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Title/Author	Effect of rearing substrate on growth performance, waste reduction efficiency and chemical composition of black soldier fly (Hermetia illucens) larvae / Meneguz, M., Schiavone, A., Gai, F., Dama, A., Lussiana, C., Renna, M., & Gasco, L.
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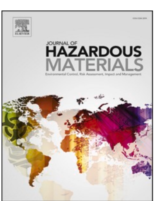

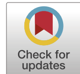

A review on recent disposal of hazardous sewage sludge via anaerobic digestion and novel composting

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ABSTRACT

The high investment cost required by modern treatment technologies of hazardous sewage sludge such as incineration and anaerobic digestion have discouraged their application by many developing countries. Hence, this review elucidates the status, performances and limitations of two low-cost methods for biological treatment of hazardous sewage sludge, employing vermicomposting and black soldier fly larvae (BSFL). Their performances in terms of carbon recovery, nitrogen recovery, mass reduction, pathogen destruction and heavy metal stabilization were assessed alongside with the mature anaerobic digestion method. It was revealed that vermicomposting and BSFL were on par with anaerobic digestion for carbon recovery, nitrogen recovery and mass reduction. Thermophilic anaerobic digestion was found superior in pathogen destruction because of its high operational temperature. Anaerobic digestion also had proven its ability to stabilize heavy metals, but no conclusive finding could confirm similar application from vermicomposting or BSFL treatment. However, the addition of co-substrates or biochar during vermicomposting or BSFL treatment may show synergistic effects in stabilizing heavy metals as demonstrated by anaerobic digestion. Moreover, vermicomposting and BSFL valorization had manifested their potentialities as the low-cost alternatives for treating hazardous sewage sludge, whilst producing value-added feedstock for biochemical industries.

1. Introduction

In general, conventional wastewater treatment plant produces two types of sewage sludge – primary and secondary sludge. Primary sludge is the solid accumulated from the physical separation process and consists of gravitational precipitates. Activated sludge, also known as secondary sludge, is the byproduct generated from the biological treatment

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plant, containing high amount of active microbes (Liew et al., 2021). In raw form, both of them are highly inhabited by pathogens and bacteria such as *E. coli*, *Salmonella* spp. and *Enterococcus* (Al-Gheethi et al., 2018).

Nomenclature

BSF	Black soldier fly
BSFL	Black soldier fly larvae
BOD	Biochemical oxygen demand
COD	Chemical oxygen demand
EPS	Extracellular polymeric substances
FAME	Fatty acid methyl ester
MEC	Microbial electrolysis cell
MPN	Most probable number
sCOD	Soluble chemical oxygen demand
SRT	Sludge residence time
TOC	Total organic carbon
TS	Total solids
VFA	Volatile fatty acids
VS	Volatile solid

The raw sludge is also loaded with high organic matter and heavy metals as shown in Table 1. The direct application of sewage sludge into soil could potentially cause leaching of organic matter and heavy metals into the soil and eventually seep into the groundwater. If it is left untreated, the sludge will undergo anaerobic digestion naturally and generate an unpleasant smell due to the release of volatile organic and inorganic compounds such as NH_3 and H_2S, being the most dominant odor gases (Zhu et al., 2016).

Table 1
Characteristics of raw sewage sludge.

Parameter	Value	Reference
pH	7.2 ± 0.7	(Metcalf et al., 2013; Gupta and Garg, 2008; Ruiz-Espinoza et al., 2012; Awasthi et al., 2020a; Koupaie et al., 2018; Babel et al., 2009; Khwairakpam and Bhargava, 2009)
Total dry solids (% of TS)	4.4 ± 3.2	(Metcalf et al., 2013; Gupta and Garg, 2008; Ruiz-Espinoza et al., 2012; Koupaie et al., 2018; Babel et al., 2009)
Volatile solids (% of TS)	58.4 ± 19	(Metcalf et al., 2013; Gupta and Garg, 2008; Ruiz-Espinoza et al., 2012; Koupaie et al., 2018; Babel et al., 2009)
TOC (% of TS)	31.7 ± 10.2	(Awasthi et al., 2020a; Babel et al., 2009; Khwairakpam and Bhargava, 2009; Hait and Tare, 2011)
Total Nitrogen (% of TS)	1.93 ± 0.81	(Metcalf et al., 2013; Awasthi et al., 2020a; Babel et al., 2009; Khwairakpam and Bhargava, 2009; Hait and Tare, 2011)
Total Phosphorus (% of TS)	1.66 ± 1.57	(Metcalf et al., 2013; Gupta and Garg, 2008; Babel et al., 2009; Khwairakpam and Bhargava, 2009)
Lead (mg/kg)	102 ± 138	(Babel et al., 2009; Tomasi Morgano et al., 2018; Liu et al., 2018; Seggiani et al., 2012; Abu Bakar et al., 2008)
Zinc (mg/kg)	1400 ± 986	(Babel et al., 2009; Tomasi Morgano et al., 2018; Liu et al., 2018; Seggiani et al., 2012; Abu Bakar et al., 2008)
Chromium (mg/kg)	225 ± 118	(Babel et al., 2009; Tomasi Morgano et al., 2018; Liu et al., 2018; Seggiani et al., 2012)
Copper (mg/kg)	299 ± 328	(Tomasi Morgano et al., 2018; Liu et al., 2018; Seggiani et al., 2012; Abu Bakar et al., 2008)
Nickel (mg/kg)	94 ± 75	(Babel et al., 2009; Tomasi Morgano et al., 2018; Liu et al., 2018; Seggiani et al., 2012; Abu Bakar et al., 2008)
Fecal coliform (MPN/g)	$1.33 \times 10^7 \pm 1.31 \times 10^7$	(Hait and Tare, 2011; Scaglia et al., 2014; Bina et al., 2004; Liu et al., 2017)
Salmonella (MPN/g)	495 ± 854	(Hait and Tare, 2011; Scaglia et al., 2014; Bina et al., 2004)

To overcome these hazards, numerous sludge treatment technologies have been introduced. They can be classified into three main categories, namely, chemical treatment, thermal treatment and biological treatment. Generally, the performances of each sludge treatment method can be assessed based on five criteria: (i) carbon recovery, (ii) nitrogen recovery, (iii) volume/weight reduction, (iv) pathogen destruction and (v) heavy metal stabilization. The advantages and limitations for each treatment are summarized as shown in Table 2. Chemical treatment is a simple treatment method that only requires pH monitoring after chemical addition. However, this method increases the dry weight of residue sludge due the additional weight contributed by the added chemicals. With chemical treatment alone, it is also impossible to recover the carbon in the form of biofuel or biomass. Thermal treatments such as incineration, pyrolysis, gasification, and supercritical water oxidation have higher weight reduction ability and are able to recover the carbon for conversion into biofuels (Liew et al., 2021; Ge et al., 2020; Lam et al., 2019). Meanwhile, biological treatment employs living organisms such as bacteria, worms, and insect larvae to accelerate the decomposition of organic matters in sewage sludge. It requires less energy to operate and could also recover carbon in different ways. It also has the ability to retain the nitrogen in the treated sludge as compared with thermal treatment where most of the nitrogen would be volatilized and removed.

Among the many technologies, incineration and anaerobic digestion are the most matured method that are exploited. The adoption rate of these technologies is still very low and it is only concentrated in developed countries such as Germany, Netherland, United States and China. This could be attributed to the high capital cost involved. For example, an incineration plant with a processing capacity of 200,000 ton/year requires a capital cost of around €122 million (Hogg, 2017). Owing to this reason, the implementation of these modern treatment methods is still very much lagging in developing countries. Therefore, to promote a wider adoption of more sustainable sewage sludge treatment technologies, the focus should be allocated on researching and introducing a low-cost sludge treatment method that can be affordably adopted globally.

Currently, there are two potentially low-cost biological treatment methods, namely vermicomposting and black soldier fly larvae (BSFL) that could be further explored to tackle the issue of sludge management. The employment could eliminate the need of installing high-temperature equipment as required by thermal treatment, thereby reducing the process complexity and capital cost (Kacprzak et al., 2017; Mayer et al., 2020). Vermicomposting has demonstrated the ability to decompose the sewage sludge three times faster than that naturally being decomposed during the disposal (Domínguez et al., 2000). In New Zealand, a large-scale application of vermicomposting to treat sewage sludge and co-substrate has been successfully established (Quintern, 2014). Meanwhile, the BSFL assimilate the organic wastes such as food wastes and animal manure, and convert the waste nutrition into insect biomass, containing high lipid and protein contents (Gold et al., 2018). Therefore, the BSFL treatment has also been deployed industrially to produce high protein poultry feed through feeding with organic wastes.

This review elucidates the current status, limitations and area of improvements for employing the biological treatments to reduce and convert sewage sludge. The performances of biological treatments, namely, anaerobic digestion, vermicomposting and BSFL valorization, are assessed with respect to carbon recovery, nitrogen recovery, mass reduction and sludge stabilization. Comparisons are made between the two low-cost solutions and the more matured anaerobic digestion method. This provides useful information to evaluate the feasibility of applying vermicomposting or BSFL treatment as a low-cost alternative to support a more sustainable sludge management process.

Table 2
Comparison among sewage sludge treatment methods (Liew et al., 2021; Bina et al., 2004; Kacprzak et al., 2017; Mayer et al., 2020; Schnell et al., 2020; Sanger et al., 2001; Teoh and Li, 2020; Massé et al., 2007).

Treatment mode	Carbon Recovery	Nitrogen Recovery	Mass Reduction	Pathogen Destruction	Heavy Metal Stabilization	Advantage	Limitation
Chemical Treatment	–	+	–	+	+	- Easiest mode of operation. - Inhibition of pathogens at pH 12. - High nitrogen retention in treated sludge	- Increasing weight of sludge residue. - High operating cost due to the use of excessive chemicals.
Thermal Treatment	+	–	+	+	+	- Carbon recovery through generation of biogas, bio-oil or biochar. - Highest volume/weight reduction. - Short treatment time. - Complete destruction of pathogens.	- High investment cost and complex technology. - High energy requirement and consumption. - Emission of pollutants, requiring air pollution control unit. - Some methods have not been proven in mass-scale. - Low nitrogen retention due to the volatilization at high temperature.
Biological Treatment	+	+	+	V	V	- Carbon recovery ability varies for different type of biological treatments. ■ Anaerobic Digestion: Biogas ■ Vermicomposting: Worms ■ BSFL: Biodiesel/Larvae - High nitrogen retention in solid residue.	- Long treatment duration. - Anaerobic digestion can stabilize heavy metal and pathogens, but inconclusive finding was reported for vermicomposting and BSFL. - Anaerobic digestion requires high investment cost and has a complex operation.

+ : Effective; -: Ineffective; V: Varies

2. Anaerobic digestion

2.1. Overview

Anaerobic digestion is a biological process that uses microbes to convert biodegradable organic matters into biogas in an oxygen-free condition (Cao and Pawłowski, 2012). It has been extensively used as a treatment method for both organic wastes and sewage sludge. The wastewater treatment plant at Italy, for instance, uses anaerobic digestion to process around 380 m^3/d of sewage sludge, while generating about 6100 m^3/d of biogas (Rittmann et al., 2008). In general, anaerobic digestion system requires a lower capital cost as compared with thermal treatment plant such as incinerator, which could cost at least 3 times more investment than anaerobic digestion (Mayer et al., 2020). However, the reaction time needed for anaerobic digestion is significantly longer as opposed to other non-biological methods.

Anaerobic digestion consists of four successive biochemical processes, namely, hydrolysis, acidogenesis, acetogenesis and methanogenesis. The first step is hydrolysis by which large complex organic polymers such as polysaccharides, proteins, starches, free oil and grease are hydrolyzed into simpler soluble substituents such as amino acids, long chain fatty acids and simple sugars. The bacteria species involved in such hydrolysis include *Clostridium, Cellulomonas, Bacteroides, Succinivibrio, Prevotella, Ruminococcus, Fibrobacter, Firmicutes, Erwinia, Acetovibrio, Microbispora* species, etc (Zhen et al., 2017). Subsequently, during acidogenesis, the acidogenic bacteria would further break down the by-products from the initial hydrolysis to shorter chain volatile fatty acids (VFA), ammonia, CO_2 and H_2S. This is completed with the help of *Peptoccus, Clostridium, Lactobacillus, Geobacter, Bacteroides, Eubacterium, Phodopseudomonas, Desulfovibrio, Desulfobacter, Sarcina* species, etc (Zhen et al., 2017). As the third step ensues, long chain organic acids are then decomposed into mainly acetic acid, H_2 and CO_2. The key bacteria involved in acetogenesis are *Syntrophobacter, Syntrophus, Pelotomaculum, Syntrophomonas,* and *Syntrophothermus* species (Cai et al., 2016). Lastly, methane is produced through the mechanism of acetoclastic and hydrogenotrophic methanogenesis. Acetoclastic methanogenesis is facilitated by *Methanosarcina* and *Methanosaeta* species through the conversion of acetate and water into methane. Meanwhile, methane can also be formed through hydrogenotrophic methanogenesis that reacts CO_2 with H_2 in the presence of *Methanobacterium* and *Methanoculleus* species (Zhen et al., 2017; Cai et al., 2016; Guo et al., 2015; Scherer and Neumann, 2013).

The duration allocated for sewage sludge to undergo anaerobic digestion, also known as sludge retention time (SRT) usually spans from 10 to 20 days. This is because most works agreed that 90% of methane yield could be obtained during the first 14 days of treatment as shown in Fig. 1 (Appels et al., 2008). A comprehensive work had investigated the effect of SRT on anaerobic digestion of sewage sludge (Nges and Liu, 2010). The SRT studied in the work ranged from 3 to 35 days. During the early stage of digester operation (SRT $<$5 days), acidogenesis culminates VFA, driving the system pH to a low value, which is unfavorable for methanogenesis. After 5 days, VFA reduces and creates a more favorable environment for methanogenesis to occur as shown in Fig. 1. Consequently, the accumulative gas production increases exponentially from 62 mL/g VS_{added} (Day 3) to 340 mL/g VS_{added} (Day 15).

2.2. Mass reduction

The ability of anaerobic digestion is reducing the mass of sewage sludge is normally reported in terms of volatile solids (VS) and total solids (TS) reduction. According to APHA standard methods (APHA, AWWA, 2005), VS refers to the organic matter that could be lost when heated up to 550 °C. Meanwhile, TS is defined as the residual material left in vessel after undergoing evaporation at 105 °C. Generally, VS is considered as the biodegradable content in sewage sludge while TS is the combination of biodegradable and non-biodegradable matter. Anaerobic digestion is able to reduce the VS content of sludge by 35–60%, depending on the operational conditions and whether a pre-treatment step is applied (Gebreeyessus and Jenicek, 2016). Meanwhile, TS reduction is around 28–30% according to the work of Chi et al. Chi et al. (2010).

2.3. Carbon recovery through biogas generation

During anaerobic digestion, VS will be degraded by different groups of bacteria, which subsequently produce biogas as the end product. Cao and Pawłowski (2012) reported that 0.8–1.2 m^3 of biogas were obtained with every kg of VS being reduced. This range was quite close to the

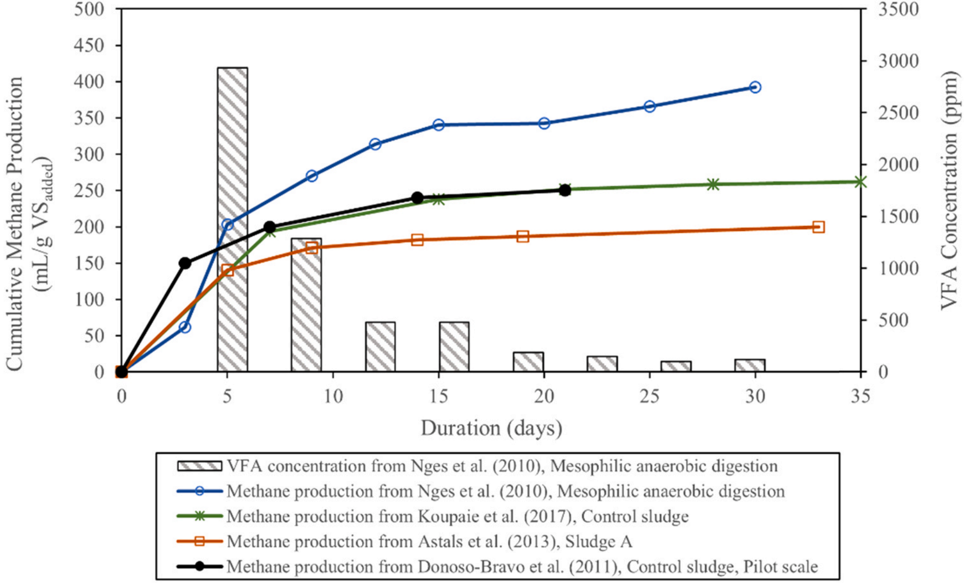

Fig. 1. Methane production and VFA concentration against SRT during anaerobic digestion processes (Nges and Liu, 2010; Hosseini Koupaie et al., 2017; Astals et al., 2013; Donoso-Bravo et al., 2011).

value reported by Appels et al. (2008), Cao and Pawłowski (2012), who stated that biogas production was around 0.75–1.12 m^3/kg VS reduced. The biogas produced is made up of 65–70 vol% of methane, followed by CO_2 at 30–35 vol% and the remaining being contributed by fractions of water vapor, H_2S and H_2 (Appels et al., 2008). The calorific value of biogas ranges between 15.9 and 27.8 MJ/Nm^3. The removal of other unwanted gaseous species is usually conducted to obtain a higher quality biogas that can be used as a fuel for engines, compressors, boilers, and vehicles. The CO_2 and H_2S can be removed via the use of activated carbon as an adsorbent, water-scrubbing system or membrane separation process (Appels et al., 2008; Degrève et al., 2001). Cryogenic separation could also be used to produce high purity methane gas (97 wt %) but at a high cost. Instead of separation, the formation of H_2S could also be inhibited with the addition of Fe^{3+} salts (Appels et al., 2008). The remaining residue after the anaerobic digestion is known as digestate. Since there is still carbon content left in digestate, energy recovery is possible through other methods (Cao and Pawłowski, 2012). However, the leftover is usually highly resistant to degradation, therefore thermal treatment methods such as pyrolysis, and gasification are normally used to treat the digestate.

