a1}
	25%	112.6±15.1^{b}	100.3±0.8^{a}	68±2.1^{a2}
	50%	87.4±7.9^{c3}	99.7±0.2^{a}	62±0.5^{a3}
G1 (9 days)	**0%**	105.6±22.0^{a124}	99.4±0.5^{a}	54±1.2^{a4}
	25%	114.7±25.5^{a13}	98.2±4.0^{a}	42±5.9^{a123}
	50%	106.6±24.7^{a135}	99.3±0.5^{a}	19±5.2^{b1236}
G2 (7 days)	**0%**	109.3±12.0^{a136}	102.6±4.1^{a}	38±10.6^{a1236}
	25%	119.6±4.3^{a13}	94.7±11.4^{a}	79±5.1^{b4568}
	50%	121.8±1.7^{a13}	91.2±11.1^{a}	46±10.1^{a1269}
G3 (8 days)	**0%**	130.4±2.5^{a13456}	99.9±6.5^{a}	2±0.6^{a12345789}
	25%	127.6±3.0^{a1345}	99.4±0.4^{a}	60±6.7^{b678}
	50%	114.4±0.3^{b134}	100.8±11.1^{a}	78±1.4^{c45679}

[1] Within a generation, mean values with different letters within a column are significantly different ($p<0.05$).
[2] Between generations, statistically different values are identified by the same number.

The inclusion of macroalgae in the feeding medium influenced the texture of the food and remain residues harder. The larvae fed on macroalgae-containing diets were darker in colour and more difficult to separate from the residues. In G0, the individual prepupal weight was significantly different in every diet under study. **Table 2** shows that in G1, the larvae had a more extended larval stage on 50%AV (*Agarophyton vermiculophyllum*) (19% prepupae), when compared with 0% and 25%AV (54% and 42% prepupae, correspondently). In G2, larvae fed 25% AV presented a statistically lower percentile of prepupae by the end of the experiment (79%), when compared with the 0%AV diet also in G2, reporting 38% of prepupae.

Survival rates above 100% can be justified by the manual picking of the 7-days-old larvae, which could have had younger or smaller larvae attached and imperceptible to the eye, that later grown and were recorded. There were not observed significant differences in the survival rate within the same generation and neither in the multigenerational comparison.

The prepupae on 0%AV in G3 was the lowest value recorded (2%) and more significantly different when compared to the others generational trials, which indicates that in G3, larvae fed with experimental diets turned into prepupae earlier that those fed with the control diet. In G2 there was a decrease in the oviposition of the flies fed with AV diet, which led to a deficit in egg production for the start of G3. For this reason, instead of 1000 7-day-old larvae, only 350 larvae were used to carry out this last experimental trial.

To evaluate the acceptability of the algae incorporation and capability of BSF larvae to convert the diets under study into body mass, it was calculated the growth rate, bioconversion rate, feed conversion rate and substrate reduction, reported in **Table 3.** Tukey's post-hoc test ($p<0.05$) detected significant differences substrate-dependent, within generations (**Tables 9 to 16, appendix)** and between **(Table 17, appendix)**.

Table 3. Growth weight (GR), bioconversion rate (BCR) and feed conversion rate (FCR) of BSF larvae fed on diets with different inclusion rates of *A. vermiculophyllum* [1,2]. Values are presented on a wet weight basis (mean ± SD; n=3).

Generation	Inclusion rate	GR (mg/larva/day)	BCR (%)	FCR (%)	Substrate reduction (%)
G0	0%	11.6±1.3^{a}	35.2±3.9^{a1}	2.87±0.00^{a1}	76.44±2.13^{a1}
	25%	10.5±1.6 ^{a}	23.9±4.7^{b2}	4.37±0.01^{a2}	66.31±2.84^{b2}
	50%	10.7±0.7 ^{a}	16.3±2.1^{b}	6.26±0.01^{a3}	59.96±2.51^{b3}
G1	0%	8.3±2.3^{a}	22.6±6.3^{a3}	4.72±0.01^{a14}	64.31±1.23^{a4}
	25%	10.5±2.4^{a}	28.6±6.5^{a4}	3.72±0.01^{a5}	58.37±1.93^{a15}
	50%	10.7±3.0^{a}	29.1±8.2^{a5}	3.75±0.01^{a6}	56.77±3.15^{a16}
G2	0%	14.8±2.4^{a}	31.4±5.2^{a6}	3.28±0.01^{a7}	87.18±1.352^{a34567}
	25%	1.9±0.5^{b}	4.0±1.1^{b1457}	27.45±0.10^{b123456}	74.75±0.50^{b6}
	50%	10.1±0.9^{ab}	21.4±1.9^{a}	4.7±0.00^{a8}	72.74±2.00^{b}
G3	0%	12.3±0.2^{a}	29.8±0.4^{a7}	3.36±0.00^{a7}	73.35±0.63^{a}
	25%	1.3±0.4^{b}	3.1±1.0^{b123456}	35.33±0.10^{b12345678}	69.92±0.77^{a}
	50%	3.3±0.2^{b}	7.1±0.3^{b1456}	14.11±0.01^{c7}	57.32±3.24^{b17}

[1] Within a generation, mean values with different letters within a column are significantly different ($p<0.05$).
[2] Between generations, statistically different values are identified by the same number.

The GR of the larvae was strongly affected by AV incorporation, when compared with the control diet, mainly on G2 (25%: 1,9 mg/day; 50%: 10 mg/day) and G3 (25%: 1,3 mg/day; 50%: 3,3 mg/day). Statistically different values were obtained for the BCR for 0%AV on G0 (35,2 mg/day) in comparison with G2 (25%: 4,0 mg/day) and G3 (25%: 3,1 mg/day; 50%: 7,1 mg/day). In fact, the inclusion of

25%AV in G2 and G3, and the inclusion rate of 50% presented the most significant differences when compared with every other multigenerational BCR values.

The growth curves of the four generations of BSF larvae are shown in **Figure 6.**

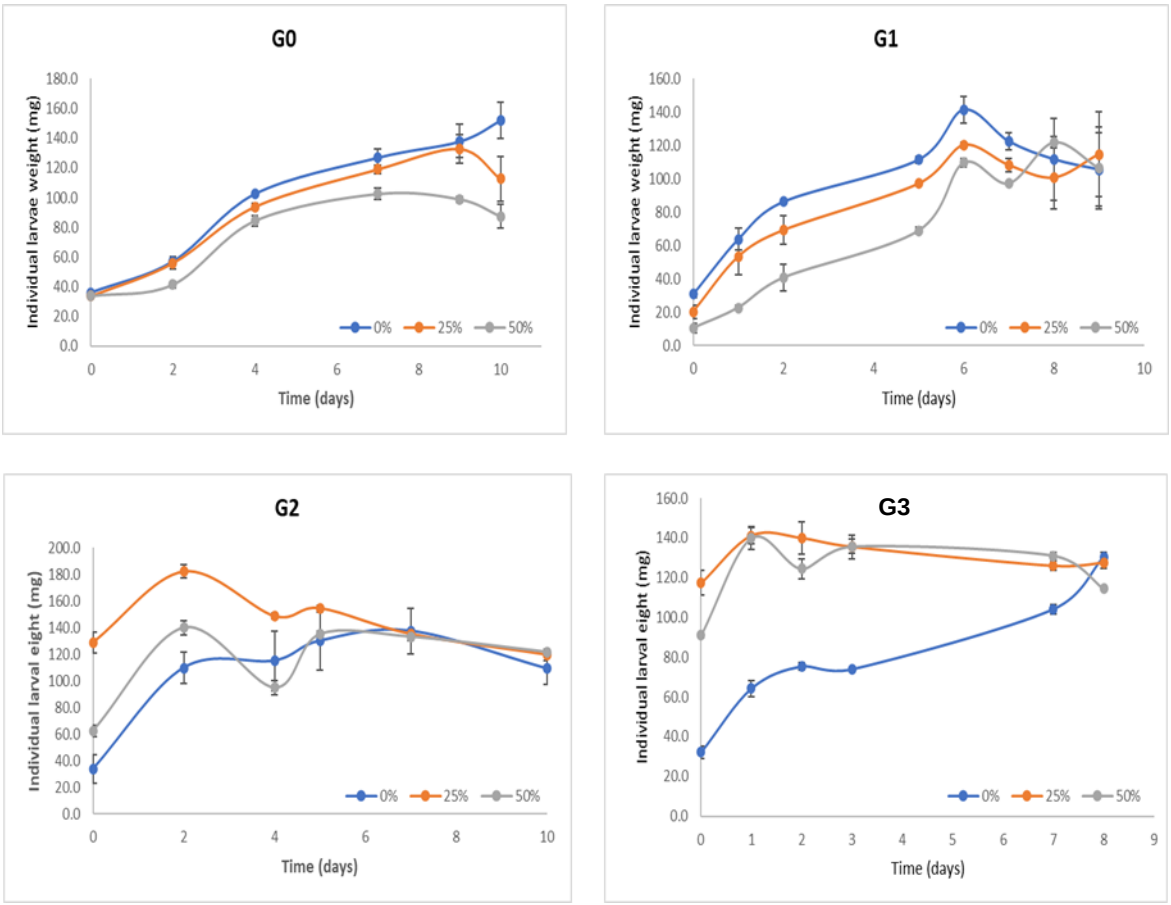

Figure 6. Growth curves of the four generations of BSF. Values at the graphics are presented as mean of individual larval weight ± standard deviation.

In **Figure 6** it can be observed in the growth curve for G0, that the larvae in the control diet presented a crescent curve until the 10th day. On the other hand, larvae under experimental diets shown a decrease on the fresh weight in the 10th day, indicating that these larvae were entering the prepupae stage by this time.

BSF larvae fed with the control diet (0%AV) kept a similar initial larval weight throughout the multigenerational experiment. In G0, larvae fed algae-containing diets stopped gaining body mass at day 9, but not in 0%AV diet. On the other

side, in G1, larvae fed with 25% and 50%AV diets had a lower initial weight which increased only until day 6. On G2 and G3 a big difference could be observed between larvae fed with control diet and those fed with diets with algae incorporation. However, the final body mass was more or less in the same range (100-130 mg). Some fluctuations in the larval weights are shown in the growth curves. These can may be related with oscillations due to biased selection of the larvae from the substrate (e.g. selection of bigger larvae or larvae on the upper part of the substrate).

3.2. Fatty acid profile

The results obtained from the GC-MS regarding the diet composition are reported in **Table 4.** A statistically different value was found in C18:2 abundance for 50%AV diet. The test for normal distribution of the data is reported in **Table 18 (appendix)** and p values (95% confidence interval) after Tukey's multiple comparison test in **Table 19 (appendix)**.

Table 4. Fatty acid composition of the diet fed to BFS larvae, in relative abundances (%)(mean ± SD; n = 3). Significant differences are represented by different letters ($p<0.05$).

Fatty acids	Long name	Diet		
		0%	**25%**	**50%**
C12:0	Lauric	–	–	–
C14:0	Myristic	0.53±0.01	–	–
C16:0	Palmitic	14.60±0.50^{a}	14.30±1.07^{a}	14.00±4.96^{a}
C18:0	Stearic	4.39±0.38^{a}	3.85±0.15^{a}	6.30±4.45^{a}
Σ SFA	**Saturated**	**19.52±5.94**	**18.16±5.23**	**20.30±3.85**
C16:1	–	–	–	–
C18:1 n-9	Oleic	27.89±0.49^{a}	28.59±0.37^{a}	19.83±7.04^{a}
Σ MUFA	**Monounsaturated**	**27.89±0.00**	**28.59±0.00**	**19.83±0.00**
C18:2 n-6	Linoleic (LA)	49.83±0.03^{a}	50.34±0.53^{a}	30.85±15.82^{b}
C18:3 n-3	α-linolenic	2.76±0.31^{a}	2.92±0.02^{a}	6.20±6.64^{a}
C20:4 n-6	Arachidonic	–	–	14.45±3.14^{a}
C22:6 n-3	Docosahexaenoic	–	–	8.38±13.61^{a}
Σ PUFA	**Polyunsaturated**	**52.59±23.54**	**53.26±1.29**	**59.87±4.87**

The diets provided to the larvae had a different profile, and the relative abundance of SFA was approximately 20% in the control diet, 18% and 20% when AV was incorporated by 25% and 50%, respectively. Saturated fatty acids were lauric

(C12:0), myristic (C14:0), palmitic (C16:0) and stearic (C18:0) acids. The monounsaturated acids found were palmitoleic 16:1 and 18:1 n-9 (10-nonadecenoic). The diet itself have the lowest MUFA abundance when 50%AV was used in the formulation. The polyunsaturated acids identified in all the experiments, including the diet, were linoleic (LA: 18:2 n-6), and α-linolenic acid (ALA: 18:3 n-3). Arachidonic acid (ARA: 20:4 n-6) was present in the diet containing 50%AV (14.45% of total fatty acids) and was found with an abundance of 6.58% in the larvae of G2 fed with that same diet. Finally, the other PUFA identified was docosahexaenoic acid (DHA: 22:6 n-3), which was only present in a very small amount in the diet substrate containing 50% of the algae.

