DIPARTIMENTO DI SCIENZE AGRARIE ALIMENTARI E AMBIENTALI

CORSO DI LAUREA IN: SCIENZE E TECNOLOGIE ALIMENTARI

MODULATION OF NUTRITIONAL VALUE OF *HERMETIA ILLUCENS* LARVAE BY FEEDING MICROALGAE- ENRICHED COFFEE SILVERSKIN: AMINO ACIDS, FATTY ACIDS AND CAROTENOIDS

Modulazione del valore nutrizionale delle larve di *Hermetia illucens* allevate su scarto di caffè arricchito in microalghe: amminoacidi, acidi grassi e carotenoidi

TIPO TESI: sperimentale

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RIASSUNTO

Le larve di mosca soldato nera (BSF), *Hermetia illucens* (Dittero, Stratiomydae), rappresentano un'alternativa sostenibile per convertire una grande quantità dello scarto di caffè chiamato coffee silverskin (CS), in preziosi ingredienti per mangimi per pesci. Lo scopo del presente lavoro di tesi è quello di valutare come la composizione chimica del substrato su cui vengono fatte crescere le larve influenza il loro valore nutrizionale. A tale proposito le larve di BSF sono state allevate su substrati a base di CS, arricchiti con diverse percentuali di microalga (5, 10% di *Arthrospira platensis).* Successivamente, sono stati analizzati il profilo in amminoacidi, in acidi grassi e in carotenoidi delle larve. I livelli dietetici di microalghe non hanno influenzato il contenuto proteico totale e il profilo amminoacidico delle larve, ad eccezione della treonina, lisina, istidina e tirosina. L'aumento dei livelli di microalga nella dieta ha determinato un aumento del contenuto lipidico larvale. L'alimentazione con il 10% di microalghe ha portato a larve con il più alto contenuto di PUFA (902,6 ± 22,4 mg/100 g larve peso secco). Un aumento significativo dei carotenoidi è stato osservato nelle larve alimentate con CS arricchito con microalghe, raggiungendo la massima bioconversione con la dieta contenente il 10% di spirulina fino a 16,0 ± 0.0 (zeaxantina) e 57,8 ± 0,4 (β-carotene) mg/kg larve peso secco, rispettivamente.

ABSTRACT

Black Soldier Fly (BSF) larvae, *Hermetia illucens* (Diptera, Stratiomydae), represent a sustainability valorization alternative to convert large quantities of coffee silverskin (Cs), an industrial waste, into valuable ingredients for aquafeed. The aim of this work was to evaluate the effect of the diet composition on the nutritional composition of larvae. For this purpose, BSF larvae was reared on diets formulated enriching CS (diet control) with different percentage of microalgae (5, 10% of *Arthrospira platensis*). The dietary levels of microalgae did not affect the larval crude protein content and the amino acid profile, except for the amounts threonine, lysine, histidine and tyrosine. The larval lipid content raised by increasing dietary microalgae levels. Feeding with 10% of microalgae led to larvae with the highest PUFA content (902,6 ± 22,4 mg/100g larvae dry weight). A significant increase of carotenoids was observed in larvae fed on Cs enriched with spirulina, reaching the maximum bioconversion on diet containing 10% of microalgae up to 16,0 ± 0.0 (zeaxanthin) and 57,8 ± 0,4 (β-carotene) mg/kg larvae dry weight.

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ACRONYMS AND ABBREVIATIONS

EFSA	European Food Safety Authority
HI	*Hermetia illucens*
BSF	Black soldier fly
BDW	Body dry weight
FA	Fatty acids
EFA	Essential Fatty Acids
FFA	Free Fatty Acids
NEFA	Non-Esterified Fatty Acids
MUFA	Monounsaturated fatty acids
PUFA	Polyunsaturated fatty acids
LC-PUFAs	Long chain polyunsaturated fatty acids
SFA	Saturated fatty acids
ALA	α-linolenic acid
AL	Linoleic acid
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
GLA	Linolenic acid
AA	Arachidonic acid
FDA	Food and Drug Administration
GRAS	Generally Recognized As Safe
CS	Coffee silverskin
FAME	Fatty acid methyl esters
LOD	Limit of detection
LOQ	Limit of quantification

1. INTRODUCTION

1.1 Food issue

As per statistics the population of the world by 2050 will reach 9 billion so with the increasing population it is necessary to increase the amount of animal feed and human food. Due to this rapid increase, the demand for meat and milk is expected to increase by 58% and 70% in 2050 (Fao 2011), therefore the production of a sufficient number of feeds for farmed animals and food for humans is a serious challenge for the future.

We have also to consider that the livestock production occupies 30% of the world's ice-free surface or 75% of all agricultural land (including crop and pasture land) and consume 8% of global human water use, mainly for the irrigation of feed crops. In addition, the livestock sector contributes approximately 14.5% of all anthropogenic greenhouse gas (GHG) emissions (7.1 Gigatonnes of CO2-equivalent per year) (Makkar et al. 2014).

Additionally, if we see the production sectors then we will notice that food production with non-sustainable techniques there is a considerable rise in the waste and by product production.

For this reason, it's important to find new sources to fix the food shortage that is likely to be happen in near future. So, many studies focused on using insect for human feed and for aquaculture.

Insects can be considered highly nutritious food source because they provide protein of high biological value, so they could be an alternative to traditional food of animal origin such as meat, fish, eggs and milk in human nutrition. They are also rich in essential amino-acids like lysine, methionine and leucine but their fatty acid composition is characterized by high amounts of saturated fatty acids.

Recently, EFSA (European Food Safety Authority) proposed a list of insect species with the greatest potential as food and feed ingredients in the EU (Committee 2015) including *Hermetia illucens*. Due to its rapid development, reduced environmental footprint, and preference for organic waste as growth substrate (Truzzi et al. 2020; Osimani et al. 2021).

1.2 *Hermetia illucens* (black soldier fly)

The scientific name of the black soldier fly is *Hermetia illucens* (HI). It is a fly saprophytic insect that belong to the order Diptera of the Stratiomyidae family (Makkar et al. 2014; Truzzi et al. 2020).

It is native from the tropical, subtropical and warm temperate zones of America. The development of international transportation since the 1940s resulted in its naturalization in many regions of the world, like Italy, Spain, Switzerland, Albania, Croatia, Portugal and others. It is now widespread in tropical and warmer temperate

regions (Makkar et al. 2014), because BSF (black soldier fly) can tolerate a broad range of environmental conditions like light, temperature and humidity (Barragan-Fonseca, Dicke, and van Loon 2017).

The BSF is a species of holometabolous insects that store energy reserves for the adult (imaginal) stage as larvae before undergoing metamorphosis, chiefly in the form of protein (40%) and fats (30%) (El-Dakar et al. 2020).

The life cycle of *H. illucens* consists of several developmental stages and includes eggs, larvae, prepupae, pupae and adults (Figure 1).

It prefers temperatures of about 28°C for oviposition (Müller et al. 2017).

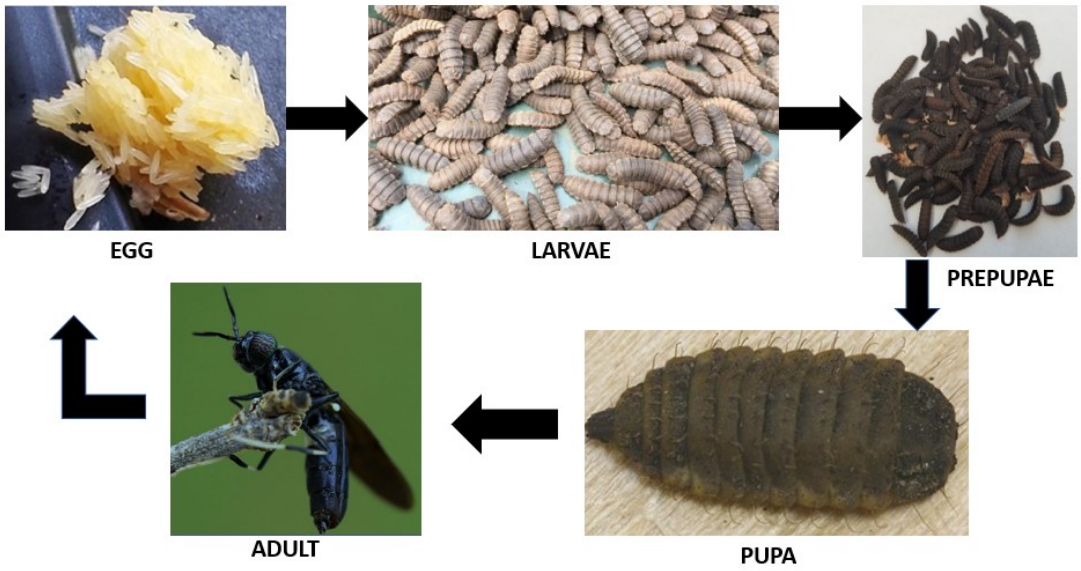

Figure 1-Life cycle of *H. illucens*

Eggs

Eggs have a colour ranging from cream white to straw yellow and are long about 1mm and weight about 0,015 mg. They hatch after about 4 days at 27°C and then they became larvae.

Larvae

Larvae have a vermiform. Before becoming prepupae the larvae pass through 6 stages. During the first stage have a white colour and are very voracious in fact start from a weight of 0.01g and a length <1 mm and arrive to a weight of 0.5g, a length of 2,5-3,5cm.

All this takes place in a time range from 2 weeks to several months, it's depended to the substrate. If the substrate is very nutritive, they reach after two weeks.

The larvae are small harmless insects suitable for mass production and several agricultural and industrial applications. They have the potential to efficiently dispose of a wide variety of organic waste materials by converting it into valuable protein-rich and fat-rich biomass thanks to them being nonselective in terms of their rearing

substrate and their efficient feed conversion ratio (Smets et al. 2020; Milanovic et al. 2021).