Cao and Pawłowski had evaluated the energy efficiencies of anaerobic digestion and pyrolysis processes on primary and activated sludges (Cao and Pawłowski, 2012). The routes of evaluation are shown in Fig. 2. Strategy 1 involved the application of anaerobic digestion to produce biogas. Strategy 1b was an extension of strategy 1, whereby pyrolysis of the digestate was conducted to further produce bio-oil and biochar. Meanwhile, Strategy 2 involved the application of pyrolysis to produce bio-oil and biochar. For better comparison of the main products from the anaerobic digestion and pyrolysis, first, a comparison of anaerobic digestion biogas and pyrolysis bio-oil was performed. In the case of 100 kg of primary sludge (VS content: 84 wt%), the energy output was higher at 1573.2 MJ in the form of biogas produced from

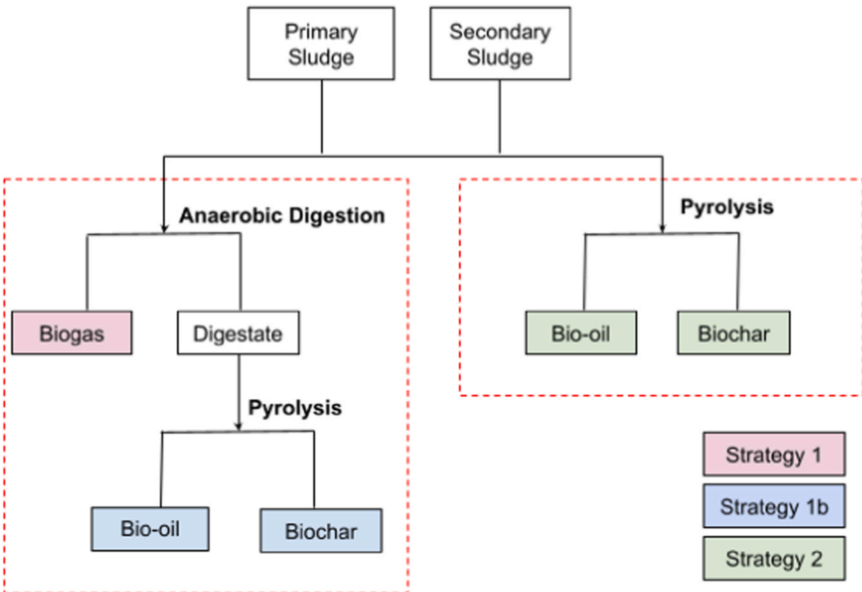

Fig. 2. Flowchart of different strategies in valorizing sewage sludge (Cao and Pawłowski, 2012).

anaerobic digestion as compared with 1554 MJ obtained in the form of pyrolysis bio-oil. Meanwhile, the 100 kg of activated sludge (VS content: 69 wt%) showed a lower energy output of 629.3 MJ in the form of biogas produced from anaerobic digestion as compared with 1147 MJ obtained in the form of pyrolysis bio-oil. This showed that anaerobic digestion was capable of generating more energy output for sewage sludge with high VS content (>84%). It also reiterates the importance of high VS content in producing more biofuel for both treatment methods. However, Cao and Pawłowski (Cao and Pawłowski, 2012) stated that the energy recovery from anaerobic digestion could be further enhanced from valorizing the residual digestate through using pyrolysis to produce bio-oil and biochar via Strategy 1b, generating an additional 581 MJ of energy output from the produced bio-oil and biochar. Hence, once integrated with a subsequent pyrolysis process (Strategy 1 +1b), anaerobic digestion showed a higher energy recovery efficiency for both high and low VS content sludges, namely, primary and activated sludges.

2.4. Nitrogen recovery

Digestate, the residual of anaerobic digestion, is commonly used as a soil fertilizer as it contains a high content of nutrients (N,P,K) and organic carbon (Hermassi et al., 2018). The N content is mainly derived from decomposition of proteins in sewage sludge. In the work of Massé et al. (2007) where swine manure was subjected to anaerobic digestion, nearly 100% of N content was retained in the digestate after the process. The amount of ammoniacal nitrogen in digestate had increased by 36.5 wt%, signifying the occurrence of conversion of nitrogenous compound into ammoniacal nitrogen. As the digestate was allowed to settle, the settled sludge cake carried 31.5 wt% of ammoniacal nitrogen, while the supernatant that was usually returned to the wastewater treatment plant contained 67.1 wt% of ammoniacal nitrogen. On the other hand, 74.5 wt% of the P content was retained in the digestate, with 24.95 wt% in the supernatant and 46.4 wt% in the settled digestate. Through the addition of magnesium at a Mg/N/P molar ratio of 1:1:1, 63–64 wt% of NH_4^+ and PO_4^{3-} could be recovered as struvite ($MgNH_4PO_4$) and bobierrite ($Mg_3(PO_4)_2$) (Hermassi et al., 2018). Other alternatives that show promising efficiency in recovering N content include air stripping, thermal drying, hydrophobic and vacuum membranes (van der Hoek et al., 2018).

2.5. Heavy metal stabilization

Besides, anaerobic digestion had been reported with ability to reduce the bioavailability of heavy metals in sewage sludge by converting them from the readily exchangeable form into the more stable form that is harder to leach into the surrounding environment (Hong et al., 2007). Generally, the metals could be classified into five different forms including exchangeable (F1), acid-soluble (carbonate bound, F2), oxidizable (organic bound, F3), reducible (Fe/Mn bound, F4) and residual (F5). F1 was the least stable form of heavy metals, as it could be readily leached into water and subsequently, being uptake by plants and animals. This is then followed by F2 that could be readily available under an acidic environment. These two forms of metals (F1 + F2) were the main contributors to the bioavailability and toxicity effect of sludge. Meanwhile, the F3, F4 and F5 forms were relatively stable in soil and had the lowest bioavailability (Hong et al., 2009; Lim et al., 2013).

However, for anaerobic digestion, Zhu et al. (2014) introduced one new fraction which is the water-soluble fraction. This refers to the heavy metals which were solubilized in the supernatant of the digestate, meanwhile F1 to F5 were used to categorize the heavy metals in the settled digestate. When taking into consideration of all the six heavy metal forms including the water-soluble heavy metal concentration in the supernatant, it was reported that most of the heavy metals tested were within China's control standards for pollutants in sludge for agricultural use (GB4284–84), except for Cu, Zn and Cd (Zhu et al., 2014). However, putting the more stable F3-F5 aside, the toxicity should only be judged by F1, F2 and the water-soluble forms. In this scenario, only Zn did not meet the standard required for acidic soil. Although the settled digestate could not be applied on acidic soil (pH<6.5) in China, it still meets a more lenient limit demarcated by the same standard GB4284–84 for the application on alkali soil (pH > 6.5). In addition, Zhang and Wang (2020) also showed that the addition of Fe-Mn modified biochar could facilitate the immobilization heavy metals. In their work, the bioavailable form (F1 + F2) of Cr, Ni and Cd reduced by 49.76%, 31.46%, and 47.69% respectively when 0.12 g of Fe-Mn modified biochar was added to every 1 g of sludge (Zhang and Wang, 2020). They attribute this to the additional active sites and functional groups of Fe-Mn modified biochar to interact with the heavy metals.

2.6. Pathogen reduction

The operating conditions of anaerobic digestion can be divided into mesophilic and thermophilic conditions. Under mesophilic condition, the temperature is in the range of 30–38 °C, while that required by thermophilic condition is higher from 50 to 57 °C (Cao and Pawłowski, 2012). Since thermophilic anaerobic digestion operates at higher temperature, it consumes more energy. However, higher temperature does improve the effectiveness in reducing pathogen. As consequence, the residual from anaerobic digestion, known as digestate, meets the pathogen standard of Class A biosolid as set by US EPA (Gebreeyessus and Jenicek, 2016). Being classified as Class A biosolid, the digestate produced from thermophilic anaerobic digestion can be suitably applied to both agricultural land and public access land such as private lawn and home garden. Meanwhile, the mesophilic anaerobic digestion can only produce a digestate that meets the criteria as Class B biosolid (Al-Gheethi et al., 2018), which is inferior as compared with Class A biosolid. It contains more pathogen and is not able to meet the more stringent standard. The application of Class B biosolid is limited to only agricultural land (USEPA, 1994). Generally, the amount of pathogen reduction increases with digestion duration. The most drastic reduction happened between day 3 and day 6, recording a 4 $\log_{10}$ reduction. Subsequently, from day 6 to day 60, only 1 $\log_{10}$ reduction was achieved (Scaglia et al., 2014). The content of *Salmonella* spp. reduced from 81 MPN/g to nil in 3 days for a thermophilic anaerobic digestion, while it took 13 days to achieve a similar result for a mesophilic anaerobic digestion, proving that the thermophilic anaerobic digestion is more superior in pathogen reduction. In both anaerobic digestion processes, no living eggs of helminths was detected (Scaglia et al., 2014).

2.7. Pre-treatment

To enhance the biogas yield from anaerobic digestion, pre-treatment of sewage sludge is often carried out. Different pre-treatment methods have been tested and proved effective, including thermal pre-treatment, chemical pre-treatment, enzymatic pre-treatment and mechanical pre-treatment. The prime objective of pre-treatment is to disintegrate the sludge in advance. This is because the sewage sludge is an unique biomass that contains extracellular polymeric substances (EPS) (Tyagi and Lo, 2011). The EPS is a gel-like structure that binds together the microbes and holds plenty of nutrients within it. It is made up of 58% of protein and 9% of polysaccharides (Mikkelsen and Keiding, 2002). By disrupting the EPS structure, more biodegradable nutrients could be released, and this enhances the potential of anaerobic digestion in extracting more biogas.

The effectiveness of pre-treatment is normally measured in terms of soluble chemical oxygen demand (sCOD) and the solubilization ratio. The sCOD increases when more bio-degradable organic matter is released into the soluble phase (Hosseini Koupaie et al., 2017). This could be in the form of intracellular biopolymers such as reducible sugars, nuclei acids, lipids or even extracellular biopolymers such as disintegrated EPS (Hosseini Koupaie et al., 2017). Although the total

chemical oxygen demand (COD) in the digester might remain unchanged, the solubility ratio will increase when the sCOD increases. A higher solubility ratio signifies that the pre-treated sewage sludge is more easily degraded and could produce a higher biogas yield.

Many different pre-treatment methods have been studied to improve the biodegradability of sewage sludge. For instance, the microwave pre-treatment of activated sludge at a heating duty of 336 kJ/kg total solid increased the sCOD content by 214% and subsequently 50% increase in the biogas production was observed during the mesophilic anaerobic digestion (Appels et al., 2013). A pilot scale study of thermal pre-treated activated sludge at 65 ^{o}C for 20 min showed a 2- to 3-fold increase in the solubility ratio and as expected, an increment of 30–40% in biogas production. Similarly, NaOH and H_2SO_4 had been tested for the chemical pre-treatment of sewage sludge. Results showed that an alkaline pre-treatment regime enhanced the sCOD more than an acidic pre-treatment. This could be due to refractory compound formation when acid is used for sludge pre-treatment, causing a reduction in the outcomes of the digester as refractory compounds do not degrade easily. In their work, a pH at 10 released the highest amount of methane, followed by that released at pH 5 and then pH 2 (Tulun and Bilgin, 2019).

2.8. Co-digestion

Co-digestion involves the anerobic digestion of sewage sludge with another organic waste. Co-digestion of sewage sludge has shown improved VS reduction, enhanced biogas yield and improved in heavy metal stabilisation. In the context of anaerobic digestion, an optimum C/N ratio for anaerobic digestion is usually between 20:1 and 30:1 (Monnet, 2003). Sewage sludge that has a low carbon-to-nitrogen (C/N) ratio within 5:1 – 16:1 is thus considered nutrient deficient for anaerobic digestion to thrive (Rynk: Robert, 1992). Low C/N ratio will lead to excess ammonia formation, thereby increasing the system pH above 8.5 which is not conducive for methanogens (Monnet, 2003). Therefore, by deploying a co-substrate, the missing nutrients in sewage sludge can be counterbalanced, resulting in improved C/N ratio for a better anaerobic digestion performance (Chow et al., 2020).

In a large-scale plant, the co-digestion of sewage sludge with organic fraction of municipal solid waste has shown positive effect on biogas production. In Frutigen, Switzerland, a full-scale study to validate the feasibility of co-digesting organic fraction of municipal solid waste with sewage sludge was conducted (Edelmann et al., 2000). Organic municipal solid waste was collected from regional supermarket chains and food wastes from hospitals, shredded and added for co-digestion. Experimental result shows that the biogas production increases by 27% with the addition of 20% solid waste into the system, without affecting the process stability.

Crude glycerol, a by-product from biodiesel production, has also been used as a co-substrate due to its high carbon content (BOD of 97080 mg/L) (Chow et al., 2015). When 1 vol% of the crude glycerol was added into an anaerobic co-digestion system, an increase of 115% in the methane yield was recorded (Fountoulakis et al., 2010). However, any higher dosage than that would reduce the alkalinity of the digester and thus culminating in the accumulation of VFA which inhibited methanogens (Razaviarani et al., 2013). Next, free oil and grease can also be co-digested with sewage sludge at optimum ratio (50:50 in terms of VS). Any higher ratio would result in an excessive accumulation of long chain fatty acids in the biomass, limiting the mass transfer and resulting in aggregation and clogging (Chow et al., 2020). In addition, co-digestion of different sludges is also possible. This is shown in the work of Babel and Sae-Tang (Babel et al., 2009), who mix sewage sludge and brewery sludge for co-digestion. As a result, the mixed digestate finally meets the pollutant limit of biosolid as set by US EPA.

In general, for an effective co-digestion, an optimum mixing ratio must be investigated as it varies according to the type of co-substrates envisaged for use. Furthermore, a proper mixing is also very important to produce a homogenous feed. Although co-digestion has shown its effectiveness in facilitating sewage sludge anaerobic digestion, it looks more like a symbiosis relationship. For instance, the VS reduction and biogas yield improves because the co-substrate is often more biodegradable. Furthermore, all the co-substrate discussed earlier has a lower heavy metal concentration, thereby creating a dilution effect for the digestate to achieve EPA standard. It is highly possible that the digestion of co-substrate alone without sewage sludge will bring more economic value.

2.9. Microbial electrolysis cell (MEC)

Lately, MEC has been paired up with anaerobic digestion to produce a higher methane yield and stabilize the anaerobic digestion system (Zhen et al., 2017; Yu et al., 2018). In MEC, exoelectrogenic bacteria consume the organic matter anaerobically and generate electrons at the anode. The electrons then travel to the cathode side with the help of an external voltage supply. At the cathode side, electrons are used by methanogens to convert CO_2 and acetic acid into methane gas. Liu et al. (2016) conducted a MEC-anaerobic digestion process of activated sludge at 0.8 V and obtained a methane production rate of 91.8 g/m^3, which is 3 times higher than that obtained from the typical anaerobic digestion process (30.6 g/m^3). This was supported by the increased population of methanogens. They reported that the highest increment was *Methanobacterium* (hydrogenotrophic bacteria), followed by *Methanosaeta* (acetrophic bacteria) and *Methanospirillum* (hydrogenotrophic bacteria) species. They also observed that the degradation rate of organic matter was higher in MEC-anaerobic digestion with a lower remaining content of polysaccharides and proteins. This accelerated rate of degradation was attributed to the growth of exoelectrogenic bacteria *Geobacter* sp., which could anaerobically decompose a variety of organic substrates (Yu et al., 2018).