In **Table 5** are reported the relative mean aliphatic FAME relative abundances (n=3) obtained from the GC-MS for the different groups of diets within separate generations. Ten fatty acids were identified: 4 SFA, 2 MUFA and 4 PUFA. BSF larvae fatty acid profile was mostly composed by saturated fatty acids, regardless of the diet under study, accounting for 41-57% of total fatty acid.

A two-way ANOVA followed by Tukey's post-hoc test was performed to identified how the diet composition and transgenerational effects impacted the results obtained. Significant differences ($p<0.05$) were found in the C12:0 abundance within G0 **(Table 21, appendix)**. A significant decrease of this FA is also observed between G2 25% and G3 50%. In G3, C18:1 content was statistically different in 50% incorporation rate, when compared with 0% and 25% **(Table 25, appendix)**. Still, related to this fatty acid, G3 25% presented a statistically lower value when compared with 50% G0.

Table 5. Fatty acid profile of BSF prepupae, in relative abundances (%) (mean ± SD; n = 3)[1,2].

	G0			G1			G2			G3		
	0%	**25%**	**50%**	**0%**	**25%**	**50%**	**0%**	**25%**	**50%**	**0%**	**25%**	**50%**
C12:0	19.63±13.06[a]*	7.60±5.65[ab]	5.04±2.94[b]	11.61±11.38	7.19±5.66	5.83±4.53	13.74±6.52	4.48±1.89*	5.83±0.96	9±2.81	7.73±6.58	2.7±3.38*
C14:0	12.85±4.06	11.69±3.08	9.05±1.65	12.59±4.22	9.70±1.96	7.85±1.39	10.65±2.35	10.94±4.36	8.69±1.80	12.11±3.47	10.29±2.79	4.86±2.86
C16:0	16.54±5.34	19.57±7.87	19.44±7.56	19.60±8.55	21.44±9.48	19.73±7.9	17.19±5.61	19.82±8.41	19.63±9.48	19.83±7.97	18.81±7.08	19.09±7.25
C18:0	8.00±5.75	8.68±5.14	9.94±4.03	9.36±4.92	11.10±3.80	10.05±4.04	9.08±4.79	8.72±5.09	9.82±1.81	9.92±4.06	9.27±4.78	14.41±0.60
Σ SFA	**57.02±4.34**	**47.53±4.68**	**43.47±5.28**	**53.16±3.83**	**49.43±5.43**	**43.47±5.33**	**50.66±3.10**	**43.95±5.60**	**43.97±5.19**	**50.85±4.26**	**46.1±4.30**	**41.06±6.74**
C16:1	6.07±4.41	6.42±4.09	6.00±4.46	5.72±4.69	5.75±4.68	5.97±4.50	6.00±4.45	6.26±4.24	6.48±2.25	5.89±4.55	6.62±3.91	6.42±4.10
C18:1 n-9	17.52±3.08	21.96±6.30	24.40±8.40*	19.21±5.69	21.60±5.93	24.02±8.57	21.08±5.39	23.78±8.50	23.33±9.63	21.98±6.07[a]	22.6±7.49[a]*	26.39±9.97[b]
Σ MUFA	**23.6±5.73**	**28.38±7.77**	**30.41±9.20**	**24.93±6.75**	**27.35±7.93**	**30.00±9.03**	**27.07±7.54**	**30.04±8.79**	**29.8±8.43**	**27.87±8.05**	**29.22±7.99**	**32.81±9.99**
C18:2 n-6	13.08±3.13	17.53±2.02	19.56±3.26	15.57±2.16	16.53±1.62	19.93±5.06	15.99±0.84	19.57±4.04	14.49±1.21	14.9±1.10	18.29±3.36	19.6±3.08
C18:3 n-3	6.31±9.86	6.55±9.65	6.56±9.65	6.34±9.83	6.69±9.53	6.60±9.61	6.27±9.89	6.44±9.75	5.17±8.02	6.38±9.80	6.39±9.8	6.53±9.68
C20:4 n-6	–	–	–	–	–	–	–	–	6.58±10.34	–	–	–
C22:6 n-3	–	–	–	–	–	–	–	–	–	–	–	–
Σ PUFA	**19.39±3.39**	**24.08±5.49**	**26.12±6.50**	**21.92±4.62**	**23.22±4.92**	**26.53±.67**	**22.26±4.86**	**26.01±6.57**	**26.23±4.10**	**21.28±4.26**	**24.69±5.95**	**26.13±6.54**

[1] In the same generation, mean values with different letters within a row are significantly different ($p<0.05$).
[2] Between generations, statistically different values are identified by one asterisk (*).

As it can be observed in **Table 5**, lauric acid C12:0 was found in higher abundance in the trials, when compared to the diet. Also, regarding G0, its value was significantly higher when the control diet was fed to the larvae, and lower abundances were found on G0 50%AV (5.04% of total fatty acids) and G2 25%AV (4.48% of total FA) and G3 50% (2.7% of total FA). The other SFA had a constant distribution throughout the experiment. The total content of SFA was higher for the control diet in every generation (G0: 57.02%, G1: 53.16%, G2: 50.66%, G3: 50.85%) and the lowest SFA abundance was found on G3 for larvae fed with 50%AV (41.60%). The C18:1 content increased with the incorporation of algae in the diet and, consequently, the MUFA content was also higher in these cases. A statistically significant increase of C18:1 abundance by 20% is reported in G3, when 50% of macroalgae was incorporated in the diet, comparatively to 0%. For PUFA, an increase of macroalgae incorporation in the diet of BSF larvae seem to have a positive impact on C18:2 content, although significant differences were not reported.

3.2.1. *Partial Least Squares – Discriminant Analysis (PLS-DA)*

In **Figure 7** is represented the 2-D scores plot between the selected principal components (PCS), after the Partial Least Squares - Discriminant Analysis (PLS-DA). A great separation between the group of larvae fed with the diet containing 50% of algae and of 0% and 25% can be observed.

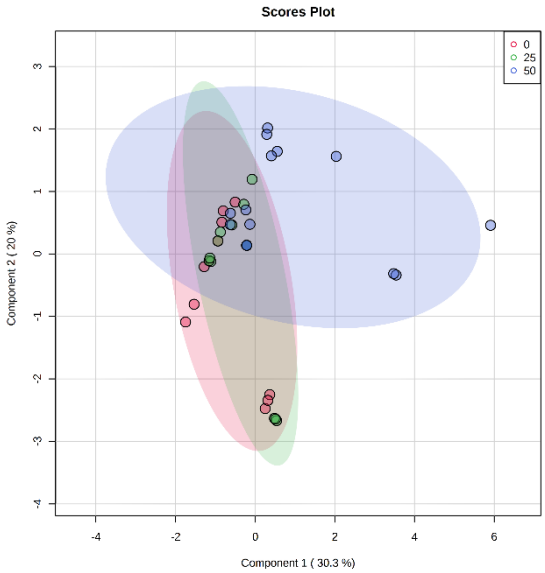

Figure 7. 2-D scores plot between the selected principal components (PCS), after the Partial Least Squares - Discriminant Analysis (PLS-DA).

3.2.2. *Clustering heatmap*

A heatmap was used to visualize the overall correlations between the fatty acids in **Figure 8.**

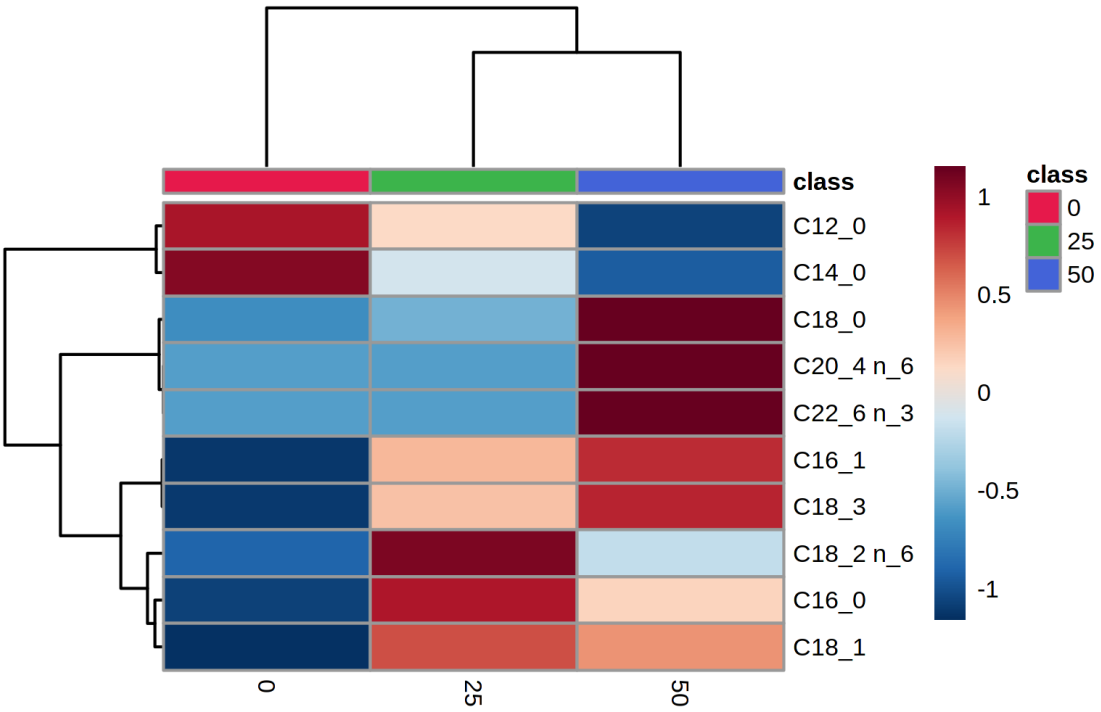

Figure 8. Correlation analysis of fatty acids – heatmap.

The analysis of **Figure 8** also allows to identify how the program has statistically grouped the different types of diet. Diets containing 25% and 50% of macroalgae had a higher correlation with MUFA and PUFA, and lower with SFA. On the other hand, the figure shows a negative correlation between the control diet and every fatty acid, with exception for C12:0 and C14:0. Additionally, a hierarchical separation between those diets containing macroalgae and the control diet is represented.

4. Discussion

The results from this study show that BSF larvae can efficiently convert diets enriched with *A. vermiculophyllum* into body mass, and this efficiently can increase along several generations.

Further, the use of the invasive algae *A. vermiculophyllum* in the diet formulations can be of great interest not only to reduce its overabundance on Ria de Aveiro and its impacts on the ecosystem but also it can provide an increase of MUFA and PUFA content to BSF diet. Marine algae are a source of bioactive compounds, e.g, antimicrobial, antitumoral and antioxidant (Jimenez-Lopez et al., 2021) and have already been studied for its incorporation in aquafeed, for example as feed for molluscs (Robertson-Andersson et al., 2008).

In this study, *A. vermiculophyllum* was collected at the beginning of the summer, in June. According to Afonso et al. (2021), summer period is when this species presents the lowest protein content and highest lipid content.
Despite initially larvae were more drawn to feed on the chicken feed parcel of the diet, and the consumption of the algae was not their first choice, a change in the colour of the larvae and of the residues was observed, indicating that the alga was being consumed. The humidity of the medium containing the macroalgae was adjusted more frequently that in the control diet, which helped the larvae to move and mix the medium, increasing their exposure to uneaten algae that was on the surface. Albeit this also had a negative downside, as larvae were stimulated to migrate and tried to escape the containers when humidity increased.