Prepupae

Before became pupa there is a stadium called prepupae. In this stadium larvae start to transform in dark brown/black colour and leave the slurry in search of a dry place to pupariate (Müller et al. 2017).

Pupa

After about 2 weeks we have the pupa.

Adult

The adult of *H. illucens* is typically black, how the picture 2 shows and the range in size is from 1.0 to 2.5 cm length.

This fly has a wasp like appearance, because it has two translucent sections on the proximal margins of the abdomen, resulting in a thread-waist appearance (Figure 2).

Figure 2- Wasp appearance of *H. illucens*

After 2 days of emerging the male start to aggregating. From this aggregation start a competition to find the best family and mating. Mated female lay eggs after 2 days and they usually have a single clutch of 400 eggs.

Adults do not have mouthparts, therefore do not eat, sting or bite and are hence not vectors for diseases. They consume only water and do not approach humans or foods and for that reason is not considered a nuisance. (Müller et al. 2017).

1.2.1 HI nutritional value

The larvae of *H. illucens* are among the most promising insects to be used as feed, for poultry and fish and animal manure (Milanovic et al. 2021; Truzzi et al. 2020). Principally due to their good capability of converting a wide variety of organic wastes, such as coffee, vegetables, fruit, fish, urban organic wastes, and animal manure into the organic matter of the larval body, (El-Dakar, Ramzy, and Ji 2021; Truzzi et al. 2020) which is mainly composed of valuable proteins (approximately 55% of body dry weight (BDW)) and lipids (approximately 35% BDW) (Proc et al. 2020; Truzzi et al. 2020; Osimani et al. 2021).

HI nutritional composition are deeply influenced by the rearing substrates, and it has been demonstrated that rearing HI larvae on a substrate based on organic waste containing desirable omega-3 fatty acids could be a suitable way to enrich the final insect biomass (Truzzi et al. 2020).

1.2.1.1 Amino acids

In chemistry amino acids are organic molecules which in their structure have both the functional amino group (-NH2) and the carboxylic group (-COOH) (Figure 3).
The isolated amino acids are presented in the form of zwitterion, because they have at the same time an acid (-COOH) and a basic ($-NH_2$) group maintaining the neutrality.

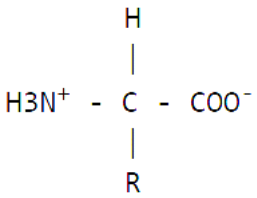

Figure 3- Structure of amino acids

The amino acids differ between them by the chemical nature of group R (side chain) depending on the chemical properties of this group, an amino acid is classified as acid, basic, hydrophilic (or polar) and hydrophobic (or apolar).
Amino acids are the constituent units of proteins and they are defined proteinogenic. In nature we know 20 proteogenic amino acids of which 11 can be synthesized by our body and they are called **non-essential amino acids**, they are: alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, tyrosine, proline, serine and histidine.
The other 9 are called **essential amino acids** because they are not synthesized by our body and must be introduced through nutrition. They are: phenylalanine, isoleucine, histidine, leucine, lysine, methionine, threonine, tryptophan and valine
There are also **semi essential amino acids**, which can be synthesised by the body, provided that other essential amino acids are supplied in excess of requirements. They are methionine and homocysteine (sulphur-containing amino acids), phenylalanine and tyrosine (aromatic amino acids), arginine, ornithine and citrulline.
The percentage of proteins in insect, in our case *H. illucens* is about 55% and it also contains all essential amino acids.

1.2.1.2 Fatty acids

Fatty acids are monocarboxylic acids with a long hydrocarbon chain. They are generally formed by a chain ranging from 4 to 30 carbon atoms, are not branched and not cyclic.

The hydrocarbon part of the fatty acid represents the hydrophobic tail, while the carboxylic group represents the hydrophilic head.

In nature we can find them in free or tied form. When found in their free form (not bound to other chemical species), fatty acids are identified with the name of **FFA (Free Fatty Acids)**, or **NEFA (Non-Esterified Fatty Acids)**. Or we can find them bound to glycerol and they form triglycerides (Figure 4).

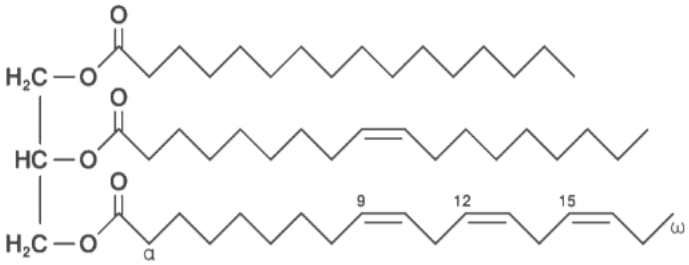

Figure 4- Structure of triglyceride

Fatty acids (FA) can be classified according to the presence or absence of double bonds. If they have only simple bonds (C-C) they are called ***Saturated FA*** such as palmitic acid (C16:0), stearic acid (C18:0), while with the presence of double bonds (C=C) they are called ***Unsaturated FA***, such us oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), arachidonic acid (C20:4).

Depending on how many double bonds are present the FA are called monounsaturated (MUFA), the ones with only one double bond, or polyunsaturated (PUFA) those have more than one double bond.

Based on the position of the first double bond from the last methyl group, FA are classified in **ω3**, **ω6** and **ω9**.

The main ω3 FA are α-linolenic acid (ALA) C18:3, eicosapentaenoic acid (EPA) C20:5, docosahexaenoic acid (DHA) C22:6.

Omega-3 FA represent important constituents of cell membranes and play essential roles in human physiology by reducing blood titres of triglyceride, lowering blood pressure and preventing the formation of arterial plaques. They also reduce the risk of patients developing psychotic disorders, including symptoms of depression, schizophrenia and bipolar disorder. In addition, reduces obesity and prevents the accumulation of fat in the liver. Omega-3 FA have also been shown to be indispensable for brain development in infants. Finally, have anti-inflammatory properties. In contrast, omega-6 FA do also have the ability to reduce blood titres of cholesterol, they are primarily metabolized by the body (El-Dakar et al. 2020).

Essential Fatty Acids (EFA) are two lipids that must introduce through the diet because our body can't synthesize them. Specifically, they are: **Linoleic acid** (**AL**) (figure 5), parent of the omega 6 FA and **α-linolenic acid** (**ALA**) (figure 6), parent of the omega 3 series.

Starting from Linoleic acid (C18:2 ω6) we can obtain arachidonic acid (20:4 ω6).

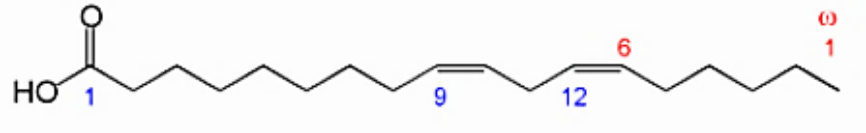

Figure 5- Structure of linoleic acid (18:2) omega-6

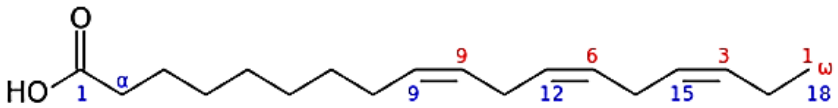

Figure 6- Structure of α-linolenic acid (18:3) omega-3

In general, HI shows high lipid content (up to 500 g/kg), but its fatty acid composition is not always optimal for animal and human nutrition and health, because characterized by low amounts of MUFA and PUFA and high amounts of saturated ones (SFA) (Truzzi et al. 2020).

BSF larvae and prepupae contain about 58-72% of SFA containing high levels of lauric, palmitic and oleic acid and about 19-40% of MUFA and PUFA (Barragan-Fonseca, Dicke, and van Loon 2017), in specially have a low content of DHA (C22:6) and EPA (C20:5) (El-Dakar et al. 2020).

The nutritional composition of HI is deeply influenced by rearing substrates. Studies have found that the accumulation of unsaturated FAs in BSF pre-pupae depends on the levels of these in their diet. Many studies have suggested that it is possible to modify the FA composition of BSF larvae by adjusting that of their rearing substrate.

A considerable number of studies have shown how an array of different types of organic waste can be used to rear BSF larvae, including poultry, cattle, horse, and sheep manure, faecal sludge, kitchen waste, and municipal organic and abattoir waste. Additionally, some studies, assessed changes in the compositions of nutrients and heavy metals in BSF larvae feeding on a marine microalga (El-Dakar et al. 2020).

1.3 Spirulina

Figure 7- Spirulina *Arthospira platensis*

Spirulina microalgae is a filamentous multicellular and is commonly called blue-green algae-cyanobacteria*; Arthospira Platensis* and *Arthospira Maxima* are cultivated worldwide. It is lives in fresh water and nowadays is cultivated worldwide such us China, India, United States, Chile, Italy, France, Cuba and Australia. Microalgae are attracting attention as the future clean energy and industrial material resources such as food, drug, cosmetics, and organic fertilizers because they can be mass-produced in a short time in various environments (J. H. Park, Lee, and Kim 2018), in fact microalgae can double their biomass ranging from 2 to 5 days on average and reach high yields without applying pesticides, herbicides or fungicides (Vaz et al. 2016).

It has bioactive compounds like protein, vitamins, essential PUFA (gamma-linolenic acid), sterols, carotenoids (beta-carotene and zeaxanthin) and chlorophyll pigments with known antioxidants activity (Vaz et al. 2016). All of these compounds make those microalgae very interesting from the health benefits point of view (Andrade et al.2018).