Researchers had shown that the effectiveness of MEC could be affected by the external voltage applied. In the work of Choi et al. (2017), different voltage supplies (0.5, 0.7, 1 and 1.5 V) were tested on the MEC of mixed substrate containing anaerobic digestion effluent and growth medium. Results showed that methane production improved with increasing voltage from 0.5 to 1 V. However, the methane production decreased at 1.5 V. Likewise, Linji et al. (2013) also reported that 0.8 V was the optimum voltage for their MEC experiment on activated sludge. Despite of the potential of MEC, so far only lab-scale MEC had been proven successful.

3. Vermicomposting

3.1. Overview

Vermicomposting, also known as vermistabilization, combines the use of earthworms and microorganisms to accelerate the organic waste degradation process. The earthworms will ingest, grind, and digest the organic waste with the help of microflora in their gut, thereby modifying the physical, biological, and chemical status of the organic waste. The excretion of earthworms is known as vermicast, is in the form of a homogenous, stable, fine, humified and microbially active organic matter. Besides, it contains nutrients such as K and P in a more readily available form for use as fertilizer to grow plants (Gupta and Garg, 2008; Huang et al., 2013; Liu et al., 2005). Furthermore, the growth of earthworm populations could also be used as a high-protein feed for farmed animals such as chickens. All these can be achieved at a low cost, making this treatment method economical and ecological to be implemented, especially in developing countries. However, vermicomposting requires an even longer treatment time as compared to anaerobic digestion, and it is not capable of generating any biofuel too. The experimental duration for vermicomposting in most studies spans from 30 days to 60 days (Gupta and Garg, 2008; Khwairakpam and Bhargava, 2009; Hait and Tare, 2011; Huang et al., 2013; Liu et al., 2005).

Vermicomposting of sewage sludge has reached the industrial-scale

application, but it is only limited to co-substrate basis, where sewage sludge is mixed with pulp mill solid. In Tasman, New Zealand, the commercial operation of vermicomposting was running based on the mixture of sewage sludge and paper mill sludge. As of 2008, the vermicomposting system was processing 2000 tons of pulp mill solid and 900 tons of municipal sewage sludge per year (Quintern, 2014). The end product was a good quality vermicast that met the criteria as New Zealand biosolid (Quintern and Morley, 2017). Nonetheless, it should be highlighted that the raw sewage sludge contained a comparatively low heavy metal concentration.

3.2. Mass reduction

Based on literature review, mass reduction in terms of VS or TS reduction is less emphasized on. The main objective of vermicomposting is to stabilize the sewage sludge by reducing the organic matter content in the sludge, thus preventing the growth of bacteria later on. This reduction of organic matter can be measured through the total organic carbon (TOC). The TOC reduces as the duration of vermicomposting increases. This is because part of the organic carbon is used up by earthworms and bacteria for respiration, converting it into CO_2 and reducing the volatile solids in the biomass (Hait and Tare, 2011). In the work of Khwairakpam and Bhargava, (2009), the TOC in sewage sludge was reduced by 10–25% in pure cultures and 10–17% in mixed cultures after 45 days. The highest TOC reduction recorded was 25.46% when pure culture of *P. excavates* was used. Meanwhile, control sewage sludge without earthworm only recorded a TOC reduction of 8.7%. Pure culture refers to the use of only one species of earthworm during vermicomposting, while mixed culture uses two or more species of earthworm. TOC reduction of 20–30% is commonly agreed by the experimental values in several studies when vermicomposting is deployed on sewage sludge (Gupta and Garg, 2008; Huang et al., 2013), which is about 2.5–3 times higher TOC reduction that the control without earthworm.

3.3. Carbon recovery through earthworm growth

In vermicomposting, earthworm ingests the carbon content in the substrate to grow and populate. Consequently, the weight of earthworms increases along with the vermicomposting duration. After 45 days of vermicomposting, the weights of earthworms are reported to increase 9.09–28.57% (Khwairakpam and Bhargava, 2009). Gupta and Garg (2008) showed that the total weight of earthworms increased steadily to a peak before losing weight due to exhausted nutrients in the substrates. For sewage sludge, the nutrient exhaustion point happened around day 35.

3.4. Nitrogen recovery

After vermicomposting of sewage sludge, the concentration of total N in the vermicast always increases due to the reduction in substrate dry mass as water is lost through evaporation and respiration via earthworm (Gupta and Garg, 2008; Huang et al., 2013). According to the work of Antoniou et al. (1990), the vermicomposting of straw pallet and cattle manure mixture showed that 76.3–79.4% of original total N content in the substrate mixture could be recovered in the vermicast. This high N content retention could be attributed to the low pH profile (5.5–7.2) throughout the vermicomposting period because high pH above 7.5 favors the formation and volatilization of NH_3 (Khwairakpam and Bhargava, 2009).

Khwairakpam and Bhargava (2009) also reported that the concentration of NH_4^+ was always higher than NO_3^- throughout the 45 days of vermicomposting. However, the concentration of NH_4^+ declined sharply between the 30th and 45th days, whereas NO_3^- started to increase. The rapid reduction of NH_4^+ at the end could be attributed to the increment of pH from an average of 5.5–7.2. The increment of pH would unfavour ammonia protonation (Oonincx DGAB et al., 2015; Pan et al., 2018). Concurrently, this could also be due to the formation of NO_3^- as it had been reported that the nitrifying bacteria only started to colonize at the later stage of composting (Bernal et al., 2009). Furthermore, the growth rate of nitrifying bacteria was also reported to be higher as the substrate approached pH 7–8 from both acidic and alkaline conditions (Watson et al., 1981).

3.5. Heavy metal stabilization

In terms of heavy metals stabilization, inconclusive findings are reported thus far. Khwairakpam and Bhargava (2009) found that the final concentration of Cu in vermicast remained unchanged, whereas Pb, Zn and Mn were reduced by around 30%, 20% and 50%, respectively. The decrease in heavy metals could be due to the accumulation of heavy metals in the chloragosomal tissue in the body of earthworm (Fischer and Molnár, 1992). It has been suggested that when earthworm was placed in heavy-metals contaminated substrate, a cation-exchange mechanism would take place in the chloragosomal tissue of earthworm; for instance, the Pb would displace Ca in the chloragosomal (Fischer and Molnár, 1992; Fischer, 1977). This could be due to the self-protective mechanism of earthworm that could prevent heavy metal in its body from entering the haemoglobin pathway (Morgan and Morgan, 1989).

A similar finding was reported by Huang et al. (2013), exhibiting that the heavy metal concentrations reduction was ranging from 9.8% to 20.5% after 30 days of vermicomposting with two different types of earthworm. In contrast, heavy metal concentrations were found to increase by more than 50% for Fe, Cu and Zn in another work that tested municipal plant primary sludge and cow dung as the feeds for vermicomposting (Gupta and Garg, 2008). Hartenstein and Hartenstein (Hartenstein and Hartenstein, 1981) explained that it could be due to the mineralization process that was accelerated in the presence of earthworm as the final heavy metal concentration was more than the concentration in the control (without earthworm).

Later, Liu et al. (2005) determined that the earthworms could absorb heavy metals into their body. This was conducted by measuring the heavy metal content in the earthworm's body before and after vermicomposting. However, there was also a maximum concentration that the earthworm could tolerate. From the experiment, this was determined to be around 500 mg/kg for Cu and 20 mg/kg for Cd in the sewage sludge. Beyond these lethal concentrations, earthworm's ability in absorbing heavy metals was reduced. Their work also showed that vermicomposting did not change the fraction of heavy metals from one form to another (Liu et al., 2005). For instance, the amounts of carbonate Cd were 10.53 wt% and 10.27 wt% with and without earthworms, respectively. Yet, when the sewage sludge and vermicast were separately added into two similar soil pots for planting Chinese cabbage, it was discovered that the Cu and Cd contents in the cabbage were lesser than that found in vermicast-raised cabbage. This proved that the vermicast could reduce the bioavailability of heavy metals, not by changing metal fractions, but by the uptake of heavy metals to itself and perhaps, changing the soil characteristics.

3.6. Pathogen reduction

Vermicomposting could also reduce most of the pathogens in sewage sludge (Khwairakpam and Bhargava, 2009). Up to 99.9% of the fecal coliform was reduced after a vermicomposting of 45 days, recording the final fecal coliform population of between 1300 and 2300 MPN/g. The reduction of pathogens is due to the antibacterial effect of the coelomic fluid released by earthworms during their physiological activities such as respiration and burrowing (Valembois et al., 1982). Although achieving a high reduction, the final fecal coliform still did not meet the pathogen requirement of US EPA Class A biosolid, which limits fecal coliform to be 1000 MPN/g. Nevertheless, the vermicast managed to

meet USEPA Class B biosolid within just 15 days of vermicomposting. The authors also showed that a mixed culture of worms was more effective in destroying pathogen as compared with a pure culture (Khwairakpam and Bhargava, 2009). In another work, pathogen level after vermicomposting had met the US EPA Class A standard, but the sample was first pre-treated with aerobic composting (Hait and Tare, 2011).

3.7. Co-substrates

Domínguez et al. (2000) pointed out that sewage sludge was not a suitable substrate for vermicomposting due to its low C/N ratio. Co-substrate can thus be deployed to increase the C/N ratio. For example Gupta and Garg (2008) mix cow dung (C/N ratio: 69.3) and sewage sludge (C/N ratio: 16.1) together for vermicomposting. This resulted in an improved TOC reduction of 62.6% as compared with 30.5% for the control (only sewage sludge). This in turn translates to a 48% weight increment for the earthworms.

Instead of introducing a different co-substrate, Huang et al. (2013) utilized vermicast itself as a co-substrate to see its effect on sewage sludge vermicomposting. Interestingly, despite the low initial C/N ratio in sludge, the C/N ratio still showed a massive reduction from 9.72 to 5.16 (*E. foetida*) and 5.08 (*B. parvus*) in just 30 days as shown in Fig. 3. The growth rate and reproduction rate of both earthworm species also increased by 28–37%. At the same time, the total N, P, K increased as much as 15.4–37.5%, while heavy metal content was reduced by 9.8–20.5%. This could be due to the effect of increased microbial activity contributed by the addition of vermicast, which is rich and diverse in bacterial microflora.

4. Black soldier fly larval valorization

4.1. Overview

The research into black soldier fly, *Hermetia illucens* was initiated around the 1950 s due to its ability as a natural control to the disease-transmitting house fly, *Musca domestica*. Black soldier fly (BSF) population could reduce the population of *Musca domestica* by 94–100% (Sheppard, 1983). BSF is non-identical to the typical household fly that it is not a pest and does not transmit diseases. BSF is an insect that is normally found feeding on decomposed organic matters such as rotting vegetables, fruits, plant litter and manure in the tropics and warm temperate regions. It has a short lifespan of about 54 days from its first emergence from cocoon to the stage it reproduces and dies. As shown in Fig. 4, the life cycle begins with the female adults ovipositing about 320–620 eggs near a decomposing organic matter source (Tomberlin et al., 2009). The eggs will normally hatch after 4 days, and the young larvae then feeds on the decomposed organic matter. Next, the larvae will undergo stages of larval development from 1st until 5th instar stages. The duration of growth varies according to the quality of food sources it has access to, ranging from 15 to 52 days (Gold et al., 2018). Next, having accumulated enough proteins and lipids for the transformation into prepupae, the larvae then stop ingesting any matter. Its mouth part slowly turns into a hook-shaped structure that allows it to move to the dry and dark place and ensconce itself for pupation. This is the 6th instar stage and the last stage of the larvae before turning into a cocoon and then emerging as a fly after about a week. The fly usually mates after 2 days of pupal emergence (Tomberlin and Sheppard, 2002). Again, after mating, it takes another two days for the female to oviposit eggs, before dying of exhaustion at a maximum of 9 days (Booth and Sheppard, 1984; Raksasat et al., 2020; Samayoa et al., 2016).

Similar to vermicomposting, it uses a combination of BSFL and microbial population to speed up the decomposition process of organic matter. During their larval stage, BSFL ingests organic matter ferociously and stores them in the form of body fat (Craig Sheppard et al., 1994). This is crucial to sustain their development during prepupae and adult stage, as they no longer consume food at that two stage. As consequence, BSFL contains high contents of lipid and protein at 47% and 41%, respectively (Guo et al., 2021). Simultaneously, solid excrement, also known as frass is discharged by BSFL and it can be used as a fertilizer.

The high protein content of BSFL makes it suitable to be used as a part of the poultry diet. There had been many researches showing positive results of using BSFL as a feedstock meal for poultry, swine and fish (Li et al., 2016; Sprangers et al., 2017). Skrivanova et al. (2007), for instance, reported that a diet of BSFL could help in managing the microbiota in the small intestine of pigs and poultry, thereby fortifying the poultry's health. Meanwhile, Kim et al. (2020) reported that a diet of BSFL oil could improve the gut health and enhance the antioxidant capacity of broiler chickens. Recently, BSFL lipid is also extracted to produce biodiesel.

Numerous kinds of organic waste, also known as substrates have been administered to grow BSFL. This includes food waste, cow manure, chicken manure, coconut endosperm, soybean curd, vegetable waste, human feces and sewage sludge. Different kinds of substrates bring about significant effect on the growth and development of BSFL (Sprangers et al., 2017; Lalander et al., 2019; Leong et al., 2016; Liu et al., 2020a). The application of BSFL treatment has reached industrial scale for numerous organic wastes. For example, InnovaFeed in France collects agricultural by-products from a starch production plant to rear

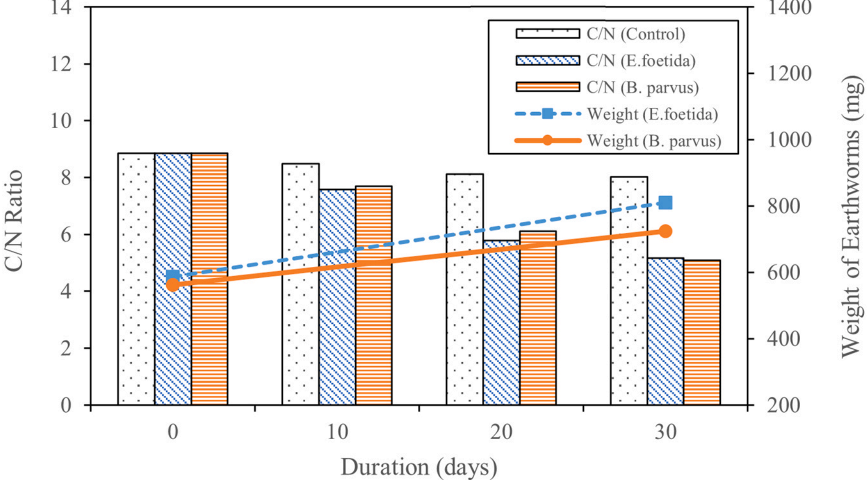

Fig. 3. Effects of vermicast addition as co-substrate on sewage sludge vermicomposting process (Huang et al., 2013).

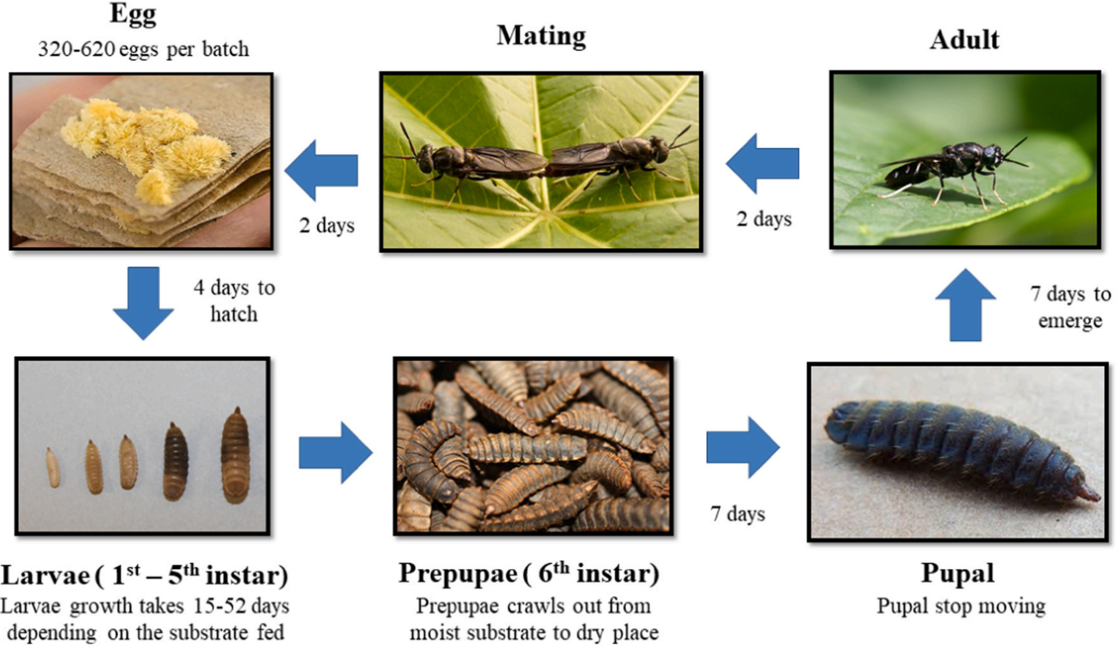

Fig. 4. Lifecycle of black soldier fly (Gold et al., 2018; Tomberlin et al., 2009; Tomberlin and Sheppard, 2002).