This study was performed in a transgenerational approach, as proposed by Bosch et al. (2019), to evaluate the effect of the diet modulation throughout the life cycle of the BSF and if there was the possibility to enhance the acceptability of the macroalgae enriched diet when was fed during multiple generations. The last two generations larvae had a more heterogeneous weight distribution, and bigger prepupae were found simultaneously with very small larvae and prepupae. This could be due to the competition with the amount of chicken feed in the diet on the earlier stages of larvae development and possibly influenced the final

individual larval weight shown in **Table 2**, which was based on subsample of 50-larvae. The prepupae from 25% and 50%AV treatments were smaller and, consequently, also the adult flies were shorter. This was most likely caused by nutritional deficit which latter lead to a lower oviposition by the female flies, phenomenon described by Honek (1993). Since a smaller quantity of eggs hatched in these cases, that number of larvae was also less than on 0%AV diets, increasing the availability of food in these containers and allowing the larvae to feed more freely until the beginning of the trial (7 days), resulting in a higher initial weight.

The strong bioconversion capacity of *H. illucens* of different substrates, reported by many authors (Giannetto et al., 2020; Lopes, Lalander, Vidotti, & Vinnerås, 2020; Nyakeri, Ogola, Ayieko, & Amimo, 2017; Surendra et al., 2020), was corroborated by this work. Overall, the results observed show that BSF larvae can efficiently convert diets enriched with AV into body mass, information supported by the constant protein content found on the larvae during the experience, whether or not they were fed with a diet containing macroalgae. However, the inclusion of algae in the diet had an impact on the individual larval weight in G0, presenting a decrease of 43% (50%AV) when compared with the control diet (0%AV). This effect was not significant in the next two generations – G1 and G2 – even though a decrease of 23% (50%AV) was observed in G3 in comparison to the larvae fed with the control diet in the same generation. The feeding medium did not have a significant effect on the survival rate of the larvae. However, it was increasingly challenging to proceed with this study as it was initially outlined: to collect sufficient amount of eggs from the flies which were fed during the larval stage with algae-containing diets. In the last generation, G3, the initial number of larvae per container had to be reduced to 350 larvae, and previous studies by Karol B. Barragan-Fonseca, Dicke, & van Loon (2018) have demonstrate the influence of larval density in bioconversion capacity, especially regarding the positive correlation between higher larval density and larval crude fat.

As expected, the diet affected the normal growth of BSF larvae (Liland et al., 2017). In G0 and G1, the larvae fed with algae had an extended larval

development, once there was less prepupae by the end of the trial, when compared with the control diet. This is a compensatory mechanism in insects described by Miller (1964). However, the opposite can be observed on G2 and G3: the larvae fed with 25% and 50%AV diets entered the prepupal stage earlier, and the BCR was lower and the FCR higher, indicating that these larval groups consumed more food to achieve a lower body mass. This phenomenon is a response to nutritional deficiencies and was already reported by Sullivan & Sokal (1963), consisting of a reduction in the number of larvae able to complete their life cycles. Although this may not seem completely applicable for this case, the number of emerging adults was in fact much lower in G3 and, in the last generation, the heterogeneity of the larvae was easily noticeable as larvae on instars I and II were observed along with instar V and prepupae stage. For example, in Bertrand et al. (2021) experiment, the enrichment of the feeding diets with a flax cake rich in polyunsaturated fatty acids led to twice longer development times of the larvae phase. When fed with an feeding media enriched in 50% with microalgae waste, BSF larvae also needed more 14 days to develop (El-Dakar, Ramzy, Ji, & Plath, 2020).

As reported by Banks, Gibson, & Cameron (2014), lower feed availability leads to a better substrate reduction. In this experiment, the total feed added was the minimum to assure the growth of the larvae, 33 mg/day/larvae, to limit the availability of the chicken feed portion in the diets and therefore assure that the larvae would eat the algae. This would account of 330 mg for the 10 days of experiment, but even when the trial duration was shorter, the same amount of feed was available. This, together with the lower protein content of the algae, could justify the slightly lower prepupal weight (87.4 to 127.6 g) when compared with other studies, on a wet weight basis: chicken manure 0.200 g (Craig Sheppard et al., 1994), food waste 0.101 mg (Nyakeri et al., 2017). The individual prepupal weight was overall consistent throughout the experiment. The lowest value was found on G0 fed 50%AV. Studies concerning the bioconversion capacity of BSF larvae have shown a decrease in the individual prepupal weight when, for example, organic waste (food waste, banana peels, brewer's waste) is incorporated in the larvae diets (Nyakeri et al., 2017), or even for oil-seed enriched diets (Bertrand et al., 2021).

Bioconversion ratio indicates the efficiency of the substrate consumption. On the other way, the feed conversion rate expresses the proportion of digested food assimilated that is converted into biomass. The analysis of the bioconversion ratio and feed conversion ratio indicate the challenge of BSF larvae to convert the substrate into body mass, for diets containing algae in G2 and G3. It is reasonable to assume that the larvae achieved a nutrient deficit and the algae acceptability decreased. Still, a lower BCR is expected when non-preferential diets are fed to *Hermetia illucens*, which is corroborated by the high FCR values (G2, 25%AV: 27.45%, G3, 25%AV: 35,33% and 50%AV: 14.11%), that point that the substrate was digestible but poor in the desirable nutrients.

An interesting option to avoid deleterious effects of the macroalgae but still benefit from its nutritional properties could be to carry out the transgenerational experiment but to only provide the diet containing *A. vermiculophyllum* in the last days of larval growth. This way it would act like a finisher diet, like it was proposed by Barroso, Sánchez-Muros, et al. (2017). Still, macroalgae are not often used in aquaculture system due to its low digestibility (Makkar et al., 2016) and lack of acceptance by the fish. For this reason, this work presents a new opportunity to feed the BSF larvae with an invasive species, as they, indeed, were able to convert this substrate. Further analysis of the nutritional content of the residues could further support these results.

In the last years, several studies reported that BSF development and nutrient content were strongly related to the quality of the substrate and, in particular, that the fat composition of the larvae is influenced by the diet supplied (Wang & Shelomi, 2017).

The lipid content of the larvae oscillated among generations. Initially, in G0, a small decrease was found in larvae fed with AV diets. In G1 and G2, the larvae fed with 25% already showed a higher lipid content and for G3, both larvae fed with 25% and 50%AV had a significant increase of its lipid content, when compared with the previous generations.

The fatty acid profile of the diet used as feed in this experiment had a high PUFA content, mainly in formulations with 25 and 50% of *A. vermiculophyllum*

incorporation. Nonetheless, BSF larvae profiles were dominated by SFA, as demonstrated by other authors (Barroso, Sánchez-muros, et al., 2017; Giannetto et al., 2020; Meneguz et al., 2018; Spranghers et al., 2017). However, the inclusion of algae in the diet led to a decreased in lauric acid content in larvae, a phenomenon that had already been reported in other few studies (Gao et al., 2019; Liland et al., 2017; Meneguz et al., 2018). Another increase was found in the MUFA content of the larvae fed with 25% and 50%AV diets, due to higher abundance of oleic acid, which doesn't seem to be provided by the diet. The abundance of PUFA was also influenced by the presence of AV in the substrate, and a general increase in linoleic and α-linolenic acids was observed. An exception to this was G2 50%AV, that presented an even lower abundance (14.49%) of linoleic acid compared to larvae fed with 0%AV. The decrease of this fatty acid can be justified by the presence of arachidonic acid (6.58% of total FA).

The use of algae as feedstuff for insects is yet to be better explored. A similar study was conducted by Liland et al. (2017), using the brown algae *Ascophyllum nodosum*. The incorporation of this brown algae by 50% led to a content of 24.5% of SFA, 22.3% MUFA and 50.7% PUFA in the feeding media. In comparison with this trial, the incorporation of *A. vermiculophyllum* by 50% led to a lower abundance of SFA and MUFA (20.3%, 19.8%, respectively) and to a higher value of PUFA (59.9%). In the larvae, 50%AV diet decreased the SFA content across the generations (43.47 to 41.47%) and increased the MUFA (30.41 to 32.81%) and PUFA (26.12 to 26.23%). In comparison, these values were also higher that the ones obtained with a study which used the brown algae *Ascophyllum nodosum*: 13.3% MUFA and 25.3% PUFA (Liland et al., 2017).

It was expected to find some content in C20:5 n-3 EPA, since this was present in the algae, although in reduced quantities (values vary according to the season from 0.11 to 0.28 % of FA) (Afonso et al., 2021). However, algae are very susceptible to oscillations in their nutrient profiles, as described by Afonso et al. (2021). Besides that, the incorporation of the fresh algae led to an even lower PUFA content. Therefore, for future works using this invasive alga, it would be important to make a preliminary trial with *A. vermiculophyllum* to assess how the

season affects its fatty acid profile and to perform the experiments freeze drying the macroalgae prior to the preparation of the diets.

Some studies described the fat accumulation in different larval stages of FBSF and a recent work by Zhu et al. (2019) observed the differences in mRNA expression patterns between V instar larvae and prepupae and come to the conclusion that the genes involved in fatty acid biosynthesis (acc and fas) were upregulated in early stages. In this recent study conducted by Giannetto et al. (2020), the profile of V instar larvae was richer in MUFA and PUFA, when compared with prepupal stage. In the prepupae stage these genes were less abundant, probably because the larvae already had enough stored energy for the next stages. For this reason, the concept presented earlier in this discussion, consisting of using the diets enriched with *A. vermiculophyllum* as a finisher diet, is again placed on the table. If fatty acids biosynthesis is upregulated earlier in the larvae stage, mono- and polyunsaturated are being converted into lauric acid to be stored, and to feed a different diet in that period seems a waste of time and resources. However, if the process was already optimized, it is a great way to upcycle the invasive macroalgae and contribute to its reduction in Ria de Aveiro lagoon.

The results obtained for the fatty acid profile of larvae fed with *A. vermiculophyllum* suggest that this could be a suitable alternative to fed BSF larvae used as feed ingredients for aquaculture fish, and as a valorization of an invasive algae, it would fit a circular economy model with strong environmentally sustainable advantages. However, the fatty acid profile modulation achieved by the incorporation of the macroalgae is not suitable as a source of n-3 polyunsaturated fatty acids needed in carnivorous fish diets, namely EPA and DHA (Lall & Dumas, 2015). However, it might be an adequate option for omnivorous fish. Regardless, in this work, a transgenerational increase of the essential fatty acid 18:2 n-6 (linoleic acid) was observed.

Nevertheless, *H. illucens* larvae presents other advantages. The high content in lauric acid provides an antimicrobial activity (Vogel et al., 2018). Palmitic, palmitoleic and oleic acids, according to the results obtained, can be partially

produced by BSF, not only by accumulation from the diet but also via biosynthesis pathways (Hoc et al., 2020). This saturated and monounsaturated fatty acids have been more and more studied as important for fish wellness and for the success of the aquaculture systems. For example, some fish (mainly freshwater) have the capacity to *de novo* synthesis. In this context, SFA (mainly short-chain) and MUFA are preferable substrates for β-oxidation aiming energy production in fish (Henderson, 1996). For this reason, it is of particular interest the dietary supplementation of SFA and MUFA, which limits the metabolic energy for these lipogenesis processes, as well and the extension of the oxidation of other fatty acids, such as n-3 PUFA, promoting their accumulation in fish tissues (Xu, Turchini, Francis, Liang, & Mock, 2020). Still, for this purpose, it would be necessary the additional supplementation of n-3 PUFA (Marques et al., 2021). Furthermore, palmitic oil was reported to have induced the production of antioxidant agents and expression of pro-inflammatory cytokines in zebrafish larvae (Cha, Hwang, Kim, & Jun, 2018). A recent study also reported the benefits of the incorporation of low concentrations of palmitic acid in fish diets as an immunostimulant, having antiviral properties (Librán-Pérez, Pereiro, Figueras, & Novoa, 2019).

Recently, the use of insect processed protein was authorized for used in poultry and pig feed formulations, in the European Union. This new step opens even more the range of *H. illucens* utilization, since its suitability as a protein and lipid source for these animals was already largely studied (Barragan-Fonseca et al., 2017; Booram, Newton, Hale, & Barker, 1977; Makkar et al., 2014a).