The use of microalgae as food products has been increasing due to concerns regarding health and safety issues, for instance replacing synthetic dyes (synthetic β-carotene has been related to lung cancer and cardiovascular diseases). In this sense, according to American Food and Drug Administration (**FDA**) and **EFSA** food products based on *Spirulina* sp. are classified as **GRAS** (Generally Recognized As Safe). In addition, clinical studies indicated that the spirulina consumption could lead to the reduction of cholesterol, protection against some types of cancers, enhance immune response, increase of intestinal lactobacilli (probiotics), protection against radiation and alternative treatment for obesity (Andrade 2018).

For Spirulina production a specific substrate is used that it is called Zarrouk's medium. This medium offers optimal biomass production, but it incurs higher costs due to the expensive components needed for concocting the medium.
Research is working to increase production efficiency and decrease costs in fact they are studying numerous alterations in the composition of Zarrouk's medium with cheaper chemical ingredients have been explored as a potential substitute for cost-effective microalgae cultivation. Industrial and processing wastes and by-products with appropriate nutrient profiles for growing spirulina are also being considered as alternative culture media (Ragaza et al. 2020).

1.3.1 Amino acids

The protein content of *Spirulina* is 50-70% of dry weight and contains all essential amino acids, approximately 35% of total amino acids specially threonine, methionine, isoleucine and leucine (Vaz et al. 2016).
These amino acids contribute to the high nutritional quality of microalgae, making them highly suitable for use as supplements or as nutraceuticals (Vaz et al. 2016; Andrade 2018).
Another interesting approach in microalgae proteins is related to bioactive peptides, which can exert hormonal effects in the physiological stages of the human body.
Biopeptides are hydrolysed proteins and they are convenient sources of protein for human nutrition because they are absorbed more efficiently through the gastrointestinal tract compared with intact protein or free amino acids (Vaz et al. 2016). In addition have antioxidant, anticoagulant, antihypertensive, immunomodulatory, antimicrobial and cholesterol lowering functions (Andrade 2018).
Spirulina sp. microalgae are also composed of phycobiliproteins, which is a group of proteins related to the photosynthesis system (phycobiliproteins show pigment properties). Phycobiliproteins are well-known due to their hepatoprotective, anti-inflammatory, immunomodulatory, anticancer and antioxidant properties. Moreover, phycobiliproteins can also be applied as labels for antibodies, receptors, and other biological molecules that classify cells activated by fluorescence and are used in immunoblotting experiments and microscopy or fluorescence diagnosis.

1.3.2 Fatty acids

Microalgae consist of approximately 30% lipids making them very interesting as food supplementary by humans. In this sense, microalgae are a source of long-chain polyunsaturated fatty acids (PUFAs) especially of the omega-6 family such as γ-linolenic acid (GLA) and arachidonic acid (AA), and omega-3 family such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Andrade 2018).

According to the World Health Organization, should be taken in portions of 200–500 mg to prevent cardiovascular disease (Molino et al. 2018).

1.3.3 Carotenoids

Carotenoids are molecules made of a long chain of carbon atoms (about 35-40 atoms and called a polynic chain), with inside double bonds and it often ending in a ring.
Carotenoids are the most common pigments in nature with colours ranging from yellow to red through orange. They are not synthesised ex novo by animals (including humans), but they are synthesized by all photosynthetic organisms and fungi, so they should be introduced throw diet.
Carotenoids are considered key molecules for life (Vílchez et al. 2011) because in humans they perform important functions such as antioxidant activity, with the aim of capturing light and above all disactivate free radicals, and they also prevention cardiovascular diseases, anti-cancer and anti-inflammatory properties and are functional for vision.
In nature there are more than 600 known types of carotenoids; they are divided into two groups carotenes and xanthophylls.

- carotenes are made of hydrocarbon chain, therefore without oxygen. Including lycopene and α-carotene and β-carotene. They have a red or orange colour.
- xanthophylls are made of hydrocarbon chain and oxygen and they are more polar than carotenes. They include astaxanthin, lutein, zeaxanthin, cryptoxanthin, violaxanthin and rubixanthin. They have a colour ranging from yellow, orange and red.

From a nutritional point of view, it is also possible to separate them in: provitamin A and not-provitamin A. α-carotene, β-carotene and β-cryptoxanthin are provitamin A carotenoids while lutein, zeaxanthin and lycopene are not-provitamin A. These six carotenoids are the most studied and, even if in the second group there are not provitamins, they always have a great interest because they have a major antioxidant activity.

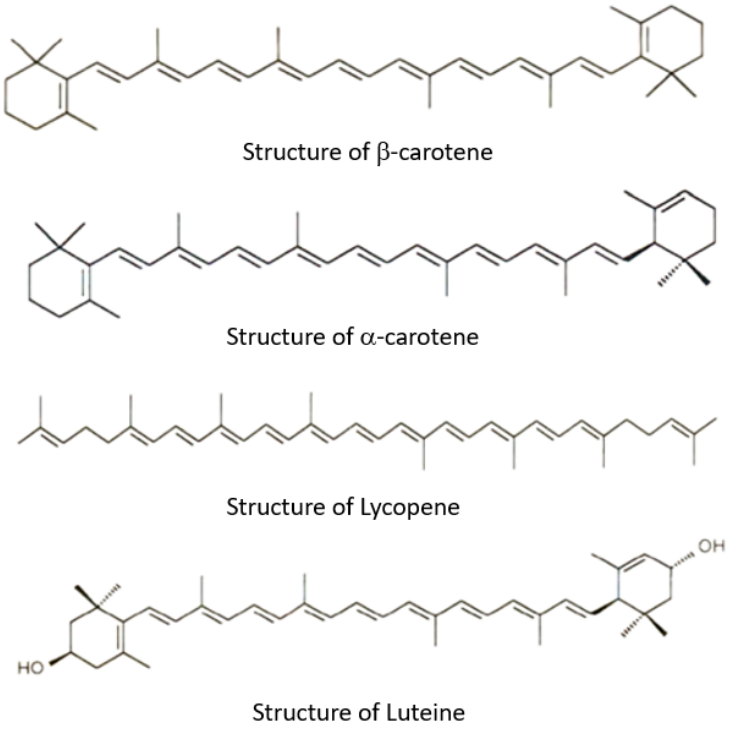

Figure 8- The chemical structure of some carotenoids

The structure of carotenoids includes double bond, more doble bonds are present and more intense is the colour of carotenoid. In fact, lycopene with 11 double bonds is the most coloured (Figure 8).

Carotenoids can be also free or esterified with other molecules like long-chain or medium-chain fatty acid or glycosides, so they change their lipophilicity and hydrophilicity respectively. Other carotenoids can also form complexes with proteins leading to increase stability and change their colours.

Spirulina contains carotenes such us β-carotene and zeaxanthin. In our body both of them have important function and antioxidant activities. β-carotene is the most carotene that have the ability to be convert into retinol (provitamin A function). Vitamin A is well recognized as a factor of great importance for child health and survival, its deficiency causes disturbances in vision and various related lung, trachea and oral cavity pathologies.

Zeaxanthin is one of the main responsible pigments for both the yellowing and the maintenance of normal visual function of the human eye macula.

Macula is a small and most important zone of retina. It is centrally located near the optic disc. At the level of the macula, the image of the object that is fixed is formed. For this reason, it represents the region that is able to provide a clear, distinct and detailed view of objects: it is important for carrying out many daily activities, such as reading, writing, driving, recognizing faces.

In the eye macula zeaxanthin absorb blue light and also attenuate pernicious photooxidative effects caused by the excess blue light, while reducing eye chromatic aberration. Due to their antioxidant properties, carotenoids protect the eye macula from adverse photochemical reactions. In people over the age 64, visual sensibility directly depends on concentration in retina of zeaxanthin (Vílchez et al. 2011).

1.4 Coffee silverskin

Figure 9- Coffee silverskin (CS)

Coffee silverskin (CS) (Figure 9) is a thin layer that is directly in contact with the coffee bean (Figure 10). It detaches from the bean when exposed to roasting high temperatures. Although silverskin represents a minor fraction of the coffee fruit, it is obtained in very high amounts, based on the millions of coffee bags roasted around the world every year (Santos et al. 2021).

During the production of coffee powder, the only part used is the bean, and all other parts, known as by-products, are discarded and can contaminate the environment, especially due to their richness in phytotoxic and/or anti-nutrient compounds (e.g. caffeine, tannins, and polyphenols) that can limit their direct use in soil or feed application (Costa et al. 2018).

For this reason, CS represents a waste, and thus a cost, for coffee companies. Thanks to the concept of circular economy, it is of great interest to value this waste. Concern with environment has grown encouraging industries to formulate more green and natural products focusing on sustainability (Santos et al. 2021).

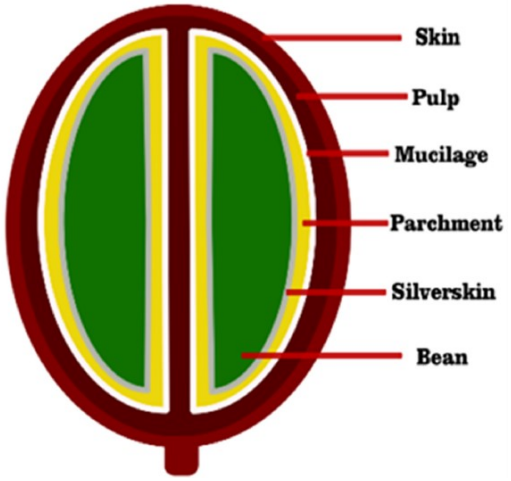

Figure 10- Structure of the coffee bean

1.4.1 Chemical composition of CS

CS is an industrial waste rich in bioactive compounds and characterized by antioxidant and potential prebiotic activities, suggesting it as ingredient for functional food. The major component in CS is dietary fiber (up to 55%), which includes insoluble (about 45%) and soluble (about 10%) fibers. Insoluble fiber including cellulose and hemi-cellulose being this last composed by xylose, arabinose, galactose and mannose (Santos et al. 2021).