BSFL (PhI et al., 2020). Subsequently, BSFL is collected as poultry feedstock as well as for oil production. However, till date, limited studies on sewage sludge as BSFL substrate have been conducted. Hence, the discussion below regarding performance of BSFL treatment will be of a combination of sewage sludge and other substrates.

4.2. Mass reduction

Mass reduction for BSFL treatment is commonly expressed in terms of dry matter reduction. Lalander et al. (2019) employed the BSFL to treat three different types of sludges, namely primary sludge, activated sludge and digested sludge. Primary and activated sludges are both raw sludge that is untreated, whereas digested sludge refers to sludge that is pretreated via anaerobic digestion. BSFL treatment on primary sludge and activated sludge could achieve 63.3% and 49.2% dry weight reductions, respectively. Meanwhile, only 13.2% dry weight reduction was recorded for digested sludge. This is because for digested sludge, most of its VS has been released during anaerobic digestion, leaving the non-biodegradable matter in the sludge, resulting in low mass reduction. In the work of Raksasat et al. (2021), a dry mass reduction of 73.34% is recorded after 27 days. Meanwhile, only 2% dry matter reduction was reported by Cai et al. (2018). This could be due to the difference in drying temperature used by all three works. For instance, Lalander et al. Lalander et al. (2019) dry the residue at 80 ^{o}C for 48 h while Cai et al. (2018) dries the residue at 105 ^{o}C for 10 min, and then 60 ^{o}C for 2 days.

To enable comparison among BSFL treatments and also other methods such as anaerobic digestion, a reasonable temperature should be unanimously agreed and used for all future works. Since anaerobic digestion often measures TS reduction, the temperature range of 103–105 ^{o}C as recommended by APHA standard is suitable for the adoption (APHA, AWWA, 2005). Furthermore, according to the thermogravimetric chart of sewage sludge, the water loss happens at the temperatures below 120 ^{o}C with most water loss occurs at 87.6 ^{o}C (Gao et al., 2014). Hence, the drying temperature should be at least above 87.6 ^{o}C. Comparing with other substrates, the BSFL treatment could reduce the dry matters of animal manure by 42–56% (Sheppard et al., 2002), food waste by 55.3% and human feces by 47.7% (Lalander et al., 2019).

4.3. Carbon recovery through BSFL growth and biodiesel production

During BSFL treatment, carbon recovery is achieved through the weight gained by BSFL. Subsequently, the heavier BSFL is harvested to be used as a poultry feedstock or to be converted into biodiesel. The characteristic of substrate affects the growth of BSFL significantly. When organic waste such as food waste, vegetable waste and abattoir wastes were fed to BSFL, the wet weight of each prepupa was within the range of 212–252 mg (Lalander et al., 2019). When animal manure was used, the wet weight of prepupa reduced to 164 mg. However, when primary sludge and activated sludge were used, the prepupa weight dropped to 137 and 145 mg. Furthermore, the time taken for the first prepupa to emerge in the food waste was only 14 days as compared to 21 and 30 days for primary sludge and activated sludge (Lalander et al., 2019) as shown in Table 3. This indicates that sewage sludge is not a suitable substrate for BSFL rearing, and it could be due to the nutrients of sludge that are rather hard to be accessed because of the EPS structure that protected the microbial population (Dohányos et al., 2004; Ruffino et al., 2015). In addition, this shows that C/N ratio is not a good indicator to determine the quality of substrates. For instance, even though the activated sludge had a similar C/N ratio as compared with the mixture of abattoir, fruit, and vegetable waste, both substrates showed a big difference in the BSFL development properties (Lalander et al., 2019). Based on the substrate characteristic, Lalander et al. (2019) inferred that VS and total N were the two important parameters that determined the quality of substrates to suit the BSFL diet.

Similar result was reported by Leong et al. (2016), who reared BSFL with fresh sewage sludge. They reported a negative growth rate of −0.07 g/day, where the prepupae weight decreased after four days of rearing. Raksasat et al. (2021) in their work, reported a 14.38 mg prepupa dry weight, which was 3 times smaller than the larva reared in the mixed substrate of sewage sludge and palm kernel expeller. Basically, most studies do not show a positive result using sewage sludge as substrate to rear BSFL.

In order to produce biodiesel, body fat can be extracted from BSFL and then undergo transesterification process. Li et al. (2011) attained 93–96% of biodiesel yield from the crude fat extracted from BSFL through acid-catalyzed esterification of free fatty acids, followed by alkaline-catalyzed transesterification. The resultant biodiesel was of

Table 3
Larval development and process efficiency of BSFL treatment using different sewage sludges.

Type of Sewage Sludge	Sewage Sludge Properties		Process Efficiency		Larval Development	Reference
	Total Volatile Solid (% of DM)	Total Nitrogen (mg/g)	Biomass Conversation Rate (% of DM)	Material Reduction (% of DM)	Observation	
Primary Sludge	77.8	4.7	2.3	63.3	Individual prepupa final weight: 137 mg Time for first prepupa: 16–21 days Survival rate: 81.0%	(Lalander et al., 2019)
Secondary Sludge	77.1	4.2	2.2	49.2	Individual prepupa final weight: 145 mg Time for first prepupa: 30 days Survival rate: 76.2%	(Lalander et al., 2019)
Digested Sludge	63.2	6.8	0.2	13.2	Individual prepupa final weight: 70 mg Time for first prepupa: 39–42 days Survival rate: 39.0%	(Lalander et al., 2019)
Sewage Sludge	–	–	1.1	73.3	Individual prepupa final weight: 14 mg Duration of experiment: 27 days	(Raksasat et al., 2021)
Sewage Sludge	–	12.7–34.1	-0.8	1.0	Total prepupae weight gain: − 500 mg Duration of experiment: 30 days	(Cai et al., 2018)
Sewage Sludge	–	–	-0.09	5.4	Total prepupae weight gain: −400 mg Duration of experiment: 8 days	(Leong et al., 2016)

DM: Dry mass

good quality meeting the European biodiesel standard (EN14214) in terms of density, viscosity, cetane number and sulfur content. Furthermore, the BSFL biodiesel had a high percentage of saturated fatty acid methyl ester (FAME) of 67.6% as compared with crop-based biodiesels such as rapeseed biodiesel (4.3%) (Li et al., 2011). This is a highly sought-after quality as saturated FAME is more chemically stable than unsaturated FAME and hence, has a higher oxidative stability as opposed to the biodiesel with high unsaturated FAME.

Based on the work of Zheng et al., the final yield of biodiesel from restaurant waste is only 2.4%. The yield of larval to restaurant waste is 6.5%, yield of body fat to larval is 39.2%, and yield of biodiesel to body fat is 93.1%. In order to produce a breakthrough in BSFL biodiesel production, it is thus crucial to overcome the limitation of the first yield, which measures how much larval weight is gained from the organic waste. Once a healthy and heavy larva is obtained, body fat extraction and subsequent transesterification process generally grants a much higher yield.

To maximize biodiesel production, Wong et al. (2019) proposed to bring forward the harvesting session of BSFL from the 6th instar stage (prepupae) to the 5th instar. They discovered that the lipid content in BSFL at the 5th instar stage was higher than that observed at the 6th instar stage, i.e., 34.23 wt% versus 25.88 wt%, respectively. This is because in the 5th instar stage, the BSFL are still actively ingesting organic substrate to grow. However, reaching the 6th instar stage, the mouth part slowly turns into a hook-shaped beak to prepare it for ensconcing itself in the pupal stage. This transformation from the 5th to the 6th instar stages takes weeks, hence consuming the lipid content stored in its body. By harvesting the BSFL earlier, a significant improvement of 32% in BSFL lipid was collected for the transesterification process (Wong et al., 2019). Furthermore, an early collection of BSFL did not affect the FAME composition from BSFL, where C12:0 was still the dominant FAME.

Additionally, it is observed that early prepupation with lower larval weight happens when a high protein substrate is fed to BSFL as compared to the protein deficient substrate (Lalander et al., 2019). To maximize biodiesel production, an optimisation study should be performed to see if the shorter development time could counter the effect of lower larval weight. This is because the total biodiesel generated per day could be manipulated by both larval weight and days required for prepupae formation. Moreover, in-depth research should also be conducted to study the effect of feeding substrates with different protein contents on the BSFL lipid yields and FAME compositions to ensure that the quality of BSFL biodiesel is not affected by the early prepupae conversion.

4.4. Nitrogen recovery

When it comes to total N content, it had been calculated that 68 wt% of the initial N content would remain in the residual biomass after 24.3 days of BSFL treatment on dairy manure (Rehman et al., 2017). This could be apprehended as some of the nitrogen content were volatilized and some had been consumed by BSFL for growth. In another experiment of BSFL treatment for substrate sewage sludge and food waste, the concentration of NH_4^+ was observed to increase and peak at day 5 before reducing (Liu et al., 2020a). The increment of NH_4^+ in food waste was, however, more tremendous, reaching 1001 mg/kg while only 438 mg/kg for sludge sample. This can be attributed to the lower pH in food waste sample, which promoted a shift in equilibrium towards ammonia protonation to form NH_4^+ ions (Pan et al., 2018; Bernal et al., 2009). Liu et al. (2020a) also plotted the changes of NO_3^- content throughout the BSFL treatment. No significant change had occurred to the concentration of NO_3^- throughout the 10 days of experiment, with NO_3^- concentration hovering between 20 and 24 mg/kg for both food waste and sewage sludge sample. This observation where NO_3^- was consistently lesser than NH_4^+ was alike with the finding for vermicomposting. The conservation of nitrogen content in sewage sludge is very critical to produce high-quality frass.

4.5. Effects of heavy metal on BSFL and heavy metal stabilization

Studies regarding the fate of heavy metals during BSFL treatment are quite limited to date, especially on sewage sludge. This is because most BSFL studies focus on food waste, restaurant waste and animal manure that are not highly contaminated with heavy metals. Thus, the leftover frass is foreseen not to have any complication with the issue of heavy metals. Nonetheless, the effects of heavy metals on BSFL growth and survivability as well as the concentrations of heavy metals in the larval body and the frass after the BSFL treatment had been investigated.

To study the effect of heavy metals on the growth BSFL, Cai et al. (2018) has prepared seven artificial diets made up of bran and wheat, with different heavy metals concentration corresponding to the sludges collected from seven different municipal plants. Based on the principal component (PC) analysis conducted (Cai et al., 2018), the PC1 that explained 39.16% of the total variability showed that high concentrations of Pb, Ni, B and Hg could retard the growth of BSFL. Meanwhile, high concentrations of Cu, Zn, Cr, Cd and Hg could slightly harm the larvae survivability as exhibited by PC2 with 28.69% of total variability. Remarkably, the combined heavy metals did not have a drastic influence on BSFL as the survival rates for all samples including the control were still high, ranging within 90–100%, while the conversion rates (weight increment in BSFL/weight reduction in substrate) were quite constant at 9–11% (Cai et al., 2018). Despite that these results were obtained based on artificial diets with emulated heavy metal content, the BSFL was proven to be highly tolerant towards the heavy metals, thus having the potential to be employed for sewage sludge treatment.

Subsequently, Cai et al. (2018) prepared a mixture of substrate containing 3:1:1 of sewage sludge, chicken manure and bran to rear the BSFL. The frass from this sample was later tested and was reported to be able to meet the fertilizer standard in China, although no exact value was given. Yet, it was worthy to highlight that the initial heavy metal contents in this mixture were already lower than the concentration set by China fertilizer standard. In addition, it was also stated that the extracted larval oil from G7 contained lesser than 1% of the heavy metal content of its body, making biodiesel production from BSFL a viable approach (Cai et al., 2018).

Next, in the work of Wu et al. (2020), they investigated the fate of Cu and Cd when BSFL was fed with wheat bran that was added with traces of heavy metals. Although Cu is a recognized as a necessary trace element for the proper growth and health of animals, no statistically significant changes on larvae weight was observed when Cu and Cd concentration was increased until 400 mg/kg and 80 mg/kg substrate, respectively. As the heavy metal concentrations in feed increased, both Cu and Cd contents in the larval body increased. Nevertheless, despite the increase of Cu content in the larval body, the content of Cu in its body was remained at an average of 30%, while 70% of the remaining Cu was consistently excreted as feces, also known as frass. The ability for an insect to excrete heavy metals as feces and exuviae had been reported as a method for the insect to regulate its internal metal concentrations (Jiang et al., 2018). In contrast, the Cd content in larval body was at the highest of 90% during low Cd feed concentration. However, as the concentration of Cd in feed increased, the composition of Cd in the larvae body decreased drastically to slightly below 50%. Despite the drastic reduction of Cd content in larvae body itself, the Cd content in larvae body was still exceptionally high (50–90%), as compared to Cu (10–30%). This was aligned with the observation of other works that BSFL had a higher bioaccumulation capacity for Cd than other heavy metals, resulting in a high Cd content in the larval body (Biancarosa et al., 2018; Diener et al., 2015). A subsequent leaching test by Wu et al. (2020) showed that residual form (F5) of Cu and Cd was always the dominant form of heavy metal in the frass for all samples. Nonetheless, the concentration of Cu in residual form seems to reach a plateau after substrate's Cu concentration exceeds 100 mg/kg. When concentration of Cu in substrate exceeds 100 mg/kg, other less stable form of Cu such as exchangeable (F1) and acid soluble (F2) starts to spike drastically.

The fate of Zn and Pb which are two common heavy metals in sewage sludge were also studied. Diener et al. (2015) reported that for a substrate with 2000 mg/kg of Zn, which resembles the concentration in sewage sludge, most Zn content was concentrated in the frass after the BSFL treatment, i.e., 3313 mg/kg. Subsequently, the Zn contents were also found in the larval exuviae at 1883 mg/kg and the larvae body at 866 mg/kg. Similarly, after feeding substrate containing Pb at 125 mg/kg, the highest Pb concentration was recorded in larval exuviae at 312.9 mg/kg, followed by frass at 267.9 mg/kg and finally larvae body at 141.7 mg/kg. These findings validated that the heavy metal content was always the highest in frass rather than larval body. Furthermore, it shows that BSFL can dispose heavy metals during moulting as exuviae, similar to terrestrial insects (Diener et al., 2015).

4.6. Pathogen reduction

The pathogen inhibition ability of BSFL had been studied by Awasthi et al. (2020b) using sewage sludge and several kinds of animal manures (chicken, cow and pig). Depending on the type of animal manures, the reduction of pathogen contents when treated with BSFL had increased by 20–60% as compared with the control without BSFL. The highest reduction of pathogen content was observed for chicken manure, where the reduction percentage increased from ~20% to ~80%. Pig manure showed the least improvement from ~30% to ~55%. Nonetheless, when the sewage sludge was tested, the reduction of pathogen decreased from ~42% to ~15%. This could be due to the increase of pathogen in the order of *Clostridiales*, *Bacillales* and *Lactobacillales* sp. However, the more commonly known pathogen including *Enterococcus faecalis* EnGen0369, *E. coli*, *Bacillus cereus* and *Staphylococcus aureus* M0406 species were all shown to reduce after BSFL treatment.

A similar observation by Liu et al. (2008) reported that BSFL could significantly reduce the *E. coli* content in dairy manure. The study was solely focusing on the reduction of *E. coli* bacteria as the dairy manure was first sterilized before inoculating 7 log cfu/g (colony forming unit/g) of *E. coli* into each sample. Depending on the amount of manure fed, 87–96.7% reduction of *E. coli* could be observed. The highest pathogen reduction of 96.7% was recorded for the sample fed with the highest manure amount while at the same time producing larvae with the highest mean weight. It could be inferred that by increasing the substrate feeding amount, it could increase the growth of BSFL and subsequently, improving its ability in *E. coli* removal. Furthermore, the effect of temperature on BSFL removing *E. coli* at between 23 °C and 35 °C was evaluated (Liu et al., 2008). Generally, the pathogen removal ability increased with increasing temperature. Indeed, at 35 °C, no *E. coli* was observed. However, the control (without BSFL) at 35 °C showed nearly a 2-fold decrease in *E. coli* content as compared with control at lower temperature. This signified that when the temperature was manipulated, the BSFL was no longer be the only variable that could contribute to the pathogen reduction, but temperature played a significant role in destroying the pathogen as well.