5. Final considerations and future perspectives

Our study concludes that the incorporation of the macroalgae *A. vermiculophyllum* in the diets of *Hermetia illucens* did not impacted the survival rate, and that the larvae could convert the macroalgae into body mass. Additionally, a modulation of the fatty acid profile was achieved. Even though the results obtained did demonstrate that *A. vermiculophyllum* was a suitable replacement of conventional n-3 PUFA sources for fish feed, the conclusions of

this study raise several other questions and open other research opportunities. Therefore, future studies are undoubtedly required and should have some considerations:

- Evaluation of fatty acid profile changes in *A. vermiculophyllum* throughout the season, to access the best time to collect the macroalgae;
- Study of other incorporation rates, up to 50%;
- Freeze-dried the macroalgae before it is fed to the larvae, to increase the concentration of desirable fatty acids

In conclusion, the macroalgae *A. vermiculophyllum* can be successfully incorporated in *H. illucens* diet and the fatty acid profile of the larvae can be modulated throughout several generations.

6. Bibliographic references

Afonso, C., Correia, A. P., Freitas, M. V., Baptista, T., Neves, M., & Mouga, T. (2021). Seasonal changes in the nutritional composition of agarophyton vermiculophyllum (Rhodophyta, gracilariales) from the center of Portugal. *Foods*, *10*(5). https://doi.org/10.3390/foods10051145

Akhtar, Y., & Isman, M. B. (2000). *Insects as an Alternative Protein Source*. *Proteins in Food Processing* (Second Edi). Elsevier Ltd. https://doi.org/10.1016/B978-0-08-100722-8.00011-5

Alder, J., Campbell, B., Karpouzi, V., Kaschner, K., & Pauly, D. (2008). Forage Fish: From Ecosystems to Markets. *Annual Review of Environment and Resources*, *33*(1), 153–166. https://doi.org/10.1146/annurev.environ.33.020807.143204

Ameixa, O., Duarte, P. M., & Rodrigues, D. P. (2019). Insects, Food Security and Sustainable Aquaculture. *Zero Hunger*, 1–11. https://doi.org/10.1007/978-3-319-69626-3_111-1

Banks, I. J., Gibson, W. T., & Cameron, M. M. (2014). Growth rates of black soldier fly larvae fed on fresh human faeces and their implication for improving sanitation, *19*(1), 14–22. https://doi.org/10.1111/tmi.12228

Barragan-Fonseca, K. B., Dicke, M., & Loon, J. J. A. van. (2017). Nutritional value of the black soldier fly (Hermetia illucens L.) and its suitability as animal feed - a review, *3*(2), 105–120. Retrieved from https://edepot.wur.nl/425799

Barragan-Fonseca, K. B., Dicke, M., & van Loon, J. J. A. (2018). Influence of larval density and dietary nutrient concentration on performance, body protein, and fat contents of black soldier fly larvae (Hermetia illucens). *Entomologia Experimentalis et Applicata*, *166*(9), 761–770. https://doi.org/10.1111/eea.12716

Barroso, F. G., Haro, C. De, Sánchez-muros, M., Venegas, E., Martínez-sánchez, A., & Pérez-bañón, C. (2014). The potential of various insect species for use as food for fi sh. *Aquaculture*, *422–423*, 193–201. https://doi.org/10.1016/j.aquaculture.2013.12.024

Barroso, F. G., Sánchez-Muros, M. J., Segura, M., Morote, E., Torres, A., Ramos, R., & Guil, J. L. (2017). Insects as food: Enrichment of larvae of Hermetia illucens with omega 3 fatty acids by means of dietary modifications. *Journal of Food Composition and Analysis*, *62*, 8–13. https://doi.org/10.1016/j.jfca.2017.04.008

Barroso, F. G., Sánchez-muros, M., Segura, M., Morote, E., Torres, A., Ramos, R., & Guil, J. (2017). Enrichment of larvae of Hermetia illucens with omega 3 fatty acids by means of dietary modifications. *Journal of Food Composition and Analysis*, *62*(March), 8–13. https://doi.org/10.1016/j.jfca.2017.04.008

Belghit, I., Liland, N. S., Gjesdal, P., Biancarosa, I., Menchetti, E., Li, Y., … Lock, E. (2019). Black soldier fl y larvae meal can replace fi sh meal in diets of sea-water phase Atlantic salmon (Salmo salar). *Aquaculture*, *503*(December 2018), 609–619. https://doi.org/10.1016/j.aquaculture.2018.12.032

Benelli, G., Canale, A., Raspi, A., & Fornaciari, G. (2014). The death scenario of an Italian Renaissance princess can shed light on a zoological dilemma: did the black soldier fly reach Europe with Columbus? *Journal of Archaeological Science*, *49*, 203–205. https://doi.org/https://doi.org/10.1016/j.jas.2014.05.015

Bentsen, H. (2017). Dietary polyunsaturated fatty acids, brain function and mental health. *Microbial Ecology in Health and Disease*. https://doi.org/10.1080/16512235.2017.1281916

Bertinetti, C., Samayoa, A. C., & Hwang, S.-Y. (2019). Effects of Feeding Adults of Hermetia illucens (Diptera: Stratiomyidae) on Longevity, Oviposition, and Egg Hatchability: Insights Into Optimizing Egg Production. *Journal of Insect Science (Online)*, *19*(1), 19. https://doi.org/10.1093/jisesa/iez001

Bertrand, H., Frédéric, F., Joachim, C., Lucien, M., Christophe, B., Giorgia, P., & Megido Rudy, C. (2021). ω3-enrichment of Hermetia illucens (L. 1758) prepupae from oilseed byproducts. *Journal of the Saudi Society of Agricultural Sciences*, (xxxx), 1–9. https://doi.org/10.1016/j.jssas.2021.01.001

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911–917. https://doi.org/10.1139/o59-099

Bondari, K., & Sheppard, D. (1981). Soldier fly larvae as feed in commercial fish production. *Aquaculture (Netherlands)*.

Booram, C. V, Newton, G. L., Hale, O. M., & Barker, R. W. (1977). Dried Hermetia Illucens Larvae Meal as a Supplement for Swine. *Journal of Animal Science*, *44*(3), 395–400. https://doi.org/10.2527/jas1977.443395x

Borrello, M., Lombardi, A., Pascucci, S., & Cembalo, L. (2016). The Seven Challenges for Transitioning into a Bio-based Circular Economy in the Agri-food Sector. *Recent Patents on Food, Nutrition & Agriculture*, *8*(1), 39–47. https://doi.org/10.2174/2212798408011603041439 39

Bosch, G., Oonincx, D. G. A. B., Jordan, H. R., Zhang, J., van Loon, J. J. A., van Huis, A., & Tomberlin, J. K. (2019). Standardisation of quantitative resource conversion studies with black soldier fly larvae. *Journal of Insects as Food and Feed*, (August), 1–16. https://doi.org/10.3920/jiff2019.0004

Buchon, N., Silverman, N., & Cherry, S. (2014). Immunity in Drosophila melanogaster — from microbial recognition to whole-organism physiology. *Nature Reviews Immunology*, *14*(12), 796–810. https://doi.org/10.1038/nri3763

Calder, P. (2003). Long-chain n-3 fatty acids and inflammation: Potential Application in Surgical and Trauma Patients. *Brazilian Journal of Medical and Biological Research = Revista Brasileira de Pesquisas Médicas e Biológicas / Sociedade Brasileira de Biofísica ... [et Al.]*, *36*, 433–446. https://doi.org/10.1590/S0100-879X2003000400004

Calder, P. C., & Yaqoob, P. (2009). Omega-3 polyunsaturated fatty acids and human health outcomes. *BioFactors*, *35*(3), 266–272. https://doi.org/10.1002/biof.42

Cha, S., Hwang, Y., Kim, K., & Jun, H. (2018). Fish and Shell fish Immunology Palmitate induces nitric oxide production and inflammatory cytokine expression in zebra fish, *79*(January), 163–167. https://doi.org/10.1016/j.fsi.2018.05.025

Collins, K. G., Fitzgerald, G. F., Stanton, C., & Ross, R. P. (2016). Looking beyond the terrestrial: The potential of seaweed derived bioactives to treat non-communicable diseases. *Marine Drugs*, *14*(3), 1–31. https://doi.org/10.3390/md14030060

Cox, M., & Nelson, D. (2000). *Lehninger Principles of Biochemistry*. *Wh Freeman* (Vol. 5). https://doi.org/10.1007/978-3-662-08289-8

Craig Sheppard, D., Larry Newton, G., Thompson, S. A., & Savage, S. (1994). A value added manure management system using the black soldier fly. *Bioresource Technology*, *50*(3), 275–279. https://doi.org/https://doi.org/10.1016/0960-8524(94)90102-3

Cummins, V. C., Rawles, S. D., Thompson, K. R., Velasquez, A., Kobayashi, Y., Hager, J., & Webster, C. D. (2017). Evaluation of black soldier fl y (Hermetia illucens) larvae meal as partial or total replacement of marine fi sh meal in practical diets for Paci fi c white shrimp (Litopenaeus vannamei). *Aquaculture*, *473*, 337–344. https://doi.org/10.1016/j.aquaculture.2017.02.022

Diener, S., Zurbrügg, C., Roa-Gutiérrez, F., Hong Dang, N., Morel, A., Koottatep, T., & Tockner, K. (2011). *Black soldier fly larvae for organic waste treatment - prospects and constraints*.

Diener, S., Zurbrügg, C., & Tockner, K. (2009). Conversion of organic material by black soldier fly larvae: establishing optimal feeding rates. *Waste Management & Research*, *27*(6), 603–610. https://doi.org/10.1177/0734242X09103838

El-Dakar, M. A., Ramzy, R. R., Ji, H., & Plath, M. (2020). Bioaccumulation of residual omega-3 fatty acids from industrial Schizochytrium microalgal waste using black soldier fly (Hermetia illucens) larvae. *Journal of Cleaner Production*, *268*, 122288. https://doi.org/10.1016/j.jclepro.2020.122288

Ewald, N., Vidakovic, A., Langeland, M., Kiessling, A., Sampels, S., & Lalander, C. (2020). Fatty acid composition of black soldier fly larvae (Hermetia illucens) – Possibilities and limitations for modification through diet. *Waste Management*, *102*, 40–47. https://doi.org/10.1016/j.wasman.2019.10.014

FAO. (2016). The State of World Fisheries and Aquaculture. *Contributing to Food Securuty and Nutrition for All*, p. 200.

FAO. (2020a). *The State of World Fisheries and Aquaculture 2020. Sustainability in action.* Rome. https://doi.org/https://doi.org/10.4060/ca9229en

FAO. (2020b). *The State of World Fisheries and Aquaculture 2020. SUstainability in action.* Rome. https://doi.org/https://doi.org/10.4060/ca9229en

Furman, D. P., Young, R. D., & Catts, P. E. (1959). Hermetia illucens (Linnaeus) as a Factor in the Natural Control of Musca domestica Linnaeus . *Journal of Economic Entomology*, *52*(5), 917–921. https://doi.org/10.1093/jee/52.5.917

Gao, Z., Wang, W., Lu, X., Zhu, F., Liu, W., Wang, X., & Lei, C. (2019). Bioconversion performance and life table of black soldier fl y (Hermetia illucens) on fermented maize straw. *Journal of Cleaner Production*, *230*, 974–980. https://doi.org/10.1016/j.jclepro.2019.05.074

Garcia-Vaquero, M., & Hayes, M. (2016). Red and green macroalgae for fish and animal feed and human functional food development. *Food Reviews International*, *32*(1), 15–45. https://doi.org/10.1080/87559129.2015.1041184

Gasco, L., Gai, F., Maricchiolo, G., Genovese, L., Ragonese, S., Bottari, T., & Caruso, G. (2018). *Feeds for the Aquaculture Sector Current Situation and Alternative Sources*. Retrieved from https://link.springer.com/content/pdf/10.1007%2F978-3-319-77941-6.pdf

Giannetto, A., Oliva, S., Ceccon Lanes, C. F., de Araújo Pedron, F., Savastano, D., Baviera, C., … Fasulo, S. (2020). Hermetia illucens (Diptera: Stratiomydae) larvae and prepupae: Biomass production, fatty acid profile and expression of key genes involved in lipid metabolism. *Journal of Biotechnology*, *307*(October 2019), 44–54. https://doi.org/10.1016/j.jbiotec.2019.10.015

Gladyshev, M. I., Arts, M. T., & Sushchik, N. N. (2009). Preliminary estimates of the export of omega-3 highly unsaturated fatty acids (EPA+DHA) from aquatic to terrestrial ecosystems BT - Lipids in Aquatic Ecosystems. In M. Kainz, M. T. Brett, & M. T. Arts (Eds.) (pp. 179–210). New York, NY: Springer New York. https://doi.org/10.1007/978-0-387-89366-2_8

Halloran, A., Roos, N., Eilenberg, J., Cerutti, A., & Bruun, S. (2016). Life cycle assessment of edible insects for food protein: a review. *Agronomy for Sustainable Development*, *36*(4), 57. https://doi.org/10.1007/s13593-016-0392-8

Hardy, R. W. (1996). Alternate protein sources for salmon and trout diets. *Animal Feed Science and Technology*, *59*(1), 71–80. https://doi.org/https://doi.org/10.1016/0377-8401(95)00888-8

Henderson, R. J. (1996). Fatty acid metabolism in freshwater fish with particular reference to polyunsaturated fatty acids. *Archiv Für Tierernaehrung*, *49*(1), 5–22. https://doi.org/10.1080/17450399609381859

Higgs, D., & Dong, F. M. (2000). Lipids and fatty acids (pp. 476–496).