The second major component present in CS is protein (about 19%), followed by about 8% of minerals (Santos et al. 2021), 6% of carbohydrates and 2% of fat (Iriondo-Dehond et al. 2019).

The major components of fat are triacylglycerols (48%), followed by free fatty acids (21%), esterified sterols (15%), free sterols (13%), and diacylglycerols (4%). Fatty acid profile changes depending on the origin. Despite this, silverskin presents a high level of saturated fatty acids ranging from 62 to 86% and low amount of polyunsaturated (10-29%) and monounsaturated (5-10%) fatty acid (Santos et al. 2021).

CS is also a source of polyphenols, particularly chlorogenic acid (CGA) (588.9 mg/100 g) of which 5-caffeoylquinic acid (the major one), 4-caffeoylquinic acid, 3-caffeoylquinic acid, 5-feruloylquinic acid, 3-feruloylquinic acid, 4-feruloylquinic acid, caffeoyltryptophan acids, etc (Santos et al. 2021), this compound have antioxidants properties.

In addition, CS contain also about 5% of melanoidins formed from the Maillard reaction during the roasting process, at the end contain small quantities of caffeine, ranging from 0,6 to 1.1% and about 5% (Santos et al. 2021; Iriondo-Dehond et al. 2019).

CS is promising as rearing substrate for insects, but it is incomplete since PUFA and MUFA are present in very low amounts. Moreover, larvae require high fatty diets to complete the development, but CS contains only around 3% of lipids. Some authors had proposed to enrich CS with different levels of microalgae (*Schizochytrium* sp or

Isochrysis sp), know to provide high contents of omega-3 and -6 PUFA and essential amino acids (Zarantoniello et al., 2020, Osimani et al., 2021; Truzzi et al., 2020)

1.5 Aquafeed

Fish is considered the healthiest meat because it contains a high amount of unsaturated fatty acids, and it has particular health benefits to children. Fish meat is also a source of valuable protein and provides a well-balanced amino acid profile for animal and human health that promotes many beneficial effects. For all these reasons, fish consumption has consistently increased, and since 2014, the world population has consumed more farmed fish than captured fish. As a result, the limited availability of fish meal has led to increased efforts to identify fish meal replacements, and these efforts include analyses of all possible physiological or metabolic consequences (Nogales-Mérida et al. 2019).

Different studies have shown that insect meals can partially or completely replace the fish and soya bean meals that are commonly used in aquaculture because insects are a rich source of proteins and their lipid content could be a source of energy and essential fatty acids for potential feed ingredients.

In particular *Hermetia illucens* larvae is considered an important candidate species to be used for animal feeds, thanks for its great potential in the waste valorization to produce, during the bioconversion process, high-value fat and proteins that currently represent a valuable source for fish feed (Giannetto et al. 2020; Belghit et al. 2019).

There are ten amino acids that are essential for fish such as arginine, histidine, isoleucine, leucine, methionine, cysteine, phenylalanine, threonine, tryptophan and valine. All of these ten amino acids are present in HI larvae. Table 1 shows the amino acid composition (%) of *H. illucens* larvae compared to meal of herring and white fish. (red: essential; green: semi-essential, black: non-essential amino acids) (Müller et al. 2017).

Table 1- Amino acids composition (%) of HI larvae compared to meal harring and white fish (Müller et al. 2017)

Amino acid	Black soldier fly [own results]	Herring meal	White fish meal
Alanine	6.2	6.3	6.3
Arginine	6.2	5.8	6.4
Aspartic acid	10.3	9.1	8.5
Cysteine	0.5	1.0	0.9
Glutamic acid	12.2	12.8	12.8
Glycine	5.4	6.0	9.9
Histidine	4.8	2.4	2.0
Isoleucine	4.8	4.5	3.7
Leucine	7.7	7.5	6.5
Lysine	7.4	7.7	6.9
Methionine	0.6	2.9	2.6
Phenylalanine	6.2	3.9	3.3
Proline	6.2	4.2	5.3
Serine	4.1	3.8	4.8
Threonine	4.5	3.9	3.9
Tryptophan	Not analyzed	1.2	0.9
Tyrosine	6.0	3.1	2.6
Valine	6.7	5.4	4.5

Compared with fish meal the content of essential amino acids (in % dry matter) in larval meal is considerably lower. However, the representation of essential amino acids in larval meal are very similar to fish meal. This is of great interest to fish feed producers because an insufficient concentration of one essential amino acid leads to an impaired resorption of the other essential amino acids (Müller et al. 2017).

Carnivorous fishes require a feed with at least 45% protein content while for herbivorous fish 15%–30% protein content suffices. Apart from the protein content the other feed components play an important role.

For the rainbow trout, for example, the optimal protein and fat content should be 35% and 15%–20%, respectively (Müller et al. 2017).

HI contains also fat characterized by low amount of PUFA and MUFA and high amount of SFA. This problem can be bypassed adjusting it by feed modulation (Hoc et al. 2021) especially using microalgae that is a source of ω3 LC-PUFAs.

As an excessive imbalance of essential fatty acids in fish feed formulation can change their fatty acid profile, it is crucial to preserve fish feed ingredients containing these ω3 LC-PUFAs of interest to keep the organoleptic quality and health benefits expected by consumers (Hoc et al. 2021).

Spirulina is of interest in the aquafeed industry as many studies used it as dietary supplement for fishes and crustaceans, even if the production cost limits its use in commercial applications. This microalga is grown using a standard substrate that is

Zarrouk's medium. Although this medium offers optimal biomass production, it incurs higher costs due to the expensive components needed for concocting the medium. Numerous alterations in the composition of Zarrouk's medium with cheaper chemical ingredients have been explored. Industrial and processing wastes and by-products with appropriate nutrient profiles for growing spirulina are also being considered as alternative culture media (Ragaza et al. 2020).

Vitamin A is an essential dietary nutrient for fish involved in physiological functions such as vision, reproduction, embryogenesis, growth and differentiation and maintenance of epithelial cells (Hernandez and Hardy 2020).

In fact, was found that several species of euphausiid and some species of penaeids have large quantities of vitamin A in the eyes (more than 90%), and not distributed in throughout the body as in vertebrates. These findings lead to believe that the main role of vitamin A in crustaceans was the production of pigments for vision.

Vitamin A is also important for reproduction in fact different authors found that fish fed on diet with supplemental vit A presented a higher gonado-somatic index than those fed on a vit A-deficient diet. This happens because during the gonad maturation there is a mobilization of vit A from eyes to gonad (Hector Hernandez et al.2010).

In addition, in 1922 was observed that fish fed a vit A-supplement to the fingerling stage showed higher growth than those maintained without any supplement (Hernandez and Hardy 2020).

2. AIM OF THE THESIS

The aim of the present study was to modulate the nutritional composition of BSF larvae in order to use the larvae as high value ingredient in aquafeed. Cs was used as basal rearing substrate for BSF larvae, and it was enriched with increasing level of 5 and 10% of marine microalga (spirulina, *Arthrospira platensis*) as sources of essential amino acids, PUFA and carotenoids with provitamin activity A.

3. MATERIALS AND METHOS

3.1 Chemicals and reagents

Fatty acids standards (>98 %; tridecanoic and nonadecanoic acid methyl esters), carotenoids standards (>95 % purity; zeaxanthin, lutein, α-carotene, β-carotene), L-amino acids analytical standards (alanine, glycine, valine, leucine, isoleucine, proline, methionine, serine, threonine, phenylalanine, aspartic acid, hydroxyproline, glutamic acid, arginine, asparagine, lysine, glutamine, histidine, tyrosine and DL-norvaline as internal standard, 98.5 % purity), derivatization agent (N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide, MTBSTFA, >99 % purity), solvents HPLC grade, hydrochloric acid (HCl), 37 %, boron trifluoride-methanol solution (BF_3-MeOH, 14% in methanol) were purchased by Merck (Darmstadt, Germany). Anhydrous sodium sulphate was purchased by ITW Company (Darmstadt, Germany). MilliQ water was purified with Millipure System (Millford, SC, USA).

3.2 Diet and insect samples

All samples were gently provided by the Entomology research group by prof. Paola Riolo, as project responsible of INSHORE project.

The main component of the insect feeding substrate consisted of coffee silverskin (Cs). Diets were formulated including two different concentrations of freeze-dried *Arthrospira platensis* (S) (5, 10%) to CS.

Insects were reared in a climatic chamber at 27 ± 1 °C, relative humidity of 65 ± 5% in continuous darkness. Six days old larvae were collected and submitted to analysis.

3.4 Extraction of total lipids and fatty acid profile

Total lipids were isolated as described by Folch, Lees, and Sloane Stanley (1957). Minced freeze-dried insects and diets (2 g) were added of tridecanoic and nonadecanoic acid methyl esters as an internal standards (500 µL of a 10 mg/mL solution in *n*-hexane), of a solution of chloroform:methanol (2:1, v/v, 40 mL), agitated (5 min) and centrifuged (3000 rpm, 10 min, 4 °C). The organic phase was washed with distilled water (5 mL), filtered through Whatman filter paper (Grade 4, 90 mm, Merck KGaA, Darmstadt, Germany) over anhydrous sodium sulphate (3 g) and evaporated with rotary evaporator (30 °C), and the fat yield was calculated.