4.7. Co-substrates

Judging by the growth of BSFL, all previous works (Lalander et al., 2019; Cai et al., 2018; Liu et al., 2020b) had shown that the sewage sludge was not a favourable substrate for BSFL. The suitability of sewage sludge as BSFL substrate, however, can be improved with the introduction of co-substrates. By gradually mixing sewage sludge and chicken manure at the ratio of 1:4, the biomass conversion rate increased from 1% to 8% (Cai et al., 2018). As a result, the BSFL weight gained had increased from 0.5 g to approximately 11 g. Next, in the work of Raksasat et al. (2021), the palm kernel expeller was tested and proved viable as a co-substrate due to its high protein and lipid contents. The optimum mixing ratio of sewage sludge and palm kernel expeller was reported to be at 2:3, in which the heaviest larva (46.9 mg) was detected in comparison to 14.38 mg when only sewage sludge was fed. However, further

increasing the proportion of palm kernel expeller would contrarily harm the larval growth as excessive protein content would induce the BSFL digestion system to carry out proteinogenic nitrogen detoxification process (Tschirner and Simon, 2015). Hence, while other nutritious organic wastes could be introduced as co-substrates, the optimization of mixing ratio is crucial for the development of BSFL.

Non-conventional organic matter such as biochar has also been tested as co-substrate. Beesigamukama et al. (2020) included 5–20 wt% of biochar into the substrate of brewery spent grain. The biochar used in the experiment was produced from the pyrolysis of rice husks. When the content of biochar was increased from 0 to 20 wt%, the weight of frass increased from 0.49 to 1.13 kg. This was accompanied by an increasing composition of nitrogen content being retained in the frass where the percentage increased from 37.1% to 56.2%. Accordingly, the increment in frass yield was mainly attributed to the lower waste degradation rate because of adding in biochar that was hard to be digested. The increased nitrogen content retained in frass could be due to the adsorption ability of NH_4^+ onto biochar (Agyarko-Mintah et al., 2017; Awasthi et al., 2016). Moreover, the concentration of nitrogen accumulated in the larvae biomass also increased from 4.8 g/100 g biomass to 11.6 g/100 g biomass (Beesigamukama et al., 2020). Not only that, the final larvae weight and waste-to-biomass conversion rate also increased with increasing of biochar amount added.

4.8. Fermentation

The quality of sewage sludge as a substrate could also improve through in-situ yeast fermentation. As shown by Wong et al. (2020), who had inoculated coconut endosperm waste with a commercial yeast species, *Saccharomyces cerevisiae*, observed an improvement in larval biomass conversion rate, growth rate and development time. When the yeast concentration was increased from 0 to 2.5 wt%, the waste-to-biomass conversion rate increased from 10% to 11.5%. Meanwhile, the growth rate increased by 30.76%, while the time needed to form prepupae was reduced from 15.5 to 13.5 days. This could be attributed to the fact that the yeast helped to breakdown the carbohydrates into monosaccharides. Another successful example in improving the substrate quality through in-situ fermentation was proven by using bacteria of the species *Bacillus* spp and *Paenibacillus polymyxa* (Rehman et al., 2019). Dairy manure, despite its high carbohydrate content, could not be served as a good substrate for BSFL due to the presence of high lignin (15.51 wt%), cellulose (26.85 wt%) and hemicellulose (14.71 wt%) contents, making the dairy manure hard for BSFL to digest. However, through the fermentation of dairy manure with the use of bacteria, the hard-to-digest carbohydrates could be degraded into simple carbohydrates thereby enhancing the nutritional value of dairy manure. Among them, the *Bacillus* strain (MRO_2) showed the highest lignin, cellulose, and hemicellulose reductions, and in turn demonstrating the greatest improvement in terms of larval weight and waste-to-biomass conversion rate. As compared with the control, the waste-to-biomass conversion rate was increased from 6.84% to 10.84%, with dry larvae mass increased from 16.35 to 25.94 g, while the larvae survival rate increased to 99.07% (Rehman et al., 2019). Analysis of primary sludge showed that 71.4 wt% of its total solids was made up of fiber-carbohydrate, which could be further breakdown into 32.2 wt% of cellulose, 2.5 wt% of hemicellulose and 13.6 wt% of lignin (Higgins et al., 1982). Hence, fermentation could be a promising method to degrade the complex carbohydrates in sewage sludge and improve its enrichment as a feeding substrate for BSFL.

5. Summary of biological treatment

These three methods of biological treatments of sewage sludge demand less energy consumption. However, the treatment duration required is much longer, ranging from 10 to 60 days, as opposed to the thermal treatment methods that only take a day or less. Nonetheless, the capital cost anticipated to build a vermicomposting and BSFL treatment systems is much lower, making them more feasible for application in developing countries. Without the involvement of high temperature, the nitrogen retention in the treated sludge residue is high, ranging from 68 to 79 wt%. Moreover, all of the biological treatments are capable of reducing pathogen to achieve at least the criteria for US EPA Class B biosolid without any pre-treatment entailed. Meanwhile, for the thermophilic anaerobic digestion, it can easily attain the standard of US EPA Class A biosolid. The mass reduction of sewage sludge happens during biological treatments, mainly through the removal of VS and water content, leaving the non-biodegradable particles to form the residue.

For carbon recovery purpose, the anaerobic digestion produces biogas and vermicomposting increases earthworm population, while BSFL treatment increases BSFL weight. Although the BSFL body lipid can be used to produce biodiesel, the yield is still very low. Furthermore, the anaerobic digestion manages to reduce the bioavailability of heavy metals, and thus stabilizing them. Vermicomposting has shown contrasting findings in terms of heavy metal stabilization, thus needing more works to conclude its performance. Meanwhile, BSFL has shown high survivability growing in heavy metals contaminated substrates. Changes in heavy metal concentrations also take place in the medium as BSFL ingest and store some of the heavy metals, as well as in the larval exuviae. Yet, no comprehensive study has investigated the bioavailability of heavy metals in frass after the BSFL treatment. A summary of the three biological treatments, including the treatment conditions, types of organic wastes, products and performances are presented in Table 4.

6. Challenges and ways forward

Anaerobic digestion of sewage sludge is a mature technology that has been deployed in many developed countries. The main limitation of the wide implementations of this technology is its high capital cost. The integration with MEC that produces more biogas also requires more investigations into identifying a cost-effective electrode for scaling up this promising technology. Although vermicomposting and BSFL valorization both show the potential in treating sewage sludge, there are still several challenges and limitations that need to be overcome as listed below.

a) Sewage sludge is not a suitable feeding substrate due to its low biodegradability and the presence of EPS, which locks all the nutrients within the gel-like structure.
b) The capabilities of vermicomposting and BSFL treatment in destroying pathogen only manage to reach the standard of US EPA Class B biosolid.
c) The capability of heavy metal stabilization is still inconclusive for vermicomposting and BSFL treatment.
d) Existing studies mainly operated in batch experimental mode. The earthworms or BSFL had to be separated manually from the residues once the treatment durations had ended. This is not sustainable for a scale-up process.
e) The earthworms and BSFL are both sensitive toward heat (Singh et al., 2019). The rearing condition should be moist for the optimum growth. Direct placement under a hot sun without shading will cause excessive evaporation of water from substrates. In contrast, the covered equipment placed under a hot sun will trap heat and raise the temperature inside the rearing system, retarding the growth of earthworms or BSFL. Hence, unlike anaerobic digestion or other thermal treatment methods of sewage sludge, vermicomposting and BSFL valorization require suitable growing environments to spur the reduction of substrates.

Common ways forward:

Table 4
Summary of biological treatment methods and their respective performances.

Treatment Method and Condition	Type of Organic Waste	Moisture Content	Generated product	Decomposed residual material	Volume/Weight Reduction	References
Anaerobic Digestion			Biogas	Digestate		
Duration: 4 weeks Temperature: 17 °C	Swine manure	NR	NR	Close to 100% N recovery in digestate. (67.1 wt% in supernatant, 31.5 wt% in settled digestate)	VS reduction: 77.3% TS reduction: 71.4%	(Massé et al., 2007)
Duration: NR Temperature: 30–57 °C	Secondary sludge	After thickening/belt filtration	Biogas composition: CH_4: 53–70% CO_2: 30–50% LHV: 23MJ/Nm^3	NR	VS reduction: 56–65.5%	(Appels et al., 2008)
Vermicomposting			Biomass (worms)	Vermicast		
Duration: 45 days Earthworm: *E. fetida*	Mixture of straw pallet and cattle manure	80–85 wt%	Biomass weight increment: ~80 wt%.	76.3–79.4% of N recovered in vermicast. Carbon content retained in vermicast: 54.5–56.5 wt%	NR	(Nigussie et al., 2016)
Duration: 45 days Earthworm: *E. fetida / E. eugeniae / P. Excavatus* or combination	Sewage sludge	50 wt%	Biomass weight increment: 9.09–28.57 wt%	TN concentration increased after vermicomposting.	TOC reduction: 10–25% in DM	(Khwairakpam and Bhargava, 2009)
BSFL Treatment (*Hermetia illucens)*			Biomass (BSFL), Biodiesel	Frass		
Duration: 32 – 51 days	Primary sludge, secondary sludge	82.5–91.7 wt%	Biomass conversion rate: 2.2–2.3% in DM Protein conversion rate: 7.8–15% in DM	NR	Weight reduction: 49.2–63.2 wt% in DM	(Lalander et al., 2019)
Duration: 24.3 days	Dairy manure	78.4 wt%	Biomass conversion rate: 6.3% in DM	68% of total nitrogen content retained in the residual.	Weight reduction: 25.8 wt% in DM 63.2 wt% of wet weight TOC reduction: 36% in DM	(Rehman et al., 2017)
Duration: 10 days	Animal manure (Cattle, pig, chicken)	65–70 wt%	Biomass Yield: 12.8–32.8 wt% Lipid Yield: 29.1–30.1 wt% Biodiesel/lipid yield: 93–96%	NR	NR	(Li et al., 2011)

NR: Not reported; DM: Dry mass

a) The effect of sewage sludge pre-treatment prior to vermicomposting and BSFL treatment should be examined. Breakdown of EPS releases more soluble nutrients into the substrate, and subsequently boost the growths of earthworms and BSFL (Dohányos et al., 2004; Ruffino et al., 2015). Furthermore, certain pre-treatments such as thermal treatment and chemical treatment are also expected to assist in pathogen reduction.
b) Continual research and optimization of blended substrates using the locally available co-substrates are the low hanging fruits that can boost the growth of both earthworms and BSFL.

Specific challenges and ways forward for BSFL valorization:

a) Current yield of biodiesel is still very low due to the low conversion rate of organic wastes into BSFL biomass. This is a limitation before BSFL treatment can be popularized for the purposes of carbon recovery and biodiesel production. The biodegradability of substrates has to be improved for attaining a better growth of BSFL. Thus, pre-treatment such as thermal treatment, enzymatic treatment, fermentation, hydrolysis, etc. should be investigated.
b) BSFL feeding on sewage sludge have a very low body weight as opposed to those feeding substrates which are more biodegradable such as fruit waste and kitchen waste. A light prepupa will later convert into a small adult fly that has less body fat. This could affect the lifespan of the BSF in terms of whether it has a sufficient stored energy to complete one whole lifecycle by mating and ovipositing thereafter. Without sufficient healthy BSF eggs, the entire sludge treating process is not sustainable and has to depend on the procurement of new BSF eggs. Thus, the sustainability of treating sewage sludge via BSFL employment needs more inclusive investigations.
c) Unconventional co-substrates such as biochar, fly ash and phosphatic rock have shown synergistic effects on stabilizing heavy metals and improving the overall performances of anaerobic digestion, thermal treatment and vermicomposting (Liew et al., 2021; Babel et al., 2009; Zhang and Wang, 2020; Suthar, 2010). Similar additives can also be vindicated in BSFL valorization.

7. Conclusions

This paper reviews extensively the performances of three biological treatment methods of sewage sludge, comprising anaerobic digestion, vermicomposting and BSFL valorization. Anaerobic digestion of sewage sludge is a mature technology that has seen numerous industrial applications. Vermicomposting of sewage sludge has also been conducted in full-scale but together with co-substrate. Meanwhile, BSFL valorization of sewage sludge is only tested in lab-scale. All these three treatment methods have high nitrogen recovery capabilities. The sewage sludge mass reductions are comparable with one another, but depending on the treatment durations in which could range between 30% and 63%. Anaerobic digestion is superior in carbon recovery, as it can generate biogas. BSFL treatment could potentially generate biodiesel if the limitation of low larval lipid yield is resolved. The residue from all three treatments can easily achieve the pathogen standard of US EPA Class B biosolid. Yet, it can be improved to Class A biosolid via thermal pre-treatment of sewage sludge at 65 °C for an hour. Effective heavy metals stabilization had been reported for anaerobic digestion, but no conclusive finding has substantiated the effectiveness of either vermicomposting or BSFL treatment. Thus far, a comparison with anaerobic

digestion shows that both vermicomposting and BSFL treatment are the potential low-cost sewage sludge treatment methods to be deployed in developing countries. Nonetheless, until the listed challenges have been addressed, more innovative solutions have to be assessed and validated prior to the executions.

CRediT authorship contribution statement

Chin Seng Liew: Conceptualization, Writing – original draft, Writing – review & editing. **Normawati M. Yunus:** Writing – review & editing. **Boredi Silas Chidi:** Writing – review & editing. **Man Kee Lam:** Writing – review & editing. **Pei Sean Goh:** Writing – review & editing. **Mardawani Mohamad:** Writing – review & editing. **Jin Chung Sin:** Writing – review & editing. **Sze Mun Lam:** Writing – review & editing. **Jun Wei Lim:** Conceptualization, Supervision, Writing – review & editing, Validation, Funding acquisition. **Su Shiung Lam:** Conceptualization, Supervision, Writing – review & editing, Validation, Funding acquisition.

Declaration of Competing Interest

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

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Biowaste treatment with black soldier fly larvae: Increasing performance through the formulation of biowastes based on protein and carbohydrates

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ABSTRACT

A key challenge for black soldier fly larvae (BSFL) treatment is its variable reliability and efficiency when applied to different biowastes. Similar to other biowaste treatment technologies, co-conversion could compensate for variability in the composition of biowastes. Using detailed nutrient analyses, this study assessed whether mixing biowastes to similar protein and non-fibre carbohydrate (NFC) contents increased the performance and reduced the variability of BSFL treatment in comparison to the treatment of individual wastes. The biowastes examined were mill by-products, human faeces, poultry slaughterhouse waste, cow manure, and canteen waste. Biowaste formulations had a protein-to-NFC ratio of 1:1, a protein content of 14–19%, and a NFC content of 13–15% (dry mass). Performance parameters that were assessed included survival and bioconversion rate, waste reduction, and waste conversion and protein conversion efficiency. In comparison to poultry feed (benchmark), vegetable canteen waste showed the best performance and cow manure performed worst. Formulations showed significantly improved performance and lower variability in comparison to the individual wastes. However, variability in performance was higher than expected for the formulations. One reason for this variability could be different fibre and lipid contents, which correlated with the performance results of the formulations. Overall, this research provides baseline knowledge and guidance on how BSFL treatment facilities may systematically operate using biowastes of varying types and compositions.

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1. Introduction

The treatment of biowaste by black soldier fly larvae (BSFL) is an emerging waste management technology (Čičková et al., 2015; Gold et al. 2018b; Zurbrügg et al., 2018). This process converts waste into larval biomass, reduces waste dry mass and generates the raw materials for the production of soil conditioner and fertilizer (Setti et al., 2019), lubricants and biodiesel (Leong et al., 2016; Li et al., 2011), pharmaceuticals (Vilcinskas, 2013) and animal feeds (Barragán-Fonseca et al., 2017; Makkar et al., 2014; Sánchez-Muros et al., 2014; Wang and Shelomi, 2017).

A key challenge for BSFL biowaste treatment is its variable reliability and efficiency. Currently, performance—as measured by bioconversion rate, larval weight, and larval biomass composition (e.g. protein and lipid content)—varies both when using the same type of biowaste (e.g. different vegetable wastes) and when treating different types (e.g. vegetable waste compared to mill by-products) (as summarised by Gold et al., 2018a). The sustainable operation of BSFL biowaste treatment facilities likely depends on the use of different waste types of varying quantity and composition. Homogenous or highly nutritious biowastes such as food industry

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by-products (e.g. bread and mill by-products) or canteen and restaurant wastes are often already used elsewhere (e.g. as animal feeds and for energy recovery). In addition, poor waste management practices, such as a lack of organic waste segregation, incentives for landfill disposal, and complex collection and transport logistics, often hinder access to high-quality wastes. Importantly, the use of different wastes from different sources will adversely affect the day-to-day operation (e.g. running over or under capacity) of BSFL treatment facilities with concurrent impacts on BSFL growth and waste treatment performance. This affects the sustainability (Mertenat et al., 2019; Smetana et al., 2019, 2016) and scalability of this technology and the down-stream application of products (e.g. live-feed for aquaculture) (Gold et al., 2018a).