Hoc, B., Genva, M., Fauconnier, M. L., Lognay, G., Francis, F., & Caparros Megido, R. (2020). About lipid metabolism in Hermetia illucens (L. 1758): on the origin of fatty acids in prepupae. *Scientific Reports*, *10*(1), 1–8. https://doi.org/10.1038/s41598-020-68784-8

Honek, A. (1993). Intraspecific variation in body size and fecundity in insects - A general relationship. *OIKOS*, *66*(3), 483–492. https://doi.org/10.2307/3544943

Imbs, A. B., Latyshev, N. A., Svetashev, V. I., Skriptsova, A. V., Le, T. T., Pham, M. Q., … Pham, L. Q. (2012). Distribution of polyunsaturated fatty acids in red algae of the genus Gracilaria, a promising source of prostaglandins. *Russian Journal of Marine Biology*, *38*(4), 339–345. https://doi.org/10.1134/S1063074012040049

Jimenez-Lopez, C., Pereira, A. G., Lourenço-Lopes, C., Garcia-Oliveira, P., Cassani, L., Fraga-Corral, M., … Simal-Gandara, J. (2021). Main bioactive phenolic compounds in marine algae and their mechanisms of action supporting potential health benefits. *Food Chemistry*, *341*, 128262. https://doi.org/https://doi.org/10.1016/j.foodchem.2020.128262

Kaushik, S. J., Covès, D., Dutto, G., & Blanc, D. (2004). Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, Dicentrarchus labrax. *Aquaculture*, *230*(1–4), 391–404. https://doi.org/10.1016/S0044-8486(03)00422-8

Khotimchenko, S. V., Vaskovsky, V. E., & Titlyanova, T. V. (2002). Fatty acids of marine algae from the pacific coast of North California. *Botanica Marina*, *45*(1), 17–22. https://doi.org/10.1515/BOT.2002.003

Kim, W., Bae, S., Park, K., Lee, S., Choi, Y., Han, S., & Koh, Y. (2011). Entomology Biochemical characterization of digestive enzymes in the black soldier fly , Hermetia illucens (Diptera : Stratiomyidae). *Journal of Asia-Pacific Entomology*, *14*(1), 11–14. https://doi.org/10.1016/j.aspen.2010.11.003

Lall, S. P., & Dumas, A. (2015). Nutritional requirements of cultured fish. In *Feed and Feeding Practices in Aquaculture* (pp. 53–109). Elsevier Ltd. https://doi.org/10.1016/b978-0-08-100506-4.00003-9

Lee, J. M., Lee, H., Kang, S., & Park, W. J. (2016). Fatty Acid Desaturases, Polyunsaturated Fatty Acid Regulation, and Biotechnological Advances. *Nutrients*, *8*(1). https://doi.org/10.3390/nu8010023

Librán-Pérez, M., Pereiro, P., Figueras, A., & Novoa, B. (2019). Antiviral activity of palmitic acid

via autophagic flux inhibition in zebrafish (Danio rerio). *Fish & Shellfish Immunology*, *95*, 595–605. https://doi.org/https://doi.org/10.1016/j.fsi.2019.10.055

Liland, N. S., Biancarosa, I., Araujo, P., Biemans, D., Bruckner, C. G., Waagb, R., … Lock, E. (2017). Modulation of nutrient composition of black soldier fly (Hermetia illucens) larvae by feeding seaweed-enriched media, 1–23.

Liu, X., Chen, X., Wang, H., Yang, Q., Rehman, K., Li, W., … Zheng, L. (2017). Dynamic changes of nutrient composition throughout the entire life cycle of black soldier fly, 1–21.

Lopes, I. G., Lalander, C., Vidotti, R. M., & Vinnerås, B. (2020). Using Hermetia illucens larvae to process biowaste from aquaculture production. *Journal of Cleaner Production*, *251*. https://doi.org/10.1016/j.jclepro.2019.119753

Lord, W. D., Goff, M. L., Adkins, T. R., & Haskell, N. H. (1994). The black soldier fly Hermetia illucens (Diptera: Stratiomyidae) as a potential measure of human postmortem interval: observations and case histories. *Journal of Forensic Sciences*, *39*(1), 215–222.

Magalhães, R., Sánchez-lópez, A., Silva, R., Martínez-llorens, S., Oliva-teles, A., & Peres, H. (2017). Black soldier fl y (Hermetia illucens) pre-pupae meal as a fi sh meal replacement in diets for European seabass (Dicentrarchus labrax). *Aquaculture*, *476*(March), 79–85. https://doi.org/10.1016/j.aquaculture.2017.04.021

Makkar, H. P. S., Tran, G., Heuzé, V., & Ankers, P. (2014a). State-of-the-art on use of insects as animal feed. *Animal Feed Science and Technology*, *197*, 1–33. https://doi.org/10.1016/J.ANIFEEDSCI.2014.07.008

Makkar, H. P. S., Tran, G., Heuzé, V., & Ankers, P. (2014b). State-of-the-art on use of insects as animal feed. *Animal Feed Science and Technology*, *197*, 1–33. https://doi.org/10.1016/j.anifeedsci.2014.07.008

Makkar, H. P. S., Tran, G., Heuzé, V., Giger-Reverdin, S., Lessire, M., Lebas, F., & Ankers, P. (2016). Seaweeds for livestock diets: A review. *Animal Feed Science and Technology*, *212*(November 2017), 1–17. https://doi.org/10.1016/j.anifeedsci.2015.09.018

Malcicka, M., Visser, B., & Ellers, J. (2018). An Evolutionary Perspective on Linoleic Acid Synthesis in Animals. *Evolutionary Biology*, *45*(1), 15–26. https://doi.org/10.1007/s11692-017-9436-5

Marques, V. H., Moreira, R. G., Branco, G. S., Honji, R. M., Rombenso, A. N., Viana, M. T., … Araújo, B. C. (2021). Different saturated and monounsaturated fatty acids levels in fish oil-free diets to cobia (Rachycentron canadum) juveniles: Effects in growth performance and lipid metabolism. *Aquaculture*, *541*, 736843. https://doi.org/https://doi.org/10.1016/j.aquaculture.2021.736843

McAlpine, J. F., Peterson, B. V, Shewell, G. E., Teskey, H. J., Vockeroth, J. R., & Wood, D. M. (Coordinators). (1981). *Manual of Nearctic Diptera. Volume 1. Monograph, Research Branch, Agriculture Canada*.

Meneguz, M., Schiavone, A., Gai, F., Dama, A., Lussiana, C., Renna, M., & Gasco, L. (2018). Effect of rearing substrate on growth performance, waste reduction efficiency and chemical composition of black soldier fly (Hermetia illucens) larvae. *Journal of the Science of Food and Agriculture*, *98*(15), 5776–5784. https://doi.org/10.1002/jsfa.9127

Miller, R. S. (1964). Larval competition in Drosophila melanogaster and Drosophila simulans. *Ecology*, *45*, 132–148.

Miranda, M., Lopez-Alonso, M., & Garcia-Vaquero, M. (2017). Macroalgae for functional feed development: Applications in aquaculture, ruminant and swine feed industries. *Seaweeds: Biodiversity, Environmental Chemistry and Ecological Impacts*, (June), 133–153.

Moutinho, S., Martínez-llorens, S., Tomás-vidal, A., Jover-cerdá, M., Oliva-teles, A., & Peres, H. (2017). Meat and bone meal as partial replacement for fi sh meal in diets for gilthead seabream (Sparus aurata) juveniles : Growth , feed ef fi ciency , amino acid utilization , and economic ef fi ciency. *Aquaculture*, *468*(999), 271–277. https://doi.org/10.1016/j.aquaculture.2016.10.024

Müller, A., Wolf, D., & Gutzeit, H. O. (2017). The black soldier fly, Hermetia illucens – a promising source for sustainable production of proteins, lipids and bioactive substances. *Zeitschrift Für Naturforschung C*, *72*(9–10), 351–363. https://doi.org/doi:10.1515/znc-2017-0030

Newton, G. L., Lacy, R. C., Kozánek, M., & Helena, C. (2015). The use of fly larvae for organic waste treatment ˇ ic, *35*, 68–80. https://doi.org/10.1016/j.wasman.2014.09.026

Nyakeri, E. M., Ogola, H. J. O., Ayieko, M. A., & Amimo, F. A. (2017). Valorisation of organic waste material: Growth performance of wild black soldier fly larvae (Hermetia illucens) reared on different organic wastes. *Journal of Insects as Food and Feed*, *3*(3), 193–202.