Fatty acid methyl esters (FAME) were obtained from total lipids through transmethylation by BF_3-MeOH reagent (Medina et al. 1992). Briefly, 20 mg of fat were added of *n*-hexane (0.5 mL), BF_3-MeOH solution (0.5 mL) and vortexed. After 15 min at 100 °C, the reaction was stopped with distilled water (0.5 mL) and the mixture was centrifuged (4500 rpm, 3 min). The organic phase was analyzed by capillary gas chromatography as reported by (Balzano, Pacetti, Lucci, Fiorini, Frega 2017)

3.5 Carotenoids determination

Carotenoids were extracted from freeze-dried samples and analyzed by liquid chromatography as reported by (Nartea et al. 2021). The quantification was performed by external calibration. Good correlation coefficients (R^2) of 0.999 were obtained in all cases. The instrumental limit of detection (LOD) and quantification (LOQ) were as follows: lutein and zeaxanthin, 5 and 16, β-carotene, 5 and 18 ng/mL.

3.6 Amino acids determination

An aliquot (20 mg) of freeze-dried sample was hydrolyzed with 6 M HCl (500 µL) at 110 °C for 24 h under vacuum. The hydrolysate was taken to dryness under nitrogen flow, reconstituted with 0.1 M HCl (5 mL) and centrifuged (4500 rpm, 3 min, 4 °C). Then, 50 µL of the extract was added of DL-norvaline as internal standard (50 µL of a 50 mg/mL solution in 0.1 M HCl), dried, added of dichloromethane (50 µL), dried again and derivatized as reported by (Jiménez-Martín et al. 2012).

All samples were injected (1 µL) in a GC/EI-MS (Thermo Scientific, USA) system, equipped with a split/splitless injector, single quadrupole and a fused silica capillary column MDN-5 (30 m, 0.25 mm, 0.25 µm, Supelco, Bellefonte, PA). The chromatographic conditions were in accordance with (Fico et al. 2018). Identification and quantification were carried as reported by Pérez-Palacios et al. (2015).

For the quantification, standard calibration curves of a mix amino acids (from 80 to 0.1 µg/mL) in 0.1M HCl were prepared in function of concentration level of each amino acid and the ratio of each amino acid peak area/norvaline (internal standard) peak area (Jiménez-Martín et al. 2012). Good correlation coefficients were obtained (R^2= 0.99-0.98). A standard stock solution (5 µg/mL) of norvaline internal standard was prepared in 0.1 M HCl. Then, 50 µL of each dilution was added of 50 µL of norvaline stock solution and dried under nitrogen flow, added of 50 µL dichloromethane, dried and derivatized (Jiménez-Martín et al. 2012).

3.7 Statistical analysis

Statistical analysis was performed using the R statistical programming. Data were compared by one-way analysis of variance (ANOVA).

4. RESULTS AND DISCUSSION

4.1 Fatty acid profile of diets

Table 2 reports the fatty acid composition of coffee silverskin diet (E) with C16:0 (palmitic acid) as the main fatty acid (32.2%), followed by C18:2 Δ9,12ω6 (γ-linolenic acid, 30.8%), C18:1 Δ9cis (oleic acid, 12.3%) and C18:0 (stearic acid, 8.9%). Diet E mainly presents saturated fatty acids (SFA, 53.1%, 168.3 mg/100g dry weight), followed by polyunsaturated fatty acids (PUFA, 30.8%, 97.5 mg/100g dry weight and monounsaturated fatty acids (MUFA, 16.1%, 51.2 mg/100g dry weight). In CS C16:0, C18:0 and C20:0 where the most abundant SFA. *A. platensis* which was used to enrich the coffee silverskin diet, was rich in palmitic (42.2%), linoleic (22.1%) and γ-linolenic acids (13.7%) (Table 3) (Figure 11). The inclusion of *A. platensis* in different percentage (5, 10%) to coffee silverskin heavy affected the fatty acid profile of the diets. A significant increase was found for palmitic and γ-linolenic acids whereas the amount of oleic acid decreased.

Table 2- Fatty acid compositions of *A. platensis* and different diets (E, A, B) expressed as mg fatty acid/100g dry weight and weight % of total fatty acid.

	Diet E			Diet A			Diet B			Diet E			Diet A			Diet B		
Fatty acid	mg/100 g dry weight									%								
C8:0	<LOD		a	14,9	±	0,0 ab	13,7	±	1,0 ab	<LOD			3,4	±	0,0	2,5	±	0,2
C14:0	10,7	±	2,2 b	8,6	±	0,2 ab	5,6	±	0,5 a	3,4	±	0,6	2,0	±	0,0	1,0	±	0,1
C15:0	8,1	±	0,2 a	9,9	±	0,6 a	13,7	±	0,5 b	2,6	±	0,1	2,3	±	0,1	2,5	±	0,1
C16:0	102,1	±	1,2 a	149,1	±	2,9 ab	193,3	±	4,9 b	32,2	±	1,1	33,9	±	0,3	35,9	±	1,2
C18:0	28,0	±	4,8 a	29,3	±	0,5 a	18,8	±	0,4 a	8,9	±	1,7	6,7	±	0,1	3,5	±	0,1
C20:0	19,3	±	2,3 a	22,3	±	0,7 ab	22,2	±	2,5 ab	6,1	±	0,6	5,1	±	0,1	4,1	±	0,4
∑ SFA	**168,3**	**±**	**1,8 a**	**234,2**	**±**	**4,9 ab**	**267,3**	**±**	**3,1 b**	**53,1**	**±**	**1,8**	**53,2**	**±**	**0,6**	**49,6**	**±**	**1,0**
C16:1Δ9	<LOD		a	7,0	±	0,5 b	12,2	±	0,4 c	<LOD			1,6	±	0,1	2,3	±	0,1
C18:1 Δ9cis	38,9	±	2,5 a	45,7	±	0,4 ab	36,4	±	2,7 a	12,3	±	0,5	10,4	±	0,2	6,8	±	0,6
C20:1	12,3	±	5,2 a	19,2	±	0,3 ab	19,1	±	1,4 ab	3,9	±	1,6	4,4	±	0,1	3,5	±	0,2
∑ MUFA	**51,2**	**±**	**7,7 a**	**72,0**	**±**	**0,2 ab**	**67,7**	**±**	**1,6 ab**	**16,1**	**±**	**2,1**	**16,4**	**±**	**0,2**	**12,6**	**±**	**0,4**
C18:2 Δ9,12ω6	97,5	±	1,2 a	127,6	±	0,6 ab	161,5	±	10,6 bc	30,8	±	0,3	29,0	±	0,4	30,0	±	1,7
C18:3 Δ6,9,12ω6	<LOD		a	6,2	±	0,0 a	41,9	±	1,4 b	<LOD			1,4	±	0,0	7,8	±	0,3
∑ PUFAω6	**97,5**	**±**	**1,2 a**	**133,8**	**±**	**0,6 b**	**203,4**	**±**	**9,2 c**	**30,8**	**±**	**0,3**	**30,4**	**±**	**0,4**	**37,8**	**±**	**1,4**

Results represents means value ± standard deviation (n = 2). SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; Cm:n Dx; m = number of carbon atoms, n = number of double bonds, x = position of double bonds. Different letters in the same row means statistical difference (ANOVA, $p<0.05$).

Table 3- Fatty acid compositions of *A. platensis* expressed ad mg fatty acid/100g dry weight and weight % of total fatty acid.

	A. platensis				
Fatty acid	mg/100g dry weight			%	
C8:0	417.5	±	16.1	12.1	± 0.1
C10:0	21.7	±	2.4	0.6	± 0.1
C16:0	1461.2	±	112.9	42.2	± 2.1
C18:0	36.3	±	3.6	1.1	± 0.1
∑ SFA	**1936.8**	**±**	**123.0**	**56.0**	**± 2.0**
C16:1Δ9	148.7	±	7.2	4.3	± 0.1
C18:1 Δ9cis	136.6	±	9.0	3.9	± 0.1
∑ MUFA	**285.3**	**±**	**16.3**	**8.2**	**± 0.2**
C18:2 Δ9,12ω6	763.4	±	36.4	22.1	± 1.7
C18:3 Δ6,9,12ω6	473.8	±	4.5	13.7	± 0.5
∑ PUFA	**1237.2**	**±**	**40.9**	**35.8**	**± 2.2**
∑ PUFAω3	**0.0**	**±**	**0.0**	**0.0**	**± 0.0**
∑ PUFAω6	**1237.2**	**±**	**40.9**	**35.8**	**± 2.2**

Results represents means value ± standard deviation (n = 2). SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; Cm:n Dx; m = number of carbon atoms, n = number of double bonds, x = position of double bonds

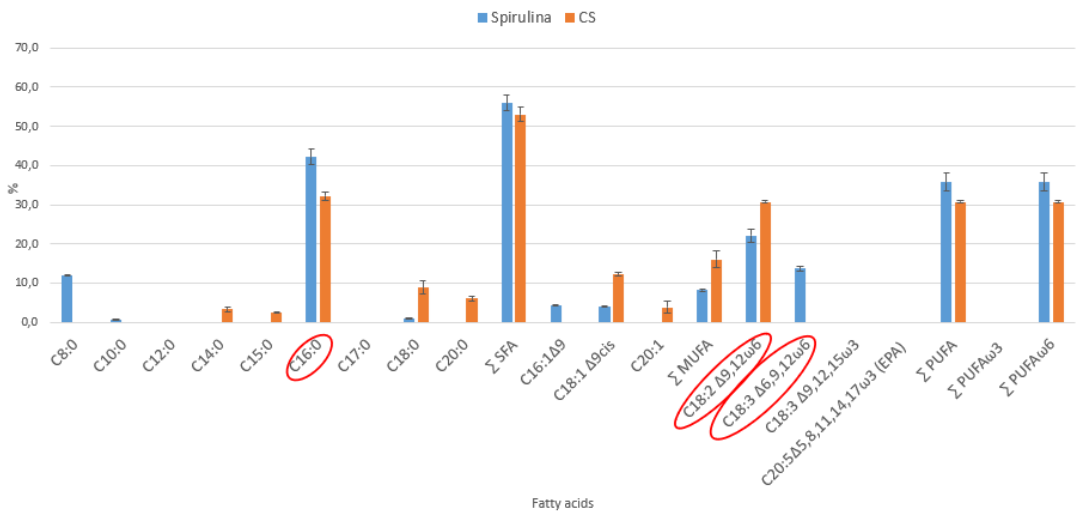

Figure 11- Fatty acids composition (% of total fatty acids) of spirulina and CS. Results represents means value ± standard deviation (n = 2).