Similar to other animal species used for production, the nutrient content of biowaste is hypothesised to have the largest influence on performance under similar operating conditions (e.g. feeding rate, larval density and temperature) (Nguyen et al., 2013; Oonincx et al., 2015a; Tinder et al., 2017). Factors determining the nutritional quality of biowaste include the density, ratio and type of nutrients it contains. Nutrients considered to be decisive include the sum of all macronutrients, organic matter, protein, non-fibre carbohydrates (NFC), fibre and lipids (Barragán-Fonseca et al., 2018a, 2018b; Gold et al., 2018a; Lalander et al., 2018). For example, manures are typically low in organic matter and fibre, restaurant and canteen wastes are rich in NFC and lipids, and fruit and vegetable wastes are low in proteins (Gold et al., 2018a). In response to these different nutritional conditions, fly larvae adjust their growth rate and nutrient accretion, with the main goal of accumulating enough reserves to complete the non-feeding life-stages of metamorphosis and adulthood (Danielsen et al., 2013; Gold et al., 2018a). Similar to other animals, an insufficient amount or an unfavourable ratio of nutrients prolongs development, reduces growth and related biomass production, and limits the efficiency of waste reduction (Danielsen et al., 2013).

BSFL feeding experiments and assessments of the midgut (the main organ involved in digestion) suggest that protein, NFC and lipids are highly digestible by BSFL and, therefore, their supply enhances performance (Barragán-Fonseca et al. 2018a; Beniers and Graham, 2019; Bonelli et al., 2019; Lalander et al., 2018). In contrast, fibre including cellulose and lignin are less digestible and tend to decrease larval growth rates (Liu et al., 2018). Among these nutrients, several studies have concluded that the protein (and amino acid) content of biowastes is most important. For example, Lalander et al. (2018) concluded that protein has the greatest impact on the development time to prepupa. Beniers and Graham (2019) also observed that protein has greater importance for larval weight than NFC. As amino acids allow larvae to proceed to the next instar (Gold et al., 2018b) and BSFL accumulate lipids during later instars (as energy reserves for later life-stages) (Liu et al., 2017), waste with a greater protein content can also increase larval lipid content. Research on the common fruit fly larvae (*Drosophila melanogaster*) indicates that fly larvae control their feeding with respect to protein and may overfeed on other nutrients such as NFC (Almeida de Carvalho and Mirth, 2017). This further emphasises that protein is so essential for development. For BSFL, protein supply may influence larval weight and lipid content when receiving low-protein and high-carbohydrate feeds as carbohydrates may be converted into body lipids (Pimentel et al., 2017; Spranghers et al. 2017). By contrast, lipids in biowaste can impede or promote larval development. Nguyen et al. (2013) suspected that excess lipids in fish offal may decrease larval development; however, results from Oonincx et al. (2015a) for food industry by-products and from Nguyen et al. (2013) for liver and kitchen waste suggest that lipids can also increase performance as the energy density of the feed is increased (Brouwer, 1965). Oonincx et al. (2015a) also observed high feed-conversion efficiencies for feeds with a high lipid and protein content. The ash content of biowaste positively correlates with larval ash content (Spranghers et al. 2017) and negatively with bioconversion rates (Lalander et al., 2018).

A reliable high-performance BSFL treatment for biowaste requires strategies that build on existing knowledge of the influence of variable waste nutrient compositions on larval performance. Similar to other biowaste treatment options such as anaerobic digestion or composting, co-conversion, i.e. the treatment of a mixture of several biowastes, could increase performance and reduce variability (Li et al., 2009). Specifically, mixing multiple biowastes can provide a more nutritious and balanced feed for larval growth. Rehman et al. (2017) and Nyakeri et al. (2019) observed that mixing cow and human manure with food wastes and food production by-products (e.g. soybean curd residue and banana peels) increased larval weight compared to these individual wastes. Similar to composting (i.e. carbon-to-nitrogen ratio) and anaerobic digestion (e.g. methane potential), a systematic approach to co-conversion based on biowaste nutrients could compensate for the variability in biowaste composition.

The formulation of appropriate biowaste mixtures based on nutrients requires the reliable determination of composition using parameters that are relevant for BSFL growth. Such an approach—and also incorporating cost considerations—is widely applied for feed formulation in commercial livestock production (McDonald et al., 2011). Barragán-Fonseca et al. (2018b) were the first to formulate feeds for BSFL with similar protein and NFC contents using combinations of food industry by-products; however, larvae still performed significantly different between these waste mixtures. These authors concluded that nutrient quality (e.g. amino acid content, type of NFC and fibre) must be considered to reduce this variability. Previous studies have not considered this sufficiently when determining biowaste composition. For example, biowaste has been characterised based on its carbon, nitrogen and protein content (using generic nitrogen-to-protein conversion factors) or the sum of other nutrients to estimate its NFC content (Barragán-Fonseca et al. 2018b; Lalander et al., 2018). Nitrogen may not, however, be an accurate measure as it may also include non-protein nitrogenous compounds of low nutritional value such as urea, ammonia, nitrate and nitrite (Chen et al., 2017). Similarly, carbon includes digestible fibre such as cellulose and lignin. The importance of these compounds is difficult to assess as the extent to which non-protein nitrogen and fibre are used by the gut microbes of BSFL is not yet known. Thus, generic nitrogen-to-protein conversion factors may overestimate protein content. In addition, carbon may greatly overestimate NFC when the ash, lipid, protein and fibre content is subtracted from 100% rather only than the sum of digestible carbohydrates such as glucose and starch.

This study aimed to assess the performance of BSFL treatment as applied to different waste formulations prepared from six types of biowaste following the determination of their respective nutritional composition. It was hypothesised that biowaste formulations with a similar protein and NFC content would increase performance and reduce variability in comparison to the individual wastes. Thereby, this research sought to generate knowledge and advice on how BSFL treatment facilities may best operate with biowaste of varying type and composition.

2. Materials and methods

2.1. Biowastes used in the feeding experiments

Six different types of biowaste were used in feeding experiments, namely mill by-products, human faeces, poultry slaughterhouse waste, cow manure, and canteen and vegetable canteen

waste. Two different batches of human faeces were used as BSFL treatment performance was unexpectedly high with the first batch. Mill by-products were obtained from a Swiss wheat-milling company. The human faeces were obtained from dry toilets separating urine and faeces at the Swiss Federal Institute of Aquatic Science and Technology (Eawag) in Dübendorf, Switzerland. The poultry slaughterhouse waste consisted of discarded body parts (feet, head, liver, stomach, and intestine) from a poultry slaughterhouse of Micarna, a leading meat processing company in Switzerland. The cow manure was obtained from a farm near Zurich, Switzerland. The vegetable waste was obtained from the Eawag canteen and consisted of a mixture of vegetables with and without salad dressing. The difference between the vegetable canteen waste and the canteen waste was that the latter had the addition of sausage and other meat offal.

Following their collection, the wastes were homogenised with a kitchen blender to mimic the pre-treatments used in BSFL treatment facilities (Dortmans et al., 2017), and moisture content was determined in duplicate with a halogen moisture analyser (BM-65, Phoenix instrument, Garbsen, Germany) The wastes were then portioned into plastic bags, frozen and stored at −20 °C until the start of the feeding experiments (Diener et al., 2009; Lalander et al., 2018; Myers et al., 2008; Nguyen et al., 2015). The wastes were thawed at 4 °C for 24 h and brought to the experimental temperature of 28 °C prior to each feeding experiment.

2.2. Composition of the experimental biowastes

Oven-dried (105 °C) wastes and poultry feed (used as a high-performance benchmark) were analysed for gross nutrient composition, moisture content and pH using standard procedures (AOAC 1997; Van Soest et al., 1991). The second batch of human faeces was only analysed for protein, lipid and organic matter content. Moisture and organic matter were determined in quintuplicate with an automatic thermogravimetric determinator (TGA-701, Leco, St. Joseph, MI, USA). Nitrogen content was determined in triplicate using a C/N analyser (Type TruMac CN, Leco Cooperation, St. Joseph, MI, USA). Fibre fractions including neutral (NDF) and acid detergent fibre (ADF) were assessed in duplicate using a fibrebag system (Fibretherm, Gerhardt Analytical Systems, Germany) according to methods 6.5.1 and 6.5.2 of the Association of German Agricultural Analytic and Research Institutes (Naumann et al., 2012). Lipids were analysed from ether extracts from freeze-dried samples by Eurofins Scientific, Schönenwerd, Switzerland, according to Regulation (EC) No 152/2009 (European Commission (EC) 2009). The extraction solvent used was petroleum ether at 40–60 °C following hydrolysis with 3 M hydrochloric acid. pH was analysed with a portable meter and pH probe (HQ40d, Hach Lange GmbH, Switzerland).

Amino acids were analysed in triplicate in freeze-dried samples (Çevikkalp et al., 2016; Kwanyuen and Burton, 2010; White et al., 1986; Zhang et al., 2009). The samples were hydrolysed at 110 °C for 16–24 h with 5 M sodium hydroxide (tryptophan) or 6 M hydrochloric acid containing 0.1% phenol (for all other amino acids). For tryptophan, the hydrolysed samples were subsequently neutralised, diluted and analysed by RP-HPLC-FLD using an Agilent 1200 series LC-system including a fluorescent detector (FLD) (Agilent Technologies, Santa Clara, USA) and a C18 analytical Pico Tag amino acid analysis column (3.9 × 150 mm) in combination with a Nova-Pak C18 guard column (3.9 × 20 mm) (Waters AG, Baden, Switzerland). The fluorescence detector was operated at an excitation wavelength of 280 nm and an emission wavelength of 340 nm. For all other amino acids, the hydrolysed amino acids were transformed into their phenylthiocarbamyl derivatives with phenyl isocyanate and analysed by RP-HPLC using an Agilent 1100 series LC-system including a diode array detector (DAD) operated at 254 nm (Agilent Technologies, Santa Clara, USA) and the same column as above. α-methyl-DL-tryptophan and L-norleucine was used as an internal standard. The HPLC results were corrected with the respective recovery rates of the internal standards. Only results with an internal standard recovery >70% were considered further. More details on amino acid analyses are included in the Supplementary Material.

Glucose and starch were determined in triplicate using freeze-dried samples with a commercial enzyme assay (Megazyme, 2019). In brief, glucose was removed from each sample with ethanol. Then, following centrifugation, the glucose concentration was determined in the supernatant and the pellet was used for starch analysis. Resistant starch was converted into maltodextrins in potassium hydroxide. Amylase and amyloglucosidase were used to hydrolyse the remaining starch into glucose. Glucose was then quantified with a spectrometer (Genesys 10S, Thermo Fisher Scientific, USA) in comparison to a glucose standard.

Protein was calculated by multiplication of the nitrogen results with specific conversion factors, namely 5.6 for poultry feed (based on results for maize and soybean meal) (Sriperm et al., 2011), 4.3 for cow manure (Chen et al., 2017), 5.4 for mill by-products (based on results for cereals) (Mariotti et al., 2008), 5.4 for canteen waste, 5.0 for vegetable canteen waste, and 5.0 for poultry slaughterhouse waste (based on results for meat, fish, cereals and vegetables) (Mariotti et al., 2008). No conversion factors were available for human faeces and so this was estimated as the ratio of the sum of all amino acids divided by the nitrogen content. Samples of human faeces (mixed with sawdust) (Nyakeri et al., 2019) and pit latrine sludge provided by Sanergy, Nairobi, Kenya, were also included in the analysis to cover the typical variability of human faeces and faecal sludge (Gold et al., 2017b, 2017a). Caloric content was estimated by multiplying the mean results for lipids, NFC and protein with their gross caloric content of 9.4, 5.4, and 4.1 kcal/g, respectively (Merrill, 1973; Wu, 2016). Hemicelluloses were determined as the difference between NDF and ADF. ADF was assumed to be a reliable estimate of cellulose and lignin content. The sum of glucose and starch was assumed to reflect the total NFC.

2.3. Formulation of the biowaste mixtures

For the feeding experiments, either the six individual wastes or six mixtures of the wastes (Table 1) were used. The mixtures were based on the composition of the biowastes and aimed to achieve a protein-to-NFC ratio of approximately 1:1 (DM) considering the low content of NFC (Barragán-Fonseca et al. 2018b; Cammack and Tomberlin, 2017). In contrast to Barragán-Fonseca et al. (2018b), no high-value ingredients such as sunflower oil or cellulose were added to balance the unavoidable variability in fibre and lipid content as this is not typically practicable for cost reasons.

The formulations were generated using Visual Basic for Applications in Microsoft Excel and were always based on mill by-products complemented with two to three other wastes. The formulations were prepared from thawed wastes on the day of feeding and were mixed thoroughly. Formulation 3 was prepared with two different batches of human faeces based on the nutrient composition of the first batch. These batches appeared to have a similar composition based on their lipid (20.9 and 19.3% DM), crude protein (20.5 and 21.8% DM) and ash (13.7 and 15.8% DM) content. In the following discussions, the two human faeces formulations are referred to as formulation F3 (1) and formulation F3 (2), respectively.

Table 2 shows the realised nutrient composition of the six formulations, calculated based on the proportions shown in Table 1 and the results of the analyses of the individual biowastes. The

Table 1
Dry mass proportion of individual wastes in the biowaste formulations (F1-F6).

Formulation	F1	F2	F3	F4	F5	F6
Mill by-products	23	37	51	60	33	65
Canteen waste	–	7	–	20	33	–
Human faeces	16	–	14	20	–	–
Poultry slaughterhouse waste	–	–	–	–	–	22
Cow manure	11	35	34	–	–	12
Vegetable canteen waste	50	21	–	–	33	–

Table 2
Mean dry mass nutrient contents of the different biowaste formulations (F1-F6) based on the percent dry mass proportion of individual wastes in the biowaste formulations (Table 1) and the composition of their constituent wastes (Table 3).

	Proteins	Non-fibre carbohydrates	Fibres	Lipids	Organic matter	Moisture content
Formulation 1 (F1)	13.8	13.6	38.5	19.0	90.5	80.8
Formulation 2 (F2)	14.0	13.0	48.7	11.2	88.9	81.5
Formulation 3 (F3)*	14.0	12.7	50.1	5.9	88.4	79.9
Formulation 4 (F4)	19.1	15.8	43.8	13.0	92.1	72.5
Formulation 5 (F5)	19.6	15.4	39.8	22.3	93.1	76.9
Formulation 6 (F6)	19.0	15.4	45.8	12.0	92.1	73.9
Mean	**16.6 (2.9)**	**14.3 (1.4)**	**44.5 (4.7)**	**13.9 (5.9)**	**90.7 (2.2)**	**77.6 (3.7)**

In parentheses: standard deviation.
* Formulation (3) 1.

formulations contained between 14 and 19% DM of protein and between 13 and 15% DM of NFC.

2.4. Feeding experiments

Feeding experiments were designed as outlined by Lalander et al. (2018) and Liu et al. (2018). Three individual sets of experiments with different batches of larvae were carried out. First with the individual wastes (experiment 1) and then with formulations 1 to 3 (experiment 2) and finally with formulations 4 to 6 (experiment 3). Larvae were obtained from the BSFL research colony at Eawag maintained according to Dortmans et al. (2017). The BSFL hatched within 24 h and were first fed *ad libitum* with poultry feed (UFA 625, UFA AG, Switzerland) for 12–14 d until they reached a mean individual weight of 3.8 ± 0.5 mg DM. The larvae had a similar content of carbon (55–56% DM), protein (36–38% DM) and ash (13–14% DM) across the experiments (Section 2.5). From these populations, 4 to 5 × 80 randomly selected larvae per treatment were manually counted and placed in plastic containers (7.5 cm diameter, 11 cm height) with individual wastes or waste formulations, giving a larval density of approximately 2 larvae/cm^2. Larvae were also freeze-dried for the analysis of larval composition. The experimental containers were covered with paper towels or mosquito nets and randomly arranged in a climate chamber (HPP 260, Memmert GmbH, Germany) providing a steady microclimate of 28 °C and 70% relative humidity. Feed was provided every 3 d. Considering the increase in the nutrient requirements of BSFL with growth (Nyakeri et al., 2019), the feeding rate was increased over the 9-day experiment from 15 to 25 and 40 mg DM/larva per day on days 0, 3 and 6, respectively. Due to the expected improved nutritional quality of the formulations, the feeding rate was lowered by 25% for each feeding in experiment two and three.