https://doi.org/10.3920/JIFF2017.0004
Oliva-Teles, A. (2012). Nutrition and health of aquaculture fish. *Journal of Fish Diseases*. https://doi.org/10.1111/j.1365-2761.2011.01333.x
Olsen, Y. (2011). Resources for fish feed in future mariculture. *Aquaculture Environment Interactions*, *1*(3), 187–200. https://doi.org/10.3354/aei00019
Oonincx, D. G. A. B., Broekhoven, S. Van, Huis, A. Van, & Loon, J. J. A. Van. (2015). Feed Conversion , Survival and Development , and Composition of Four Insect Species on Diets Composed of Food By-Products, (December). https://doi.org/10.1371/journal.pone.0144601
Pereira, H., Polo, C., Rešek, E., & Engelen, A. (2012). Polyunsaturated Fatty Acids of Marine Macroalgae : Potential for Nutritional and Pharmaceutical Applications, 1920–1935. https://doi.org/10.3390/md10091920
Ristić, V., & Ristić, G. (2003). [Role and importance of dietary polyunsaturated fatty acids in the prevention and therapy of atherosclerosis]. *Medicinski pregled*, *56*(1–2), 50–53. https://doi.org/10.2298/mpns0302050r
Robertson-Andersson, D., Potgieter, M., Hansen, J., Bolton, J., Troell, M., Anderson, R., … Probyn, T. (2008). Integrated seaweed cultivation on an abalone farm in South Africa. *Journal of Applied Phycology*, *20*, 579–595. https://doi.org/10.1007/s10811-007-9239-7
Rueness, J. (2005). Life history and molecular sequences of Gracilaria vermiculophylla (Gracilariales, Rhodophyta), a new introduction to European waters. *Phycologia*, *44*(1), 120–128. https://doi.org/10.2216/0031-8884(2005)44[120:LHAMSO]2.0.CO;2
Salomone, R., Saija, G., Mondello, G., Giannetto, A., Fasulo, S., & Savastano, D. (2017). Environmental impact of food waste bioconversion by insects : Application of Life Cycle Assessment to process using Hermetia illucens. *Journal of Cleaner Production*, *140*, 890–905. https://doi.org/10.1016/j.jclepro.2016.06.154
Sargent, J., McEvoy, L., Estevez, A., Bell, G., Bell, M., Henderson, J., & Tocher, D. (1999). Lipid nutrition of marine fish during early development: Current status and future directions. *Aquaculture*, *179*(1–4), 217–229. https://doi.org/10.1016/S0044-8486(99)00191-X
Saunders, G. W. (2009). Routine DNA barcoding of Canadian Gracilariales (Rhodophyta) reveals the invasive species Gracilaria vermiculophylla in British Columbia. *Molecular Ecology Resources*, *9*(SUPPL. 1), 140–150. https://doi.org/10.1111/j.1755-0998.2009.02639.x
Sheppard, D. C., Tomberlin, J. K., Joyce, J. A., Kiser, B. C., & Sumner, S. M. (2002). Rearing Methods for the Black Soldier Fly (Diptera: Stratiomyidae). *Journal of Medical Entomology*, *39*(4), 695–698. https://doi.org/10.1603/0022-2585-39.4.695
Simopoulos, A. P., & DiNicolantonio, J. J. (2016). The importance of a balanced ω-6 to ω-3 ratio in the prevention and management of obesity. *Open Heart*, *3*(2), e000385–e000385. https://doi.org/10.1136/openhrt-2015-000385
Sprangers, T., Ottoboni, M., Klootwijk, C., Ovyn, A., Deboosere, S., Meulenaer, B. De, … Smet, S. De. (2017). Nutritional composition of black soldier fly (. *Journal of the Science of Food and Agriculture*, (97), 2594–2600.
Spranghers, T., Ottoboni, M., Klootwijk, C., Ovyn, A., Deboosere, S., De Meulenaer, B., … De Smet, S. (2017). Nutritional composition of black soldier fly (Hermetia illucens) prepupae reared on different organic waste substrates. *Journal of the Science of Food and Agriculture*, *97*(8), 2594–2600. https://doi.org/10.1002/jsfa.8081
St-Hilaire, S., Cranfill, K., Mcguire, M., Mosley, E., Tomberlin, J., Newton, L., … Irving, S. (2007). Fish Offal Recycling by the Black Soldier Fly Produces a Foodstuff High in Omega3 Fatty Acids. *Journal of The World Aquaculture Society - J WORLD AQUACULT SOC*, *38*, 309–313. https://doi.org/10.1111/j.1749-7345.2007.00101.x
Stanley-Samuelson, D. W., Jurenka, R. A., Cripps, C., Blomquist, G. J., & de Renobales, M. (1988). Fatty acids in insects: Composition, metabolism, and biological significance. *Archives of Insect Biochemistry and Physiology*, *9*(1), 1–33. https://doi.org/10.1002/arch.940090102
Sullivan, R. L., & Sokal, R. R. (1963). The Effects of Larval Density on Several Strains of the House Fly. *Ecology*, *44*, 120–130.
Surendra, K. C., Tomberlin, J. K., Huis, A. Van, Cammack, J. A., Heckmann, L. L., & Kumar, S. (2020). Rethinking organic wastes bioconversion : Evaluating the potential of the black soldier fly (Hermetia illucens (L .)) (Diptera : Stratiomyidae) (BSF). *Waste Management*, *117*, 58–80. https://doi.org/10.1016/j.wasman.2020.07.050
Swinscoe, I., Oliver, D. M., Gilburn, A. S., Lunestad, B., Lock, E., Ørnsrud, R., & Quilliam, R. S.

(2018). Seaweed-fed black soldier fly (Hermetia illucens) larvae as feed for salmon aquaculture : assessing the risks of pathogen transfer, *1*(1), 1–13. https://doi.org/10.3920/JIFF2017.0067
Swinscoe, I., Oliver, D. M., Ørnsrud, R., & Quilliam, R. S. (2020). The microbial safety of seaweed as a feed component for black soldier fly (Hermetia illucens) larvae. *Food Microbiology*, *91*(January), 103535. https://doi.org/10.1016/j.fm.2020.103535
Tacon, A. G. J., & Metian, M. (2009). Fishing for Aquaculture: Non-Food Use of Small Pelagic Forage Fish—A Global Perspective. *Reviews in Fisheries Science*, *17*(3), 305–317. https://doi.org/10.1080/10641260802677074
Tomberlin, J. K., & Sheppard, D. C. (2002). Factors Influencing Mating and Oviposition of Black Soldier Flies (Diptera: Stratiomyidae) in a Colony. *Journal of Entomological Science*, *37*(4), 345–352. https://doi.org/10.18474/0749-8004-37.4.345
Trichet, V. V. (2010). Nutrition and immunity : an update, 356–372. https://doi.org/10.1111/j.1365-2109.2009.02374.x
Tschirner, M., Fisheries, I., Kloas, W., & Fisheries, I. (2017). Increasing the Sustainability of Aquaculture Systems : Insects as Alternative Protein Source for Fish Diets, (December). https://doi.org/10.14512/gaia.26.4.10
Turchini, G. M., Torstensen, B. E., & Ng, W.-K. (2009). Fish oil replacement in finfish nutrition. *Reviews in Aquaculture*, *1*(1), 10–57. https://doi.org/https://doi.org/10.1111/j.1753-5131.2008.01001.x
van Huis, A. (2013). Potential of Insects as Food and Feed in Assuring Food Security. https://doi.org/10.1146/annurev-ento-120811-153704
van Huis, A., & Oonincx, D. G. A. B. (2017). The environmental sustainability of insects as food and feed. A review. *Agronomy for Sustainable Development*, *37*(5). https://doi.org/10.1007/s13593-017-0452-8
van Huis, A., Van Itterbeeck, J., Klunder, H., Mertens, E., Halloran, A., Muir, G., & Vantomme, P. (2013). *EDIBLE INSECTS future prospects fo food and feed security* (Vol. 171).
Vogel, H., Müller, A., Heckel, D. G., Gutzeit, H., & Vilcinskas, A. (2018). Nutritional immunology: Diversification and diet-dependent expression of antimicrobial peptides in the black soldier fly Hermetia illucens. *Developmental and Comparative Immunology*, *78*, 141–148. https://doi.org/10.1016/j.dci.2017.09.008
Vongvichith, B., Morioka, S., Sugita, T., Phousavanh, N., Phetsanghanh, N., Chanthasone, P., … Nakamura, S. (2019). Evaluation of the efficacy of aquaculture feeds for the climbing perch Anabas testudineus: replacement of fishmeal by black soldier fly Hermetia illucens prepupae. *Fisheries Science*, (0123456789). https://doi.org/10.1007/s12562-019-01381-5
Wang, Y.-S., & Shelomi, M. (2017). Review of Black Soldier Fly (Hermetia illucens) as Animal Feed and Human Food. *Foods*, *6*(10), 91. https://doi.org/10.3390/foods6100091
Watanabe, T. (1982). Lipid nutrition in fish, *73*(1).
Wilson, R. . (2002). *Fish nutrition / edited by John E. Halver and Ronald W. Hardy.* (3 rd). San Diego, Calif. : Academic Press, c2002.
Xu, H., Turchini, G. M., Francis, D. S., Liang, M., & Mock, T. S. (2020). Progress in Lipid Research Are fish what they eat ? A fatty acid ' s perspective. *Progress in Lipid Research*, *80*(July), 101064. https://doi.org/10.1016/j.plipres.2020.101064
Zarantoniello, M., Randazzo, B., Truzzi, C., Giorgini, E., Marcellucci, C., Vargas-abúndez, J. A., … Olivotto, I. (2019). OPEN A six-months study on Black Soldier Fly (Hermetia illucens) based diets in zebrafish, (May), 1–12. https://doi.org/10.1038/s41598-019-45172-5
Zárate, R., el Jaber-Vazdekis, N., Tejera, N., Pérez, J. A., & Rodríguez, C. (2017). Significance of long chain polyunsaturated fatty acids in human health. *Clinical and Translational Medicine*, *6*(1). https://doi.org/10.1186/s40169-017-0153-6
Zheng, L., Li, Q., Zhang, J., & Yu, Z. (2012). Double the biodiesel yield : Rearing black soldier fl y larvae , Hermetia illucens , on solid residual fraction of restaurant waste after grease extraction for biodiesel production. *Renewable Energy*, *41*, 75–79. https://doi.org/10.1016/j.renene.2011.10.004
Zhu, Z., Rehman, K. U., Yu, Y., Liu, X., Wang, H., Tomberlin, J. K., … Zheng, L. (2019). De novo transcriptome sequencing and analysis revealed the molecular basis of rapid fat accumulation by black soldier fly (Hermetia illucens, L.) for development of insectival biodiesel. *Biotechnology for Biofuels*, *12*(1), 1–16. https://doi.org/10.1186/s13068-019-1531-7

7. Appendix

Table 6. Test for normal distribution - Shapiro-Wilk test performed for protein and lipid contents of BSF larvae and in the diet (n=3).

	Protein	Lipid
G0		
P value	0,4633	0,1
Passed normality test (alpha=0.05)?	Yes	Yes
P value summary	ns	ns
G1		
P value	0,1105	0,7876
Passed normality test (alpha=0.05)?	Yes	Yes
P value summary	ns	ns
G2		
P value	0,1799	0,8011
Passed normality test (alpha=0.05)?	Yes	Yes
P value summary	ns	ns
G3		
P value	0,1909	0,1377
Passed normality test (alpha=0.05)?	Yes	Yes
P value summary	ns	ns
Diet		
P value	0,9763	0,1348
Passed normality test (alpha=0.05)?	Yes	Yes
P value summary	ns	ns

Table 7. Two-way ANOVA statistical results relative to the lipid content, calculated for a 95% confidence interval (p=0.05). CI=confidence interval; ns= non-significant; *=significant differences.

Tukey's multiple comparisons test	95,00% CI of diff,	Summary	Adjusted P Value
G0			
0%G0 vs. 25%G0	9,423 to 16,22	****	<0,0001
0%G0 vs. 50%G0	10,22 to 17,02	****	<0,0001
25%G0 vs. 50%G0	-2,597 to 4,197	ns	0,8077
G1			
0%G1 vs. 25%G1	-16,73 to 1,434	ns	0,1032
0%G1 vs. 50%G1	-3,914 to 14,25	ns	0,3172
25%G1 vs. 50%G1	3,736 to 21,90	**	0,007
G2			
0%G2 vs. 25%G2	-11,74 to 3,000	ns	0,3333
0%G2 vs. 50%G2	-4,340 to 10,40	ns	0,622
25%G2 vs. 50%G2	0,02994 to 14,77	*	0,049
G3			
0%G3 vs. 25%G3	-19,68 to -5,797	**	0,0011

0%G3 vs. 50%G3	-18,66 to -4,777	**	0,0022
25%G3 vs. 50%G3	-5,923 to 7,963	ns	0,9175

Table 8. Two-way ANOVA statistical results relative to the lipid content, in a transgenerational assessment ($p<0.05$). CI=confidence interval; ns= non-significant; *=significant differences

Lipid	95,00% CI of diff,	Summary	Adjusted P Value
0%G0 vs. 25%G0	3,904 to 21,74	***	0,0006
0%G0 vs. 50%G0	4,704 to 22,54	***	0,0002
0%G0 vs. 0%G1	-2,026 to 15,81	ns	0,2795
0%G0 vs. 25%G1	-9,676 to 8,156	ns	>0,9999
0%G0 vs. 50%G1	3,144 to 20,98	**	0,0015
0%G0 vs. 0%G2	7,634 to 25,47	****	<0,0001
0%G0 vs. 25%G2	3,264 to 21,10	**	0,0013
0%G0 vs. 50%G2	10,66 to 28,50	****	<0,0001
0%G0 vs. 0%G3	12,77 to 30,61	****	<0,0001
0%G0 vs. 25%G3	0,03433 to 17,87	*	0,0483
0%G0 vs. 50%G3	1,054 to 18,89	*	0,0167
25%G0 vs. 50%G0	-8,116 to 9,716	ns	>0,9999
25%G0 vs. 0%G1	-14,85 to 2,986	ns	0,4999
25%G0 vs. 25%G1	-22,50 to -4,664	***	0,0002
25%G0 vs. 50%G1	-9,676 to 8,156	ns	>0,9999
25%G0 vs. 0%G2	-5,186 to 12,65	ns	0,9494
25%G0 vs. 25%G2	-9,556 to 8,276	ns	>0,9999
25%G0 vs. 50%G2	-2,156 to 15,68	ns	0,3054
25%G0 vs. 0%G3	-0,04567 to 17,79	ns	0,0523
25%G0 vs. 25%G3	-12,79 to 5,046	ns	0,9355
25%G0 vs. 50%G3	-11,77 to 6,066	ns	0,9933
50%G0 vs. 0%G1	-15,65 to 2,186	ns	0,3116
50%G0 vs. 25%G1	-23,30 to -5,464	****	<0,0001
50%G0 vs. 50%G1	-10,48 to 7,356	ns	>0,9999
50%G0 vs. 0%G2	-5,986 to 11,85	ns	0,9916
50%G0 vs. 25%G2	-10,36 to 7,476	ns	>0,9999
50%G0 vs. 50%G2	-2,956 to 14,88	ns	0,4922
50%G0 vs. 0%G3	-0,8457 to 16,99	ns	0,11
50%G0 vs. 25%G3	-13,59 to 4,246	ns	0,8095
50%G0 vs. 50%G3	-12,57 to 5,266	ns	0,9563
0%G1 vs. 25%G1	-16,57 to 1,266	ns	0,1571
0%G1 vs. 50%G1	-3,746 to 14,09	ns	0,6954
0%G1 vs. 0%G2	0,7443 to 18,58	*	0,0234
0%G1 vs. 25%G2	-3,626 to 14,21	ns	0,6653
0%G1 vs. 50%G2	3,774 to 21,61	***	0,0007
0%G1 vs. 0%G3	5,884 to 23,72	****	<0,0001