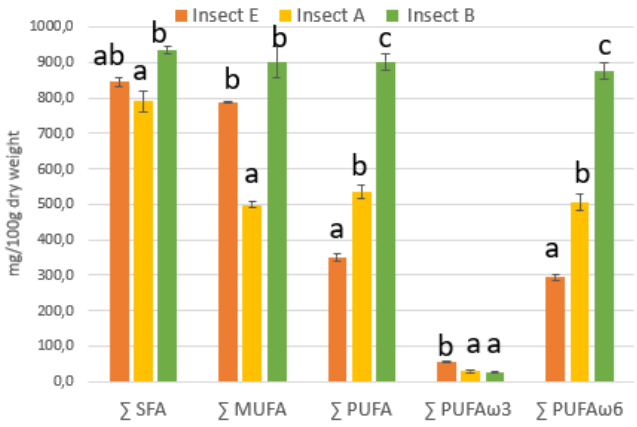

Figure 12- Fatty acids composition of insects, SFA; MUFA and PUFA. Results represents means value ± standard deviation (n = 2). Different letters mean statistical difference (ANOVA, p<0.05), calculated for compound.

4.2 Fatty acid profile of insects

The fatty acid profile of HI larvae reared on coffee silverskin substrate without *A. platensis* was characterized by the dominance of SFA (C12:0 > C16:0 > C18:0 > C14:0), reflecting the profile of coffee silverskin diet (Table 4). C18:3 Δ6,9,12ω6 (γ-linolenic acid), C18:3 Δ9,12,15ω3 (α-linolenic acid) and C20:5Δ5,8,11,14,17ω3 (EPA, eicosapentaenoic acid) were found exclusively in HI, not being present in the coffee diet (E). Thus, fatty acid composition of HI E reflects in part the composition of the correspondent rearing substrate. The enrichment of coffee silverskin substrate with increasing % of *A. platensis* (5, 10%) positively affected the fatty acid profile of HI larvae. In general, an increasing trend was found for fatty acids amounts in function of % of microalgae inclusion in the diet of larvae. 10% of microalgae addition to coffee silverskin gave the best results by generating HI with the highest contents of SFA, MUFA and PUFA, 934, 900,7 and 902,6 mg/100g dry weight, respectively (Figure 12). EPA fatty acid was highly present in larvae rearing with the nutritionally poorest substrate (diet E). The highest content of lauric acid (C12:0) was found in Insect B and it is considered a positive indicator for the goodness of the growing substrate, in terms of carbohydrates (Spranghers et al. 2017).

Our results confirmed that the inclusion of microalgae in a pour substrate of lipids and proteins such as Cs (Vargas et al. 2018; Costa et al. 2018) enhanced the lipid accumulation in the insect body. In particular, lauric acid increment were in compliance with (Truzzi et al. 2020), obtained for HI grown on Cs enriched with *Schizochytrium sp* or *Isochrysis sp* microalgae (5-25%). The best results in our experiment were recorded at 10% level of spirulina inclusion to CS. Several studies have demonstrated the great influence of lipid composition of the substrate on the lipid profile and content of insects (Tomberlin et al. 2002; Nguyen et al., 2013;

Spranghers et al. 2017; Truzzi et al. 2020). We demonstrated that HI is a good bioconvertor of essential fatty acids, such as γ-linolenic, from microalgae and organic waste (Cs). Spirulina is of interest in the aquafeed industry as many studies used it as dietary supplement for fishes and crustaceans, even if the production cost limits its use in commercial applications (Ragaza et al. 2020). For reference, *Schizochytrium sp,* added to Cs to reduce the environmental impact of aquafeed production, is more convenient (Truzzi et al. 2020), but spirulina nutritional value could be a valuable solution once its cost production will be lowered. Ragaza et al. (2020) concluded that a small inclusion levels (1–10%) of spirulina increased the nutritional value of aquaculture diets (not insect-based) improved growth and health performance of fish.

Table 4- Fatty acid compositions of different insects (E, A, B) expressed as mg fatty acid/100g dry weight and weight % of total fatty acid.

	Insect E			Insect A			Insect B			Insect E			Insect A			Insect B		
Fatty acid	mg/100g dry weight									%								
C10:0	10,0	±	0,4 a	12,0	±	0,6 a	24,1	±	2,7 b	0,5	±	0,0	0,7	±	0,1	0,9	±	0,1
C12:0	351,2	±	8,0 a	366,2	±	19,0 a	584,9	±	24,5 b	17,7	±	0,6	20,1	±	0,4	21,4	±	1,0
C14:0	74,9	±	1,6 ab	66,8	±	6,5 ab	61,2	±	17,0 a	3,8	±	0,0	3,7	±	0,2	2,2	±	0,6
C15:0	33,5	±	0,9 b	25,3	±	2,0 ab	19,5	±	1,5 a	1,7	±	0,0	1,4	±	0,1	0,7	±	0,1
C16:0	250,4	±	10,7 bc	228,1	±	3,7 b	172,8	±	8,7 a	12,6	±	0,4	12,5	±	0,2	6,3	±	0,3
C17:0	18,7	±	2,5 a	27,4	±	0,7 b	26,5	±	2,1 b	0,9	±	0,1	1,5	±	0,1	1,0	±	0,1
C18:0	82,5	±	5,5 c	54,1	±	0,6 b	33,7	±	1,0 a	4,2	±	0,2	3,0	±	0,1	1,2	±	0,0
C20:0	25,2	±	0,0 c	11,3	±	0,2 a	11,4	±	0,4 a	1,3	±	0,0	0,6	±	0,0	0,4	±	0,0
∑ SFA	**846,5**	**±**	**12,8 ab**	**791,3**	**±**	**29,0 a**	**934,0**	**±**	**10,6 b**	**42,7**	**±**	**0,2**	**43,4**	**±**	**0,2**	**34,1**	**±**	**0,5**
C16:1Δ9	253,6	±	6,2 a	293,1	±	8,6 a	386,8	±	25,0 b	12,8	±	0,5	16,1	±	1,0	14,1	±	0,9
C18:1 Δ9cis	533,4	±	4,4 b	205,5	±	17,9 a	513,8	±	19,2 b	26,9	±	0,1	11,3	±	0,6	18,8	±	0,6
∑ MUFA	**787,0**	**±**	**1,8 b**	**498,6**	**±**	**9,3 a**	**900,7**	**±**	**44,2 b**	**39,7**	**±**	**0,5**	**27,3**	**±**	**0,3**	**32,9**	**±**	**1,5**
C18:2 Δ9,12ω6	289,2	±	8,3 a	421,8	±	13,7 a	668,2	±	5,7 b	14,6	±	0,3	23,1	±	0,0	24,4	±	0,3
C18:3 Δ6,9,12ω6	5,8	±	0,3 a	83,3	±	8,9 b	208,0	±	17,1 c	0,3	±	0,0	4,6	±	0,3	7,6	±	0,7
C18:3 Δ9,12,15ω3	29,5	±	0,7 b	17,2	±	1,4 a	20,2	±	1,7 a	1,5	±	0,1	0,9	±	0,1	0,7	±	0,1
C20:5Δ5,8,11,14,17ω3 (EPA)	26,6	±	3,2 c	12,7	±	2,4 b	6,2	±	1,3 ab	1,3	±	0,1	0,7	±	0,2	0,2	±	0,0
∑ PUFA	**351,0**	**±**	**10,5 a**	**535,1**	**±**	**18,9 b**	**902,6**	**±**	**22,4 c**	**17,7**	**±**	**0,3**	**29,3**	**±**	**0,1**	**33,0**	**±**	**1,0**
∑ PUFAω3	**56,1**	**±**	**2,5 b**	**29,9**	**±**	**3,7 a**	**26,4**	**±**	**0,4 a**	**2,8**	**±**	**0,1**	**1,6**	**±**	**0,3**	**1,0**	**±**	**0,0**
∑ PUFAω6	**294,9**	**±**	**8,0 a**	**505,1**	**±**	**22,6 b**	**876,2**	**±**	**22,8 c**	**14,9**	**±**	**0,2**	**27,7**	**±**	**0,4**	**32,0**	**±**	**1,0**

Results represents means value ± standard deviation (n = 2). SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; Cm:n Dx; m = number of carbon atoms, n = number of double bonds, x = position of double bonds. Different letters in the same row means statistical difference (ANOVA, $p<0.05$).

4.3 Carotenoids

To design a balanced aquafeed, not only protein and lipid fraction should be considered, but also vitamins such as vitamin A. Vitamin A is an essential dietary nutrient for fish involved in physiological functions such as vision, reproduction, embryogenesis, growth and differentiation and maintenance of epithelial cells (Hernandez and Hardy 2020).

A. platensis microalgae is rich in carotenoids such as zeaxanthin (non-provitamin A) and β-carotene (provitamin A) as reported in Table 5, while coffee silverskin substrate shown only low amount of lutein. The enrichment of HI substrate with microalgae biomass produced in all samples a significant increase of zeaxanthin and β-carotene, reaching the highest (16,04 and 57,78 mg/kg dried weight, respectively) bioconversion in sample HI B reared on 10% of *A. platensis* + 90% coffee silverskin.