In contrast to previous studies, which have typically terminated experiments after the first appearance of prepupae (Bosch et al., 2019; Lalander et al., 2018), all experiments were terminated after 9 d, before the appearance of prepupae. Prepupae are richer in chitin and lipids and, therefore, not optimal for animal feed applications (Nyakeri et al., 2019). Larvae were manually separated from the residue, cleaned with tap water, and dried with paper towels. Subsequently, larvae were manually counted, weighed and freeze-dried. Residues were dried in a laboratory oven at 80 °C. Both the dried larvae and the residues were then weighed and stored at 4 °C.

2.5. Analysis of larval composition

The dried larvae were milled and treatment replicates were combined equally by mass. Samples were then analysed in triplicate for DM, carbon and nitrogen content using the same analysers as for the wastes. Larval protein content was calculated as the nitrogen content × 4.67 following Janssen et al. (2017). Carbon content was divided by the total amount of organic matter; as lipids typically contain more carbon than proteins and carbohydrates (Brouwer, 1965), the ratio of carbon-to-organic matter was used as an indicator of larval lipid content.

2.6. Determination of the performance of BSFL treatment

Larval counts, and residue and larvae dry weights, were used to calculate five BSFL performance parameters. First, larval survival rates were calculated using Eq. (1) as the ratio of larvae at the end ($larvae_{end}$) and the beginning ($larvae_{beg}$) of the experiments (Van Der Fels-Klerx et al., 2016).

$$\text{Survival rate } (\%) = \frac{larvae_{end}}{larvae_{beg}} \times 100 \tag{1}$$

Waste reduction was calculated using Eq. (2) as the ratio of residue dry mass ($residue_{mass}$) to the dry mass of total feed ($feed_{mass}$) provided (Diener et al., 2009):

$$\text{Waste reduction } (\%\ DM) = \left(1 - \frac{residue_{mass}(g)}{feed_{mass}(g)}\right) \times 100 \tag{2}$$

The bioconversion rate was calculated using Eq. (3), for which the larval dry weight gain ($larval_{gain}$) was calculated as the difference between the final larval dry weight and the initial larval dry weight multiplied by the number of larvae at the end of the experiment:

$$\text{Bioconversion rate } (\%\ DM) = \frac{larvae_{gain}(g)}{feed_{mass}(g)} \times 100 \tag{3}$$

Waste conversion efficiency (Liu et al., 2018), also called efficiency of conversion of ingested/digested food (Diener et al., 2009; Oonincx et al., 2015b), was calculated using Eq. (4):

$$\text{Waste conversion efficiency } (\%\ \text{DM}) = \frac{larvae_{gain}(g)}{feed_{mass}(g) - residue_{mass}(g)} \times 100 \tag{4}$$

Finally, the protein conversion efficiency was calculated using Eq. (5) as the ratio of the amount of larval protein accumulated ($protein_{gain}$) to feed provided ($feed_{mass}$). Larval protein accumulated was calculated as the difference between the amount of final larval protein and the initial larval protein multiplied by $larvae_{end}$. The amount of larval protein was calculated by multiplying the larval protein content with the larval weight:

$$\text{Protein conversion efficiency } (\%\ \text{DM}) = \frac{protein_{gain}(g)}{feed_{mass}(g)} \tag{5}$$

2.7. Performance benchmark

As in previous research, poultry feed (60% moisture content) was fed to larvae in parallel to the individual biowastes and biowaste formulations as a high-performance benchmark (Lalander et al., 2018). As shown in Fig. 1, the results for poultry feed varied between experiments but no single experiment stood out as being different across all of the performance parameters. Even though larvae had a similar weight and composition at the start of the experiment, variability between experiments could be due to differences in age, feeding rates or other confounding factors (e.g. differences in airflow in the climate chamber due to varying numbers of containers).

To ensure a consistent basis for comparison between the three experiments, and between the individual wastes and waste formulations, performance parameters were also expressed as percentage differences (Fig. 1) in comparison to the results for the poultry feed using Eq. (6). For this, the results for each performance parameter ($Performance_{treatment}$) were subtracted from the median result obtained using the poultry feed ($performance_{benchmarkl}$) over all three experiments:

$$\text{Performance in } \% \text{ to benchmark} = \frac{performance_{treatment} - median\ performance_{benchmark}}{median\ performance_{benchmark}} \times 100 \tag{6}$$

Even though all of the parameters were corrected by the mass of total feed provided, variations in feeding rate, which differed between experiments, could influence performance results. Therefore, poultry feed and mill by-products were also fed to larvae at two different feeding rates: 27 mg DM/larva per day as used in experiment 1 and 20 mg DM/larva per day as used in experiments 2 and 3. This comparison, included in Supplementary Fig. S1, did not reveal an influence of feeding rate on performance, thus justifying the comparisons across the experiments.

2.8. Data analyses and statistics

Data were analysed using R software and RStudio version 1.1.463 (RStudio Inc., Boston, MA, USA). The mean, median, standard deviation, and range (difference of maximum and minimum) of the biowaste composition and performance parameters were calculated. Significance of differences in mean nutrient composition and mean performance parameters between the individual wastes and the waste formulations were tested using analysis of variance (ANOVA) followed by pairwise Tukey post-hoc comparisons. Due to the small size sample size per group (n = 3–5), normality and homogeneity of variance were assessed visually by residuals versus fits and Q-Q plots (Ricci et al., 2019). These graphs are shown in Supplementary Figs. S2–S4. A conservative p-value of <0.01 was chosen to declare significance due to the small sample size per group, which could lead to misinterpretation of model assumptions. The Mann–Whitney *U* test ($p < 0.05$) used to identify significant differences between the distributions of performance parameters of all the individual wastes (n = 29) in comparison to all the waste formulations (n = 29). The Levene and Shapiro-Wilk test ($p < 0.05$) identified that the data in those two groups violated the assumptions for parametric tests. Following visual assessment of normality (Supplementary Fig. S5), Pearson correlation coefficients ($p < 0.01$) were also calculated to identify linear dependencies between biowaste composition and feeding experiment results.

3. Results and discussion

3.1. Biowaste composition

Nutrient composition varied significantly between the biowastes (Table 3). The results for amino acids are included in Supplementary Table S1. The biowastes showed large variability

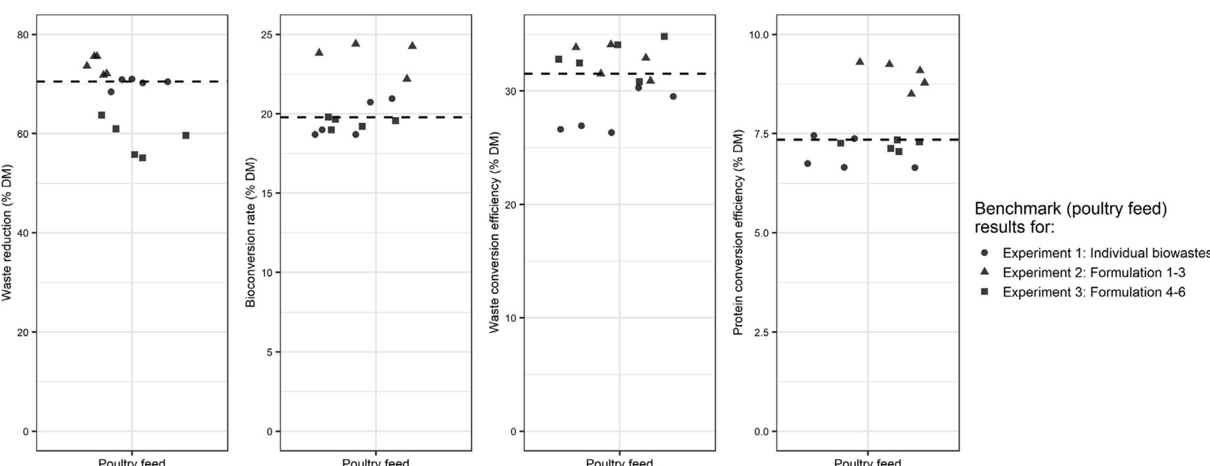

Fig. 1. Performance of BSFL fed on poultry feed used as a high-performance benchmark in the three experiments. Medians are shown as dashed lines. Performance results of the biowastes and formulations were expressed relative to these medians using Eq. 6.

Table 3
Mean nutrient composition of individual biowastes as percent of dry mass, moisture content in percent, pH, and caloric content as kcal per 100 g dry biowaste.

Wastes	pH	Moisture content	Protein	Non-fibre carbohydrates			Fibre			Lipids	Organic matter	P:NFC* ratio	Caloric** content
				Total	Glucose	Starch	Total	Cellulose & lignin	Hemicellulose				
Mill by-products	6.2 (0.1)	70.0	14.5^{d} (0.3)	23.2^{b} (0.2)	1.7^{b} (0.0)	21.2^{b} (0.6)	51.7 (0.9)	22.1 (1.0)	29.6 (1.9)	3.0	93.8^{c} (1.3)	1:2	211
Canteen waste	4.3 (0.0)	74.0 (1.2)	32.2^{b} (0.8)	7.5^{d} (0.7)	3.5^{a} (0.4)	4.0^{d} (0.4)	36.2 (1.4)	22.8 (0.6)	13.4 (0.9)	34.9	93.0^{c} (0.7)	4:1	501
Human faeces (1)	6.0 (0.0)	76.7 (0.9)	20.1^{c} (0.9)	1.7^{e} (0.1)	1.0^{bc} (0.0)	0.7^{e} (0.1)	27.9 (0.6)	19.5 (0.3)	8.4 (0.6)	20.9	86.4^{b} (0.3)	12:1	288
Poultry slaughterhouse waste	5.7 (0.1)	66.7 (1.2)	37.3^{a} (0.5)	0.3^{e} (0.1)	0.2^{d} (0.1)	0.1^{e} (0.0)	20.8 (1.9)	9.3 (0.9)	11.5 (2.7)	42.9	94.0^{c} (1.3)	152:1	557
Cow manure	7.2 (0.1)	87.0 (0.2)	11.1^{e} (0.4)	1.8^{e} (0.6)	0.7^{cd} (0.3)	1.0^{e} (0.4)	58.4 (0.4)	40.9 (1.7)	17.4 (1.2)	4.4	80.7^{a} (0.5)	7:1	96
Vegetable canteen waste	3.8 (0.0)	82.7 (0.1)	12.1^{e} (0.1)	15.5^{c} (0.9)	3.7^{a} (0.3)	11.6^{c} (0.6)	31.5 (1.8)	24.0 (1.5)	7.5 (0.3)	28.9	92.4^{c} (0.5)	1:1	404
Mean	**5.5 (1.3)**	**74.5 (10.0)**	**21.2 (10.2)**	**8.3 (9.2)**	**1.8 (1.5)**	**6.4 (8.4)**	**37.7 (14.5)**	**23.1 (10.2)**	**14.6 (8.2)**	**22.5 (16.3)**	**90.1 (5.4)**	**29:1**	**343(177)**
Poultry feed (benchmark)	5.7 (0.0)	60.0	19.1^{c} (0.7)	28.5^{a} (0.8)	0.5^{cd} (0.2)	27.5^{a} (1.4)	22.0 (1.0)	8.6 (0.0)	13.5 (1.1)	4.8	98.2^{ab}(4.0)	1:2	274

In parenthesis: standard deviation for samples where $n \geq 3$ and differences between analyses where n = 2.
results with no shared letter are significantly different from each other.
* P:NFC = ratio of protein to non-fibre carbohydrates (NFC).
** gross caloric content of protein, NFC, and lipids.

with respect to protein content, which was highest in poultry slaughterhouse waste, canteen waste and human faeces, and lowest in cow manure, vegetable canteen waste and mill by-products. Protein quality may also differ. In contrast to the other wastes, the protein in human faeces and cow manure was likely protein from gut microbial biomass (Rose et al., 2015).

This was the first study in which nitrogen-to-protein conversion factors were determined for human faeces and faecal sludge. Human faeces collected in Zurich and pit latrine sludge collected in Nairobi had conversion factors of 3.9 and 3.8, respectively. These conversion factors are comparable to those for animal manures in the range of 2.8–4.3 (Chen et al., 2017). In comparison, human faeces collected in Nairobi had less non-protein nitrogen, with a conversion factor of 5.2. Potential reasons for this difference could be the differing diets between the residents of Zurich and Nairobi (Rose et al., 2015) or storage conditions (e.g. temperatures) leading to the volatilisation of nitrogen. Overall, these results confirm that multiplying nitrogen results with the generic factor of 6.25 (i.e. the inverse of the mean nitrogen content of protein) can greatly overestimate true protein (i.e. amino acid) content (Mariotti et al., 2008). This is well established but has not been implemented even in recent BSFL research (Lalander et al., 2018; Liu et al., 2018).

Wastes were low in glucose, starch and total NFC. NFC was highest in the mill by-products and vegetable canteen waste. The addition of meat to the vegetable canteen waste increased protein content from below 15 to over 30% DM but concurrently decreased NFC content by half. Cow manure, human faeces, and slaughterhouse waste had almost no NFC. This was expected as animal tissue contains only very small amounts of glycogen and most NFC is digested or fermented in the gut of humans and animals (Riesenfeld et al., 1980). The sum of glucose and starch was much lower than when NFC was calculated as the difference between DM and ash, protein, fibre, and lipids. For example, the calculated value for human faeces was 17% DM compared to 1.7% DM for glucose plus starch (Rose et al., 2015; Spranghers et al., 2017). This indicates that there are either large amounts of non-sugar-non-starch-non-fibre organic matter or that there is an accumulation of analytical error in the gross nutrient measurements, or both. Overall, in the present study, low NFC was the reason why the protein-to-carbohydrate ratios in the waste formulations did not exceed 1:1 and that the mean NFC contents did not exceed 14% DM. The corresponding values described by Barragán-Fonseca et al. (2018b) and Cammack and Tomberlin (2017) were 1:1 to 1:2 and 21–30% DM, respectively.

The content of lipids, fibre and ash also varied among the wastes. Poultry slaughterhouse waste had a high lipid content and low ash and fibre content; the opposite was true for cow manure and mill by-products. The addition of meat to the canteen waste markedly increased the lipid content, while both of the canteen wastes were low in ash. Also, both batches of human faeces were rich in lipids, with values exceeding 20% DM. These results are high considering values ranging from 2 to 21% DM have been reported in the literature. The lipid content of the human faeces and pit latrine sludge samples collected in Nairobi were 9.4 and 16.6% DM, respectively. This suggests that the amount of lipids in faecal sludge can vary depending on management practices (e.g. residence time in the containment and the addition of sawdust) (Gold et al., 2017b), the presence of unabsorbed lipids, endogenous lipid losses (e.g. bile) and microbial processes (Aylward and Wood, 1962; Rose et al., 2015). Overall, the data show that the cow manure, human faeces, and poultry slaughterhouse wastes had low amounts of digestible nutrients and high protein-to-NFC ratios, whereas both the canteen wastes were rich in digestible nutrients and had a high caloric content However, it is unknown how much of this energy can be harnessed by the fly larvae. Similar to mill by-products, the vegetable canteen waste was balanced or slightly

NFC biased with proteins and NFC ratios of 1:1 and 1:2, respectively. Thus, these wastes were expected to perform best in feeding experiments when offered alone (Barragán-Fonseca et al. 2018b; Cammack and Tomberlin, 2017). For the other biowastes, mixing those with complementary nutrient compositions was expected to be advantageous.

3.2. Treatment performance of individual biowastes

All individual biowastes supported the development of BSFL (Table 4). The mean survival rates were 90–99% and were not significantly different between the biowaste types. They were also comparable to those found in previous research, where survival rates were shown to be above 80% (summarised by Rehman et al., 2017). Lalander et al. (2018) reported survival rates for different biowastes in the range of 81 to 100%, except for wastewater sludge which supported survival rates of only 39 to 81%. These results suggest that the experimental conditions applied in the present study were suitable and confirms that BSFL can develop on a wide variety of biowastes. That said, BSFL treatment performance varied widely between the different biowastes (Fig. 2). Mean performances values were significantly different among most of the biowastes and those that performed best were not always the same for each performance parameter. Waste reduction and protein conversion efficiency were lower for all of the wastes in comparison to the poultry feed, and cow manure had the poorest performance in all of the performance parameters.