0%G1 vs. 25%G3	-6,856 to 10,98	ns	0,9996
0%G1 vs. 50%G3	-5,836 to 12,00	ns	0,9875
25%G1 vs. 50%G1	3,904 to 21,74	***	0,0006
25%G1 vs. 0%G2	8,394 to 26,23	****	<0,0001
25%G1 vs. 25%G2	4,024 to 21,86	***	0,0005
25%G1 vs. 50%G2	11,42 to 29,26	****	<0,0001
25%G1 vs. 0%G3	13,53 to 31,37	****	<0,0001
25%G1 vs. 25%G3	0,7943 to 18,63	*	0,0222
25%G1 vs. 50%G3	1,814 to 19,65	**	0,0072
50%G1 vs. 0%G2	-4,426 to 13,41	ns	0,8447
50%G1 vs. 25%G2	-8,796 to 9,036	ns	>0,9999
50%G1 vs. 50%G2	-1,396 to 16,44	ns	0,1745
50%G1 vs. 0%G3	0,7143 to 18,55	*	0,0241
50%G1 vs. 25%G3	-12,03 to 5,806	ns	0,9865
50%G1 vs. 50%G3	-11,01 to 6,826	ns	0,9996
0%G2 vs. 25%G2	-13,29 to 4,546	ns	0,866
0%G2 vs. 50%G2	-5,886 to 11,95	ns	0,989
0%G2 vs. 0%G3	-3,776 to 14,06	ns	0,7028
0%G2 vs. 25%G3	-16,52 to 1,316	ns	0,1636
0%G2 vs. 50%G3	-15,50 to 2,336	ns	0,3437
25%G2 vs. 50%G2	-1,516 to 16,32	ns	0,1919
25%G2 vs. 0%G3	0,5943 to 18,43	*	0,0274
25%G2 vs. 25%G3	-12,15 to 5,686	ns	0,9819
25%G2 vs. 50%G3	-11,13 to 6,706	ns	0,9993
50%G2 vs. 0%G3	-6,806 to 11,03	ns	0,9995
50%G2 vs. 25%G3	-19,55 to -1,714	**	0,008
50%G2 vs. 50%G3	-18,53 to -0,6943	*	0,0246
0%G3 vs. 25%G3	-21,66 to -3,824	***	0,0006
0%G3 vs. 50%G3	-20,64 to -2,804	**	0,0022
25%G3 vs. 50%G3	-7,896 to 9,936	ns	>0,9999

Table 9. Test for normal distribution - Shapiro-Wilk test performed on the crude weight values of BSF larvae in G0 (n=3).

	G0		
	0%	**25%**	**50%**
P value	0.4782	0.4895	0.1191
Passed normality test (alpha=0.05)?	Yes	Yes	Yes
P value summary	ns	ns	ns

Table 10. Two-way ANOVA statistical results relative to the effect of the rearing diets on several parameters (growth rate, bioconversion rate, individual larval weight, survival rate, % prepupae and feed conversion ratio) for G0 ($p<0.05$). CI=confidence interval; ns= non-significant; *=significant differences.

G0			
Tukey's multiple comparisons test	**95,00% CI of diff,**	**Summary**	**Adjusted P Value**
Growth rate (mg/larva/day)			
0%G0 vs. 25%G0	-9,603 to 11,80	ns	0.9658
0%G0 vs. 50%G0	-9,803 to 11,60	ns	0.977
25%G0 vs. 50%G0	-10,90 to 10,50	ns	0.9989
BCR (%)			
0%G0 vs. 25%G0	0,5974 to 22,00	*	0.0366
0%G0 vs. 50%G0	8,197 to 29,60	***	0.0003
25%G0 vs. 50%G0	-3,103 to 18,30	ns	0.206
Individual larval weight			
0%G0 vs. 25%G0	28,70 to 50,10	****	<0,0001
0%G0 vs. 50%G0	53,90 to 75,30	****	<0,0001
25%G0 vs. 50%G0	14,50 to 35,90	****	<0,0001
Survival rate (%)			
0%G0 vs. 25%G0	-12,00 to 9,403	ns	0.9526
0%G0 vs. 50%G0	-11,40 to 10,00	ns	0.986
25%G0 vs. 50%G0	-10,10 to 11,30	ns	0.9897
Prepupae (%)			
0%G0 vs. 25%G0	-6,703 to 14,70	ns	0.6354
0%G0 vs. 50%G0	-0,702 to 20,70	ns	0.071
25%G0 vs. 50%G0	-4,703 to 16,70	ns	0.3669
Feed conversion ratio (FCR)			
0%G0 vs. 25%G0	-12,20 to 9,203	ns	0.9375
0%G0 vs. 50%G0	-14,09 to 7,313	ns	0.721
25%G0 vs. 50%G0	-12,59 to 8,813	ns	0.9027

Table 11. Test for normal distribution - Shapiro-Wilk test performed on the crude weight values of BSF larvae in G1 (n=3).

		G1	
	0%	**25%**	**50%**
P value	0.5596	0.2307	0.3006
Passed normality test (alpha=0.05)?	Yes	Yes	Yes
P value summary	ns	ns	ns

Table 12. Two-way ANOVA statistical results relative to the effect of the rearing diets on several parameters (growth rate, bioconversion rate, individual larval weight, survival rate, % prepupae and feed conversion ratio) for G1 ($p<0.05$). CI=confidence interval; ns= non-significant; *=significant differences.

G1			
Tukey's multiple comparisons test	**95,00% CI of diff,**	**Summary**	**Adjusted P Value**
Growth rate (mg/larva/day)			
0%G1 vs. 25%G1	-23,19 to 18,79	ns	0.9645
0%G1 vs. 50%G1	-23,39 to 18,59	ns	0.9579
25%G1 vs. 50%G1	-21,19 to 20,79	ns	0.9997
BCR (%)			
0%G1 vs. 25%G1	-26,99 to 14,99	ns	0.7658
0%G1 vs. 50%G1	-27,49 to 14,49	ns	0.7315
25%G1 vs. 50%G1	-21,49 to 20,49	ns	0.9981
Individual larval weight			
0%G1 vs. 25%G1	-30,09 to 11,89	ns	0.545
0%G1 vs. 50%G1	-21,99 to 19,99	ns	0.9926
25%G1 vs. 50%G1	-12,89 to 29,09	ns	0.617
Survival rate (%)			
0%G1 vs. 25%G1	-19,79 to 22,19	ns	0.9893
0%G1 vs. 50%G1	-20,89 to 21,09	ns	>0,9999
25%G1 vs. 50%G1	-22,09 to 19,89	ns	0.991
Prepupae (%)			
0%G1 vs. 25%G1	-8,994 to 32,99	ns	0.353
0%G1 vs. 50%G1	14,01 to 55,99	***	0.0007
25%G1 vs. 50%G1	2,006 to 43,99	*	0.0292
Feed conversion ratio (FCR)			
0%G1 vs. 25%G1	-19,99 to 21,99	ns	0.9926
0%G1 vs. 50%G1	-20,02 to 21,96	ns	0.993
25%G1 vs. 50%G1	-21,02 to 20,96	ns	>0,9999

Table 13. Test for normal distribution - Shapiro-Wilk test performed on the crude weight values of BSF larvae in G2 (n=3).

		G2	
	0%	**25%**	**50%**
P value	0.0333	0.7474	0.1434
Passed normality test (alpha=0.05)?	No	Yes	Yes
P value summary	*	ns	ns

Table 14. Two-way ANOVA statistical results relative to the effect of the rearing diets on several parameters (growth rate, bioconversion rate, individual larval weight, survival rate, % prepupae and feed conversion ratio) for G2 ($p<0.05$). CI=confidence interval; ns= non-significant.

G2			
Tukey's multiple comparisons test	**95,00% CI of diff,**	**Summary**	**Adjusted P Value**
Growth rate (mg/larva/day)			
0%G1 vs. 25%G1	-23,19 to 18,79	ns	0.9645
0%G1 vs. 50%G1	-23,39 to 18,59	ns	0.9579
25%G1 vs. 50%G1	-21,19 to 20,79	ns	0.9997
BCR (%)			
0%G1 vs. 25%G1	-26,99 to 14,99	ns	0.7658
0%G1 vs. 50%G1	-27,49 to 14,49	ns	0.7315
25%G1 vs. 50%G1	-21,49 to 20,49	ns	0.9981
Individual larval weight			
0%G1 vs. 25%G1	-30,09 to 11,89	ns	0.545
0%G1 vs. 50%G1	-21,99 to 19,99	ns	0.9926
25%G1 vs. 50%G1	-12,89 to 29,09	ns	0.617
Survival rate (%)			
0%G1 vs. 25%G1	-19,79 to 22,19	ns	0.9893
0%G1 vs. 50%G1	-20,89 to 21,09	ns	>0,9999
25%G1 vs. 50%G1	-22,09 to 19,89	ns	0.991
Prepupae (%)			
0%G1 vs. 25%G1	-8,994 to 32,99	ns	0.353
0%G1 vs. 50%G1	14,01 to 55,99	***	0.0007
25%G1 vs. 50%G1	2,006 to 43,99	*	0.0292
Feed conversion ratio (FCR)			
0%G1 vs. 25%G1	-19,99 to 21,99	ns	0.9926
0%G1 vs. 50%G1	-20,02 to 21,96	ns	0.993
25%G1 vs. 50%G1	-21,02 to 20,96	ns	>0,9999

Table 15. Test for normal distribution - Shapiro-Wilk test performed on the crude weight values of BSF larvae in G3 (n=3).

		G3	
	0%	**25%**	**50%**
P value	0.8954	0.5833	0.3526
Passed normality test (alpha=0.05)?	Yes	Yes	Yes
P value summary	ns	ns	ns

Table 16. Two-way ANOVA statistical results relative to the effect of the rearing diets on several parameters (growth rate, bioconversion rate, individual larval weight, survival rate, % prepupae and feed conversion ratio) for G3 ($p<0.05$). CI=confidence interval; ns= non-significant; *=significant differences.