Table 5- Carotenoids composition of Spirulina, diets (E, A, B) and insects (E, A, B).

mg/kg dried weight	**zeaxanthin**		**lutein**		**β-carotene**	
Spirulina	147,20 ± 0,08		<LOD		523,33 ± 8,54	
Diets						
E	<LOD		9,21 ± 0,00	c	<LOD	
A	37,63 ± 0,17	a	8,94 ± 0,02	b	126,76 ± 2,06	a
B	69,43 ± 0,52	b	9,11 ± 0,07	c	228,51 ± 1,57	b
Insects						
E	1,20 ± 0,01	a	2,11 ± 0,01	a	<LOD	
A	5,46 ± 0,04	b	2,07 ± 0,02	a	14,48 ± 0,67	a
B	16,04 ± 0,03	c	3,04 ± 0,03	b	57,78 ± 0,39	b

Results represents means value ± standard deviation (n = 3). Different letters in the same row means statistical difference (ANOVA, $p<0.05$), calculated for diet and for insect, respectively.

Our results (Figure 13) demonstrated that HI are good bioconvertors of carotenoids with provitamin A activity (β-carotene) and non (zeaxanthin). Spirulina carotenoids profile was found in agreement with literature, as β-carotene and zeaxanthin were found the major carotenoids (Park et al. 2018). The bioaccumulation reached a maximum of 25% of β-carotene and of 23% of zeaxanthin, when rearing HI on the diet composed by 10% of spirulina. Borel et al. (2021) found that black soldier fly larvae can bioaccumulate significant amounts of provitamin A, recycling them from fruits and vegetable waste.

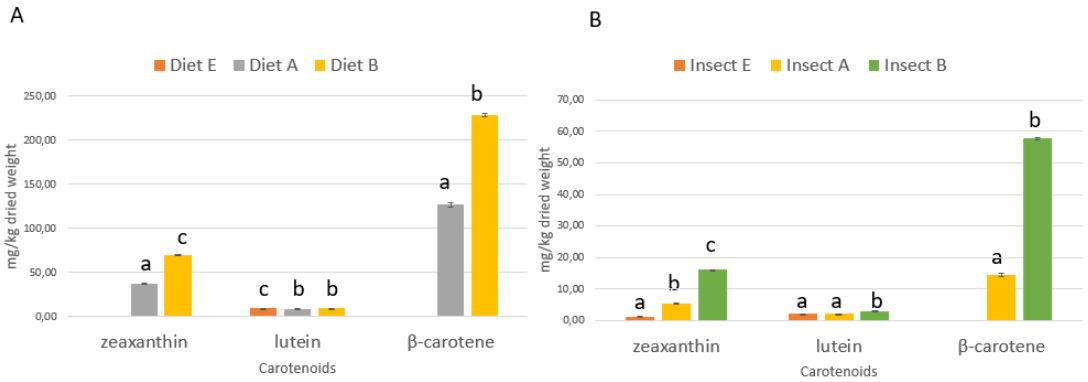

Figure 13- Carotenoids composition of diet (A) and insect (B). Results represents means value ± standard deviation (n = 3). Different letters mean statistical difference (ANOVA, p<0.05), calculated for diet and for insect, respectively.

4.4 Amino acid profile

Along fish dietary requirements, the essential amino acids are as follows: threonine, valine, leucine, isoleucine, methionine, tryptophan, lysine, histidine, arginine, phenylalanine. The non-essential amino acids cystine and tyrosine can be synthetized from methionine and phenylalanine, respectively, thus the dietary requirement for those non-essential amino acids is dependent on the levels of the respective non-essential amino acids within the diet (FAO 1987).

The rearing coffee silverskin substrate showed an amino acid profile different to *A. platensis* microalgae biomass (Figure 14). The ranking based on % profile was aspartic acid > leucine > glutamic acid > valine > lysine for *A. platensis* and hydroxyproline > arginine > proline > valine > leucine for diet E. The inclusion of microalgae biomass to coffee sikverskin substrate generated diets with higher amounts of amino acids with respect to diet E, not in all cases statistically significant, but with a linear increasing trend. The sum of essential amino acids for fish feeding went up from 53.7±1.2 (diet E) to 118.7±10.7 g/kg dried weight (diet B with 10% of microalgae addition). Diet B showed the highest content of essential amino acids such as leucine, valine, phenylalanine, threonine, lysine, histidine, tyrosine and isoleucine (Table 6).

Figure 15 reports the amino acid composition of HI larvae, which in general did not varied among insects reared on substrates with increasing levels of microalgae. The inclusion of microalgae biomass to substrate of coffee silverskin did not affect the total amino acid content. Anyway, significant variations were recorded for some essential amino acids such as lysine, histidine and tyrosine, as HI B reared on diet B reported higher values than HI E, corresponding to diet E without *A. platensis*. The sum of non-essential amino acid value showed a significant difference between HI E and HI B (80.3±8.9 vs 104.1±8.8 g/kg dried weight), reflecting the difference in terms of alanine, proline, serine, aspartic and glutamic acid. The essential amino acids, histidine and tyrosine accounted for 13,8±1,4 (HI B) and 11,6±0,6 (HI E) g/kg dried

weight, while the essential amino acids valine and lysine accounted for 35,0±3,5 (HI B) and 31,1±2,7 (HI E) g/kg dried weight.

In agreement with our results, Müller, Wolf, and Gutzeit (2017) have found no evidence that the amino acid composition can be altered significantly by different feeding regimes. Our outcomes showed that the dominant amino acid in HI E was proline (32,5±7,7 g/kg dried weight) while for HI A and HI B samples the dominant ones was valine accounted for 32,1±3,8 and 35,0±3,5 g/kg dried weight, respectively. Differently, Liland et al. (2017) demonstrated that the most abundant amino acids in larvae reared on seaweed were aspartic acid (non-essential) and glutamic acid (essential amino acids). The predominance of glutamic acid was also found by Müller, Wolf, and Gutzeit (2017) and by Giannetto et al. (2020).

The choice of substrate (organic waste) significantly affected the concentration of limiting and non-limiting amino acids in HI, but in general and for the most limiting amino acids like lysine, methionine, isoleucine and tyrosine no significant substrate effect was found (Shumo et al. 2019). In conclusion, the amino acid profile of HI was not affected by the inclusion of different percentage of spirulina (5, 10%) in the HI substrate, but in general the concentrations of total essential and non-essential amino acids increased (not significantly) at the increasing level of spirulina in HI diet.

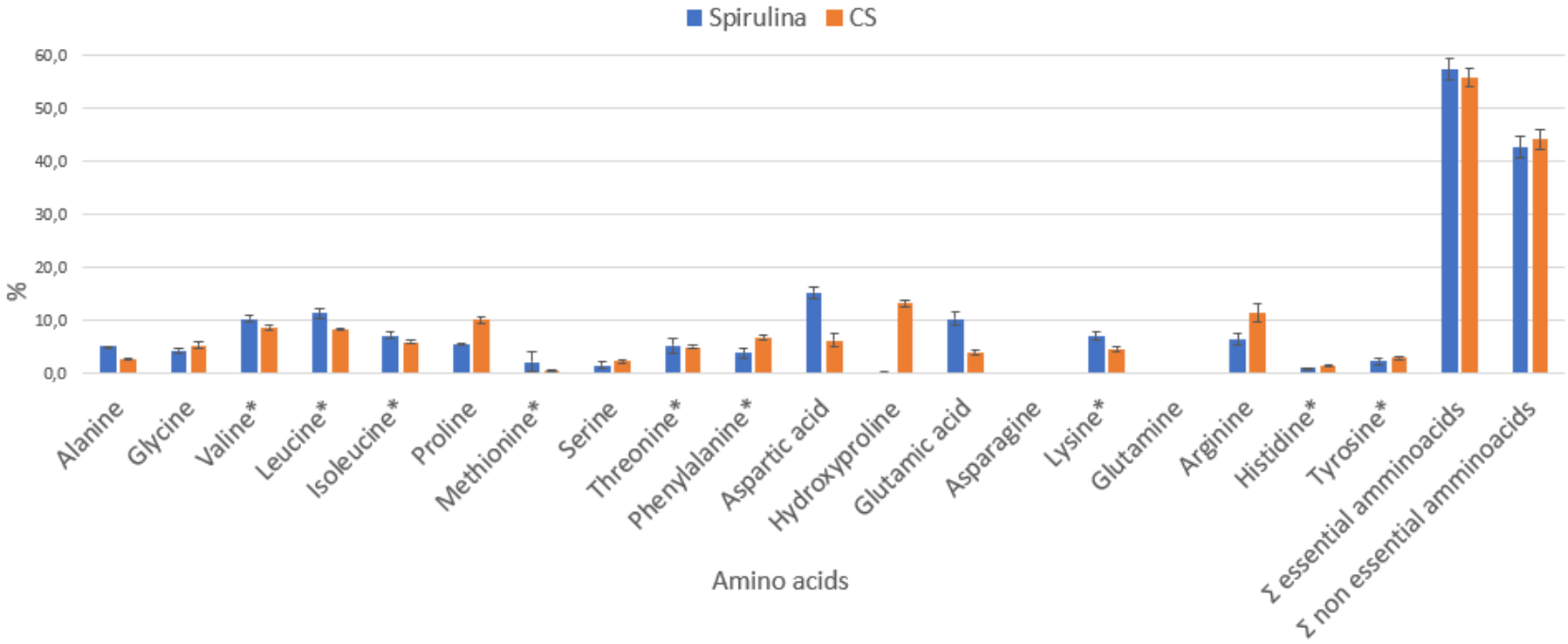

Figure 14- Amino acids composition (%) of Spirulina and CS. Results represents means value ± standard deviation (n = 2).

Table 6- Amino acids profile of different diets (E, A, B) for HI larvae rearing, at different % of *A. platensis*. Data are reported as mean of two replicates ± standard deviation (SD), expressed as g/kg dried weight and as % of the weight of total amino acids. LOD, limit of detection.

	A. platensis			Diet E				Diet A				Diet B			
g/kg dried															
Alanine	82.6	±	10.2	2.6	±	0.5	a	4.2	±	0.4	ab	7.6	±	0.5	bc
Glycine	63.5	±	4.9	5.2	±	1.4	a	7.9	±	0.7	ab	13.8	±	1.0	ac
Valine*	172.8	±	15.2	8.4	±	0.0	a	10.7	±	1.6	ab	17.7	±	0.3	bc
Leucine*	189.9	±	20.1	8.0	±	0.5	a	12.6	±	0.1	ab	19.5	±	1.0	bc
Isoleucine*	107.0	±	2.3	5.8	±	0.1	a	7.5	±	1.0	ab	11.8	±	0.1	ac
Proline	88.0	±	10.3	9.7	±	0.1	a	14.9	±	0.4	ab	22.7	±	5.6	ac
Methionine*	14.3	±	0.7	0.5	±	0.3	a	0.1	±	0.1	a	1.9	±	0.4	a
Serine	31.7	±	1.6	2.3	±	0.6	a	2.7	±	0.0	a	4.2	±	0.4	a
Threonine*	68.7	±	4.3	4.9	±	0.7	a	3.4	±	0.5	a	4.7	±	0.5	a
Phenylalanine*	51.8	±	5.3	6.5	±	1.2	a	8.7	±	0.7	ab	10.0	±	0.3	ac
Aspartic acid	254.1	±	22.0	6.1	±	2.1	a	9.8	±	0.7	ab	13.6	±	1.4	b
Hydroxyproline	1.2	±	0.1	12.8	±	0.2	a	7.7	±	1.6	a	10.7	±	0.9	a
Glutamic acid	178.8	±	31.2	3.8	±	0.9	a	6.9	±	0.8	ab	10.2	±	1.0	bc
Asparagine	<LOD			<LOD				<LOD				<LOD			
Lysine*	105.2	±	6.5	4.4	±	0.2	a	6.7	±	1.1	ab	10.3	±	1.5	ac
Glutamine	0.2	±	0.0	<LOD				<LOD				<LOD			
Arginine*	115.9	±	9.3	10.9	±	1.5	a	29.7	±	6.6	a	35.2	±	9.9	a
Histidine*	17.4	±	0.7	1.4	±	0.1	a	1.7	±	0.3	a	2.8	±	0.8	ab
Tyrosine*	45.5	±	1.3	2.9	±	0.6	a	4.3	±	0.4	ab	4.7	±	0.3	ac
Σ essential aminoacids	**892.9**	**±**	**51.8**	**53.7**	**±**	**1.2**	**a**	**85.4**	**±**	**11.0**	**ab**	**118.7**	**±**	**10.7**	**bc**

Σ non essential aminoacids	**663.7**	**±**	**16.9**	**42.7**	**±**	**4.1**	**a**	**54.1**	**±**	**1.4**	**a**	**82.7**	**±**	**4.9**	**ab**
Total	1589.6	±	81.6	96.4	±	7.6	a	139.5		12.3	b	201.4		5.8	c
%															
Alanine	5.1	±	0.1	2.7	±	0.2	a	3.0	±	0.0	ab	3.7	±	0.1	b
Glycine	4.3	±	0.5	5.4	±	0.7		5.6	±	0.0		6.8	±	0.7	
Valine*	10.4	±	0.7	8.7	±	0.5		7.6	±	0.5		8.8	±	0.1	
Leucine*	11.4	±	0.8	8.3	±	0.1	ab	9.0	±	0.9	b	9.7	±	0.8	b
Isoleucine*	7.2	±	0.6	6.0	±	0.3		5.4	±	0.2		5.8	±	0.1	
Proline	5.6	±	0.1	10.1	±	0.6		10.7	±	0.6		11.3	±	3.1	
Methionine*	2.2	±	1.8	0.5	±	0.2		0.0	±	0.1		1.0	±	0.2	
Serine	1.6	±	0.5	2.4	±	0.3		1.9	±	0.2		2.1	±	0.3	
Threonine*	5.3	±	1.4	5.1	±	0.3		2.5	±	0.6		2.4	±	0.3	
Phenylalanine*	3.8	±	0.8	6.7	±	0.5		6.2	±	0.1		5.0	±	0.3	
Aspartic acid	15.3	±	1.0	6.3	±	1.2		7.0	±	0.1		6.8	±	0.5	
Hydroxyproline	0.2	±	0.2	13.3	±	0.6	b	5.6	±	1.7	a	5.3	±	0.6	a
Glutamic acid	10.4	±	1.2	4.0	±	0.5	a	5.0	±	0.1	a	5.0	±	0.4	a
Asparagine	<LOD			<LOD				<LOD				<LOD			
Lysine*	7.1	±	0.7	4.6	±	0.4		4.8	±	0.4		5.1	±	0.6	
Glutamine	<LOD			<LOD				<LOD				<LOD			
Arginine	6.5	±	1.1	11.4	±	1.7		21.2	±	2.9		17.4	±	4.4	
Histidine*	1.1	±	0.0	1.5	±	0.1		1.2	±	0.1		1.4	±	0.4	
Tyrosine*	2.4	±	0.7	3.0	±	0.3		3.1	±	0.0		2.3	±	0.1	
Σ essential amminoacids	**57.3**	**±**	**2.0**	**55.8**	**±**	**1.8**		**61.1**	**±**	**2.5**		**58.9**	**±**	**3.6**	
Σ non essential amminoacids	**42.7**	**±**	**2.0**	**44.2**	**±**	**1.8**		**38.9**	**±**	**2.5**		**41.1**	**±**	**3.6**	

*essential amino acids for fish. Different letters in the same row (excluding *A. platensis*) mean statistical difference (one-way ANOVA, p>0.05).

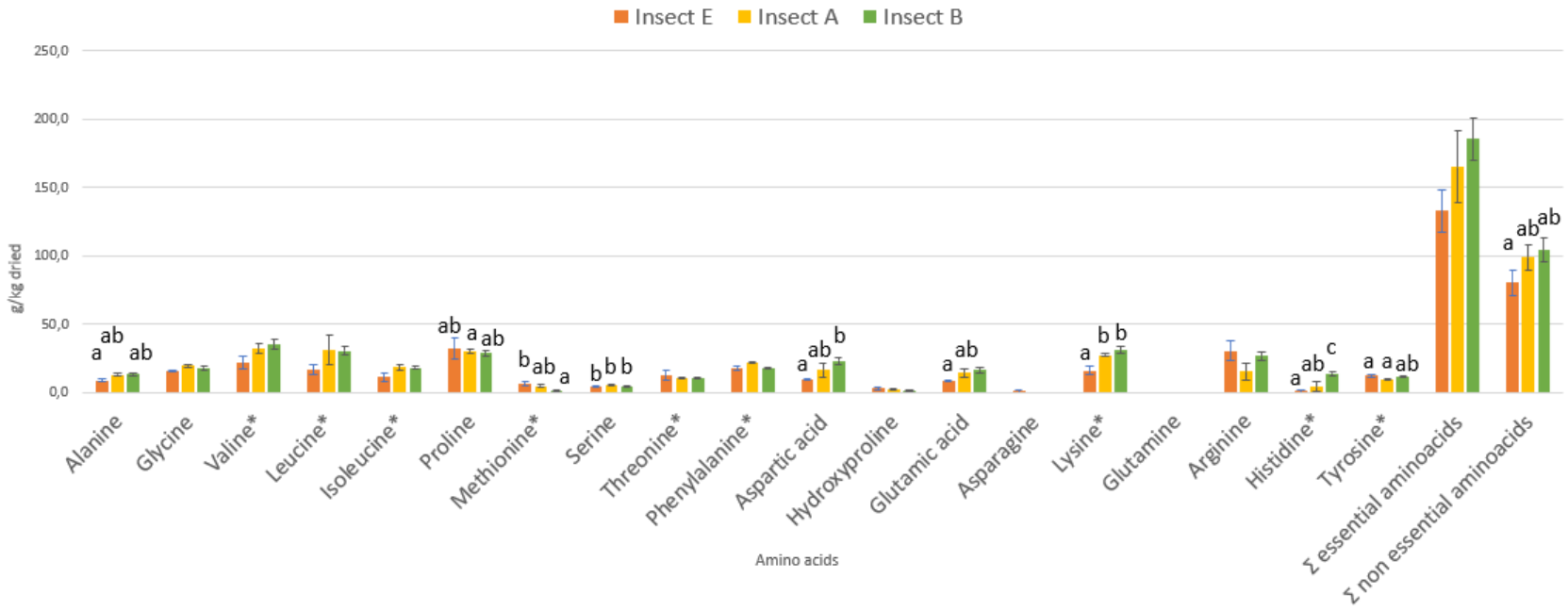

Figure 15- Amino acids composition of HI larvae reared whit different percentage of Spirulina. Data are reported as mean of two replicates ± standard deviation (SD), expressed as g/kg dried weight. Different letters mean statistical difference (one-way ANOVA, p>0.05), calculated per amino acid.

5. CONCLUSIONS

The fatty acid profile of HI larvae reared on coffee silverskin substrate without *A. platensis* was characterized by the dominance of SFA. The inclusion of 10% of microalga *A. platensis* to the substrate allowed to enrich the larvae in PUFA and MUFA.

Furthermore, the enrichment of HI substrate with *A. platensis* produced in all samples a significant increase of zeaxanthin and β-carotene, in particular the sample HI B, with 10% of spirulina, the highest bioconversion was reached.

In addition thanks to the use of this microalgae, sample HI B reported higher values for some essential amino acids such as lysine, histidine and tyrosine than HI E.

The BSF larvae used in this study can be successfully grown on diets composed of coffee by-products enriched with microalgae, in addition HI is good bioconvertor of essential γ-linolenic acid from microalgae and organic waste (CS).

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