G3			
Tukey's multiple comparisons test	**95,00% CI of diff,**	**Summary**	**Adjusted P Value**
Growth rate (mg/larva/day)			
0%G3 vs. 25%G3	3,828 to 18,17	**	0.0018
0%G3 vs. 50%G3	1,828 to 16,17	*	0.0111
25%G3 vs. 50%G3	-9,172 to 5,172	ns	0.7756
BCR (%)			
0%G3 vs. 25%G3	19,53 to 33,87	****	<0,0001
0%G3 vs. 50%G3	15,53 to 29,87	****	<0,0001
25%G3 vs. 50%G3	-11,17 to 3,172	ns	0.3706
Individual larval weight			
0%G3 vs. 25%G3	-4,372 to 9,972	ns	0.6101
0%G3 vs. 50%G3	8,828 to 23,17	****	<0,0001
25%G3 vs. 50%G3	6,028 to 20,37	***	0.0002
Survival rate (%)			
0%G3 vs. 25%G3	-6,672 to 7,672	ns	0.9841
0%G3 vs. 50%G3	-8,072 to 6,272	ns	0.9495
25%G3 vs. 50%G3	-8,572 to 5,772	ns	0.8825
Prepupae (%)			
0%G3 vs. 25%G3	-65,17 to -50,83	****	<0,0001
0%G3 vs. 50%G3	-83,17 to -68,83	****	<0,0001
25%G3 vs. 50%G3	-25,17 to -10,83	****	<0,0001
Feed conversion ratio (FCR)			
0%G3 vs. 25%G3	-39,14 to -24,80	****	<0,0001
0%G3 vs. 50%G3	-17,92 to -3,578	**	0.0022
25%G3 vs. 50%G3	14,05 to 28,39	****	<0,0001

Table 17. Two-way ANOVA statistical results relative to the effect of the rearing diets on several parameters (growth rate, bioconversion rate, individual larval weight, survival rate, % prepupae and feed conversion ratio) in a transgenerational assessment ($p<0.05$). CI=confidence interval; ns= non-significant; *=significant differences.

	Growth rate (mg/larva/day)	BCR (%)	Individual larval weight (mg)	Survival rate (%)	Prepupal (%)	FCR (%)
0%G0 vs. 25%G0	>0,9999	0.695	<0,0001	>0,9999	>0,9999	>0,9999
0%G0 vs. 50%G0	>0,9999	0.0476	<0,0001	>0,9999	0.8329	>0,9999
0%G0 vs. 0%G1	>0,9999	0.5337	<0,0001	>0,9999	0.0746	>0,9999
0%G0 vs. 25%G1	>0,9999	0.9907	<0,0001	>0,9999	<0,0001	>0,9999
0%G0 vs. 50%G1	>0,9999	0.9952	<0,0001	>0,9999	<0,0001	>0,9999
0%G0 vs. 0%G2	>0,9999	>0,9999	<0,0001	>0,9999	<0,0001	>0,9999
0%G0 vs. 25%G2	0.859	<0,0001	<0,0001	0.9998	0.985	0.0015
0%G0 vs. 50%G2	>0,9999	0.3877	<0,0001	0.9659	0.0006	>0,9999
0%G0 vs. 0%G3	>0,9999	0.9984	0.0105	>0,9999	<0,0001	>0,9999
0%G0 vs. 25%G3	0.8045	<0,0001	0.0017	>0,9999	0.6093	<0,0001
0%G0 vs. 50%G3	0.9471	0.0001	<0,0001	>0,9999	0.9958	0.7021
25%G0 vs. 50%G0	>0,9999	0.9718	0.001	>0,9999	0.9958	>0,9999
25%G0 vs. 0%G1	>0,9999	>0,9999	0.985	>0,9999	0.3651	>0,9999
25%G0 vs. 25%G1	>0,9999	0.9995	>0,9999	>0,9999	0.0006	>0,9999
25%G0 vs. 50%G1	>0,9999	0.9988	0.9958	>0,9999	<0,0001	>0,9999
25%G0 vs. 0%G2	0.9998	0.9745	>0,9999	>0,9999	<0,0001	>0,9999
25%G0 vs. 25%G2	0.9329	0.028	0.985	0.9977	0.7299	0.0042
25%G0 vs. 50%G2	>0,9999	>0,9999	0.8969	0.9036	0.0082	>0,9999
25%G0 vs. 0%G3	>0,9999	0.9964	0.082	>0,9999	<0,0001	>0,9999
25%G0 vs. 25%G3	0.8969	0.0168	0.2624	>0,9999	0.9591	<0,0001
25%G0 vs. 50%G3	0.9813	0.1291	>0,9999	>0,9999	0.8329	0.8557
50%G0 vs. 0%G1	>0,9999	0.9937	0.0677	>0,9999	0.9591	>0,9999
50%G0 vs. 25%G1	>0,9999	0.5716	0.0002	>0,9999	0.0265	>0,9999
50%G0 vs. 50%G1	>0,9999	0.5086	0.0408	>0,9999	<0,0001	>0,9999
50%G0 vs. 0%G2	0.9999	0.2533	0.0087	>0,9999	0.0023	>0,9999
50%G0 vs. 25%G2	0.922	0.5716	<0,0001	0.9992	0.1183	0.0134
50%G0 vs. 50%G2	>0,9999	0.999	<0,0001	0.9379	0.1801	>0,9999
50%G0 vs. 0%G3	>0,9999	0.4228	<0,0001	>0,9999	<0,0001	>0,9999
50%G0 vs. 25%G3	0.8826	0.459	<0,0001	>0,9999	>0,9999	<0,0001
50%G0 vs. 50%G3	0.977	0.8969	0.0003	>0,9999	0.1801	0.9643
0%G1 vs. 25%G1	>0,9999	0.9958	0.9036	>0,9999	0.6093	>0,9999
0%G1 vs. 50%G1	>0,9999	0.9918	>0,9999	>0,9999	<0,0001	>0,9999
0%G1 vs. 0%G2	0.9918	0.922	>0,9999	>0,9999	0.1801	>0,9999
0%G1 vs. 25%G2	0.9928	0.0555	0.3651	0.9995	0.0011	0.0052
0%G1 vs. 50%G2	>0,9999	>0,9999	0.1661	0.9514	0.9591	>0,9999
0%G1 vs. 0%G3	>0,9999	0.9813	0.0013	>0,9999	<0,0001	>0,9999
0%G1 vs. 25%G3	0.985	0.0348	0.0082	>0,9999	0.9958	<0,0001
0%G1 vs. 50%G3	0.9992	0.2186	0.922	>0,9999	0.0023	0.8833
25%G1 vs. 50%G1	>0,9999	>0,9999	0.9554	>0,9999	0.0044	>0,9999
25%G1 vs. 0%G2	0.9998	>0,9999	0.9984	0.9998	>0,9999	>0,9999
25%G1 vs. 25%G2	0.9329	0.0015	0.9993	>0,9999	<0,0001	0.0027
25%G1 vs. 50%G2	>0,9999	0.9813	0.9832	0.985	>0,9999	>0,9999
25%G1 vs. 0%G3	>0,9999	>0,9999	0.2026	>0,9999	<0,0001	>0,9999
25%G1 vs. 25%G3	0.8969	0.0008	0.4961	>0,9999	0.0746	<0,0001
25%G1 vs. 50%G3	0.9813	0.0111	>0,9999	>0,9999	<0,0001	0.7955

50%G1 vs. 0%G2	0.9999	>0,9999	>0,9999	>0,9999	0.0452	>0,9999
50%G1 vs. 25%G2	0.922	0.0011	0.4837	0.9996	<0,0001	0.0028
50%G1 vs. 50%G2	>0,9999	0.969	0.2443	0.9554	0.0003	>0,9999
50%G1 vs. 0%G3	>0,9999	>0,9999	0.0026	>0,9999	0.1183	>0,9999
50%G1 vs. 25%G3	0.8826	0.0006	0.015	>0,9999	<0,0001	<0,0001
50%G1 vs. 50%G3	0.977	0.0082	0.9659	>0,9999	<0,0001	0.7985
0%G2 vs. 25%G2	0.4961	0.0002	0.8045	0.9626	<0,0001	0.002
0%G2 vs. 50%G2	0.9995	0.8329	0.5463	0.6831	0.9591	>0,9999
0%G2 vs. 0%G3	>0,9999	>0,9999	0.0141	>0,9999	<0,0001	>0,9999
0%G2 vs. 25%G3	0.4228	0.0001	0.0644	>0,9999	0.0082	<0,0001
0%G2 vs. 50%G3	0.671	0.0019	0.999	>0,9999	<0,0001	0.7489
25%G2 vs. 50%G2	0.9514	0.0988	>0,9999	>0,9999	<0,0001	0.0051
25%G2 vs. 0%G3	0.7945	0.0007	0.7522	0.9988	<0,0001	0.0021
25%G2 vs. 25%G3	>0,9999	>0,9999	0.9591	0.9995	0.0452	0.9633
25%G2 vs. 50%G3	>0,9999	>0,9999	0.9988	0.9952	>0,9999	0.442
50%G2 vs. 0%G3	>0,9999	0.9426	0.9329	0.9276	<0,0001	>0,9999
50%G2 vs. 25%G3	0.922	0.0644	0.9969	0.9514	0.3651	<0,0001
50%G2 vs. 50%G3	0.9881	0.3323	0.977	0.8672	<0,0001	0.8818
0%G3 vs. 25%G3	0.7299	0.0003	>0,9999	>0,9999	<0,0001	<0,0001
0%G3 vs. 50%G3	0.91	0.0053	0.1801	>0,9999	<0,0001	0.7577
25%G3 vs. 50%G3	>0,9999	>0,9999	0.459	>0,9999	0.0746	0.0132

Table 18. Test for normal distribution - Shapiro-Wilk test performed for the FAME relative abundances of fatty acids in the diets (n=3).

	C16:0	C18:0	C18:1	C18:2	C18:3
P value	0,9914	0,4033	0,1358	0,044	0,0785
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	No	Yes
P value summary	ns	ns	ns	*	ns

Table 19. Two-way ANOVA statistical results relative to the FAME relative abundances of C18:2 fatty acid present in the diet (where significant differences were found p<0.05). CI=confidence interval; ns= non-significant; *=significant differences.

Tukey's multiple comparisons test			
C18:2	**95,00% CI of diff,**	**Summary**	**Adjusted P Value**
0%D vs. 25%D	-10,27 to 9,243	ns	0,9912
0%D vs. 50%D	9,221 to 28,73	****	<0,0001
25%D vs. 50%D	9,732 to 29,24	****	<0,0001
C20:4			
0%D vs. 25%D	-9,754 to 9,754	ns	>0,9999
0%D vs. 50%D	-24,20 to -4,694	**	0,0022
25%D vs. 50%D	-24,20 to -4,694	**	0,0022

Table 20. Test for normal distribution - Shapiro-Wilk test performed for the FAME relative abundances in BSF larvae - G0 (n=3).

	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
P value	0,3146	0,5767	0,0721	0,3013	0,6752	0,6834	0,5934	0,0496
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
P value summary	ns	ns	ns	ns	ns	ns	ns	*

Table 21. Two-way ANOVA statistical results relative to the FAME relative abundances of C12:0 fatty acid in G0 (where significant differences were found p<0.05). CI=confidence interval; ns= non-significant; *=significant differences

Tukey's multiple comparisons test			
C12:0	**95,00% CI of diff.**	**Summary**	**Adjusted P Value**
0%G0 vs. 25%G0	-0,3702 to 24,43	ns	0,059
0%G0 vs. 50%G0	2,185 to 26,99	*	0,0176
25%G0 vs. 50%G0	-9,847 to 14,96	ns	0,8725

Table 22. Test for normal distribution - Shapiro-Wilk test performed for the FAME relative abundances in BSF larvae – G1 (n=3).

	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
P value	0,4319	0,7589	0,1182	0,201	0,7756	0,9904	0,4017	0,4741
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
P value summary	ns	ns	ns	ns	ns	ns	ns	ns

Table 23. Test for normal distribution - Shapiro-Wilk test performed for the FAME relative abundances in BSF larvae - G2 (n=3).

	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
P value	0,2588	0,2275	0,121	0,8814	0,6321	0,2975	0,5571	0,2244
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
P value summary	ns	ns	ns	ns	ns	ns	ns	ns

Table 24. Test for normal distribution - Shapiro-Wilk test performed for the FAME relative abundances in BSF larvae - G3 (n=3).

	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
P value	0,3647	0,4658	0,5176	0,5184	0,2223	0,0826	0,5209	0,1477
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
P value summary	ns	ns	ns	ns	ns	ns	ns	ns

Table 25. Two-way ANOVA statistical results relative to the FAME relative abundances of C18:1 fatty acid in G3 (where significant differences were found p<0.05). CI=confidence interval; ns= non-significant; *=significant differences

Tukey's multiple comparisons test			
C18:1	**95,00% CI of diff,**	**Summary**	**Adjusted P Value**
0%G3 vs. 25%G3	-10,41 to 11,71	ns	0,9889
0%G3 vs. 50%G3	-23,74 to -1,622	*	0,0211
25%G3 vs. 50%G3	-24,39 to -2,272	*	0,0146