Using vegetable canteen waste and mill by-products resulted in the highest waste reduction even though values were still 17 to 20% lower than for the poultry feed. This could be due to the high NFC content of these two wastes, which are easily digested and absorbed into the haemolymph of fly larvae (Bonelli et al., 2019; Pimentel et al., 2018). However, comparison of the waste reduction and larval weight results of mill by-products and human faeces demonstrate that this higher waste reduction did not necessarily result in higher larval weight. The level of waste reduction in the mill by-products exceeded the sum of easily digestible nutrients which are assumed to be reflected by the sum or protein, NFC and lipids (Table 3) based on the morphofunctional features of the BSFL midgut reported by Bonelli et al. (2019). This suggests that some fibre, likely hemicelluloses, were decomposed during BSFL treatment. Gold et al., 2018b also observed some decomposition of hemicelluloses in BSFL treatment with artificial diets, but this happened to a much smaller extend than that reported by Rehman et al. (2017) with cow manure. Such differences in digestibility have not yet been considered in biowaste formulation and could lead to unexpected performance results when designing formulations based on the glucose and starch content of NFC alone.

Human faeces supported a bioconversion rate that was comparable to the poultry feed (and this was higher for human faeces (1) and lower for human faeces (2)), despite having a much lower waste reduction. This was due to an 85% higher waste conversion efficiency than with the poultry feed. BSFL showed a significantly lower performance using poultry slaughterhouse waste than human faeces. The lowest performance was found for cow manure, which was low in protein, NFC, and lipids. In contrast, the human faeces and poultry slaughterhouse wastes were high in protein and lipids. These results thus suggest that NFC is less important for larval development than high overall nutrient content.

The protein conversion efficiencies were less variable and trends were different in comparison to the other performance parameters. Human faeces (1) and vegetable canteen waste had the highest bioconversion rate but not the highest protein conversion efficiency. This was due to varying larval composition arising from the different wastes and their associated larval weights. Larval protein content was notably higher when fed on mill by-products and canteen waste than on human faeces and vegetable canteen waste (Table 4). Larvae fed with human faeces and vegetable canteen waste likely incorporated more lipids, as indicated by a higher proportion of carbon in organic matter in comparison to the mill by-products and the canteen waste. Larva growing on human faeces contained the most ash.

Considering these findings, the most promising biowaste thus depends on the objective of the BSFL treatment. Mill by-products and vegetable canteen waste performed best with respect to waste treatment whereas human faeces and vegetable canteen waste were more favourable with respect to larval biomass production efficiency. The most protein per unit of biowaste was produced using the mill by-products and the canteen waste. Thus, for facilities targeting insect protein meal production, these wastes would be favourable. Poultry slaughterhouse waste and cow manure resulted in generally poor performance. However, not all wastes

Table 4
Mean performance of BSFL treatment on the different biowastes and formulations.

	Survival rate %	Larval weight mg DM	Waste reduction % DM	Bioconversion rate % DM	Larval biomass composition: Protein % DM	Ash % DM	Carbon % OM*
Individual wastes							
Mill by-products	96.2 (1.5)	41.7 (0.9)	56.4 (1.2)	14.9 (0.3)	42.1 (0.4)	7.3	58.0 (0.7)
Canteen waste	92.3 (3.1)	44.2 (5.9)	37.9 (3.8)	15.3 (2.1)	36.1 (0.3)	5.2	62.8 (0.3)
Human faeces (1)	99.1 (0.6)	58.8 (1.7)	39.1 (1.5)	22.7 (0.6)	26.7 (0.4)	13.6	65.8 (0.1)
Human faeces (2)	96.2 (2.5)	50.2 (1.2)	48.6 (0.3)	18.8 (0.8)	27.1 (0.1)	13.1	65.1 (0.6)
Poultry slaughterhouse waste	90.7 (2.9)	39.4 (0.7)	30.7 (4.7)	13.4 (0.5)	31.5 (0.7)	4.1	64.6 (0.4)
Cow manure	89.8 (7.5)	14.3 (0.4)	12.7 (0.9)	3.8 (0.2)	36.2 (0.2)	23.1	56.1 (0.3)
Vegetable canteen waste	97.5 (2.7)	59.1 (2.6)	58.4 (1.4)	22.7 (1.1)	24.5 (0.2)	5.1	65.4 (0.1)
Poultry feed (benchmark)	97.9 (2.1)	55.6 (5.1)	67.7 (6.9)	21.0 (2.4)	36.3 (0.8)	12.2	60.4 (0.7)
Waste formulations							
F 1	99.8 (0.6)	64.2 (1.1)	64.1 (0.6)	31.8 (0.6)	25.2 (0.3)	8.0	65.8 (0.2)
F 2	97.8 (3.7)	39.1 (0.3)	51.1 (0.7)	20.9 (0.9)	33.9 (0.5)	11.4	60.9 (0.4)
F 3 (1)	100.0 (0.0)	29.7 (1.2)	45.3 (1.1)	16.4 (0.7)	38.7 (0.3)	16.1	56.9 (0.3)
F 3 (2)	99.7 (0.6)	29.2 (2.0)	49.2 (1.6)	14.5 (1.1)	39.0 (0.6)	15.9	56.9 (0.6)
F 4	98.0 (1.4)	48.9 (2.4)	58.3 (1.1)	22.9 (1.1)	36.9 (0.3)	8.3	61.7 (0.5)
F 5	97.0 (3.4)	62.8 (1.6)	65.2 (2.0)	30.9 (1.6)	28.6 (0.3)	4.9	65.3 (0.7)
F 6	99.0 (1.0)	39.8 (2.1)	56.6 (0.7)	19.8 (1.1)	38.1 (0.3)	8.1	61.0 (0.2)

In parenthesis: standard deviation for samples where $n \geq 3$.
* OM = organic matter.

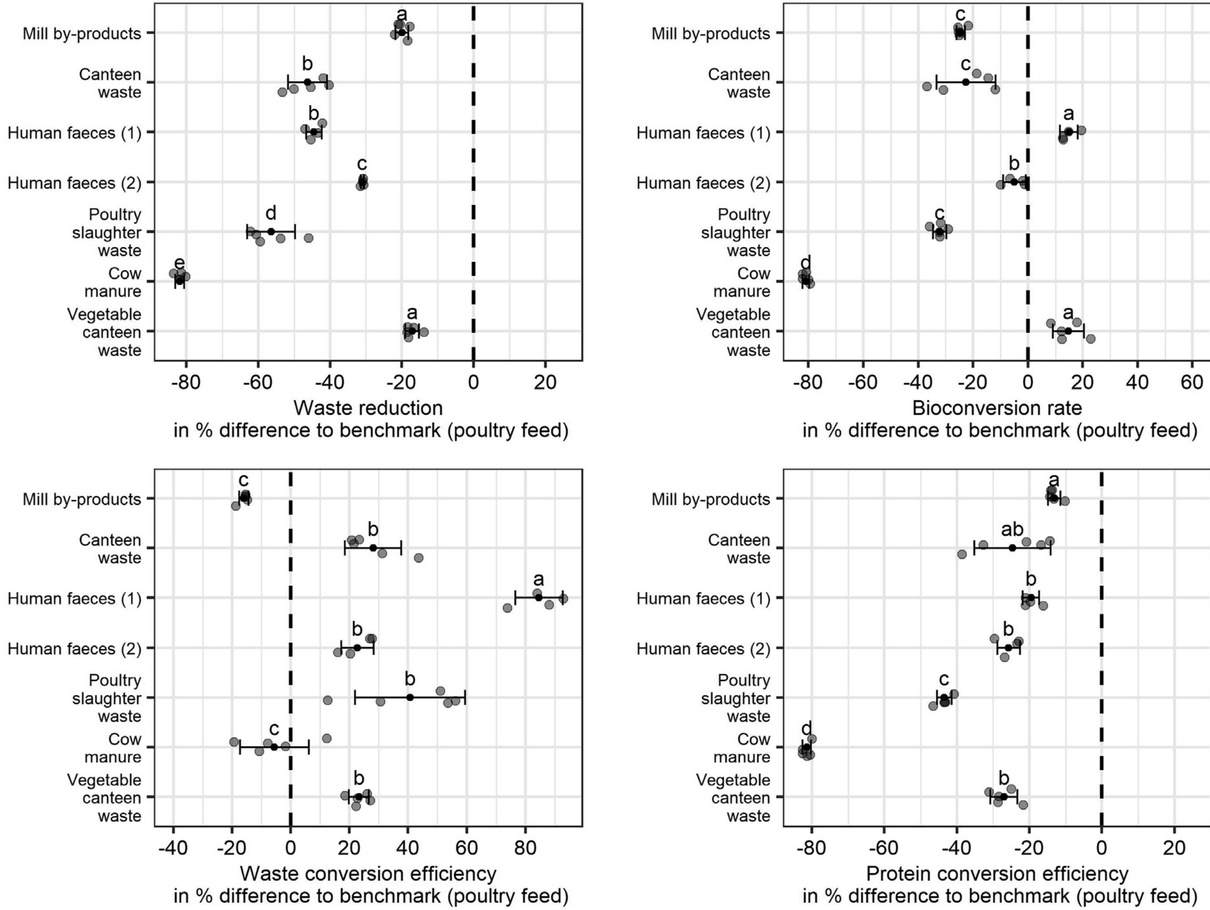

Fig. 2. Effects of the different individual wastes on waste reduction (top, left), bioconversion rate (top, right), waste conversion efficiency (bottom, left) and protein conversion efficiency (bottom, right) in comparison to the benchmark poultry feed (dashed vertical line). Means, standard deviations and results per replicate are displayed. Performance results with no shared letter are significantly different from each other. All results are given in dry mass.

can be employed in BSFL treatment facilities for animal feed production given legal resolutions (Lähteenmäki-Uutela et al., 2017). For example, in the European Union, only mill by-products and pre-consumer wastes (in nutrient composition similar to canteen wastes) can currently be used (European Commission (EC), 2017).

Food, restaurant, and canteen wastes also resulted in the highest—and animal manures the lowest—BSFL treatment performance in previous studies (Lalander et al., 2018; Nyakeri et al., 2019; Oonincx et al., 2015b). Lalander et al. (2018) reported a bioconversion rate of 14% DM for food waste in comparison to 15–23% DM for canteen wastes in this study. The corresponding values for waste reduction were 55% DM in comparison to 38–58% DM. For human faeces, the bioconversion rate was 11% DM as reported by Lalander et al. (2018) compared with 19–23% DM in the present study, and waste reduction data were 48% DM in comparison to 39–49% DM, respectively. In contrast to the present study, Lalander et al. (2018) observed a higher BSFL performance using slaughterhouse waste compared to food waste and human faeces. Values for waste reduction in the literature for cow manure range from 29 to 58% DM (Miranda et al., 2019; Myers et al., 2008; Rehman et al., 2017) and bioconversion rates range from 2 to 6% DM (Miranda et al., 2019; Rehman et al., 2017). This compares with a 13% DM waste reduction and a 4% DM bioconversion rate observed in the present study. These differences confirm that predicting larval performance exclusively based on the type of biowaste is not reliable and can lead to greatly over- or underestimated performance. Such variation is likely to result not only from variable biowaste composition (i.e. nutrient and microbial numbers and communities) but also differences in experimental setups. To help address this, international standards for BSFL feeding experiments could allow for better comparisons across studies.

3.3. Treatment performance of biowaste formulations

The performance of the BSFL grown on the different waste formulations was significantly different despite targeting a similar protein and NFC content and ratio (Table 4; Fig. 3). Overall, using a formulation significantly increased performance compared to individual wastes. Distributions were different between the waste formulations and individual wastes for survival rate, waste reduction, bioconversion rate and protein conversion efficiency but not for waste conversion efficiency.

Feeding BSFL with the waste formulations resulted in higher survival rates in comparison to the individual wastes, and ranged from 97 to 100%. Despite a 25% lower feeding rate, the median larval weight was 43.5 mg DM for the formulations and 40.1 mg DM for the individual wastes. The median of the survival rate was 99% for the formulations and 95% for the individual biowastes. Individual wastes resulted in the median waste reduction and bioconversion rate being lower, by 45.4 and 25.0%, respectively, compared to poultry feed. In comparison, the median waste reduction was only 18.5% lower—and the bioconversion rate even 8.6% higher—for the formulations compared to the poultry feed. The median protein conversion efficiency was 28.5% lower for the individual biowastes and 8.4% higher for the formulations comparison to the poultry

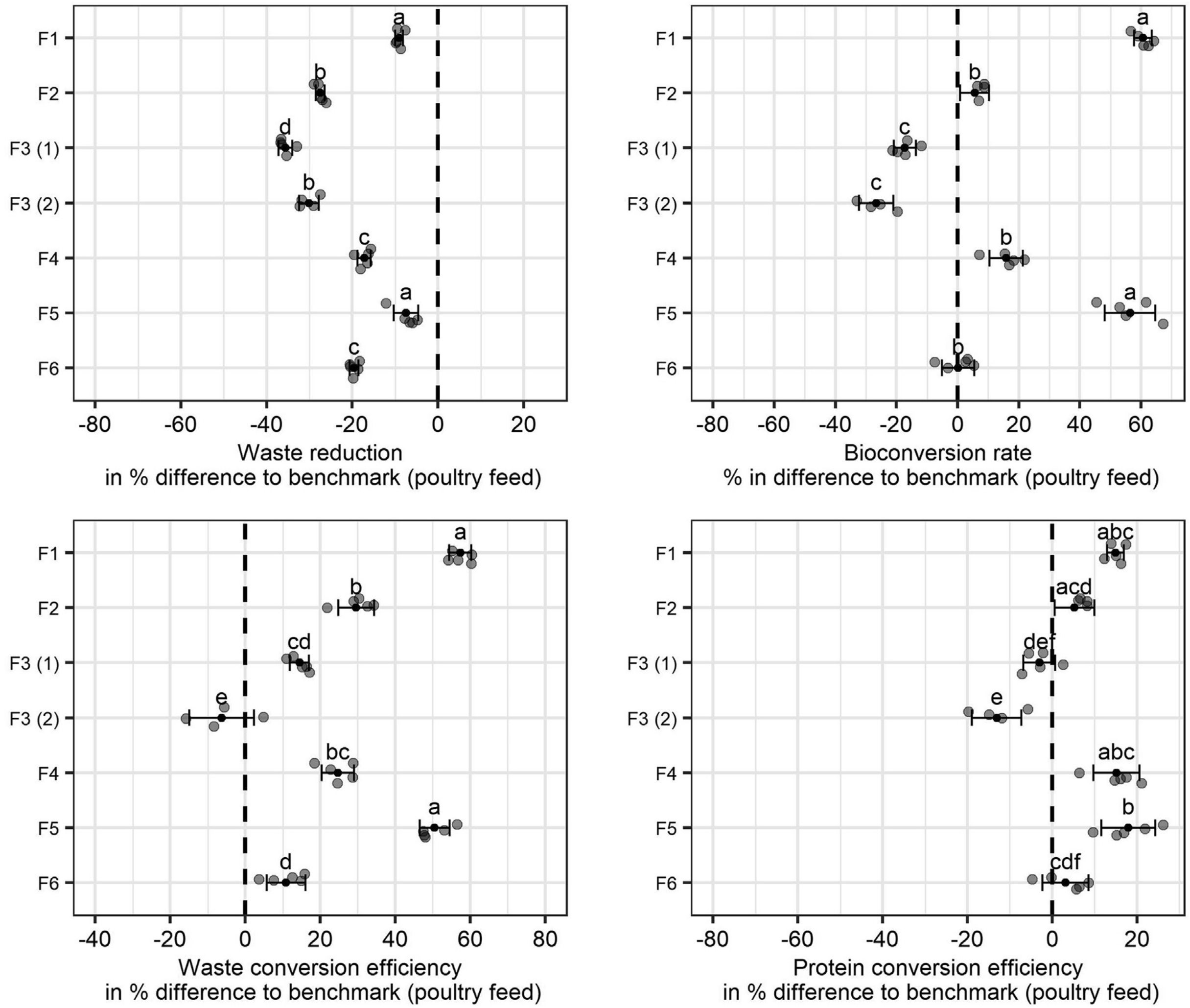

Fig. 3. Effects of the different waste formulations on the waste reduction (top, left), bioconversion rate (top, right), waste conversion efficiency (bottom, left) and protein conversion efficiency (bottom, right) of BSFL in comparison to poultry feed (dashed vertical line). Means, standard deviations and results per replicate are displayed. Performance results with no shared letter are significantly different from each other. All results are given in dry mass.

feed. These results suggest that the performance of BSFL treatment facilities can be increased by designing biowaste mixtures based on similar protein and NFC contents.

By comparing Figs. 2 and 3 it also becomes apparent that the use of formulations decreased the variability in performance. In comparison to the poultry feed, the results for the formulations had a range of 28% for waste reduction, 87% for bioconversion rate, 64% for waste conversion efficiency and 31% for protein conversion efficiency. In comparison, individual biowaste produced a range of 65% for waste reduction, 96% for bioconversion rate, 101% for waste conversion efficiency and 68% for protein conversion efficiency. This suggests that formulating different biowastes based on their initial nutrient composition can improve the reliability of BSFL treatment facilities. Although formulations, on average, contained less NFC, protein and lipids and more fibre than the individual wastes (see Tables 2 and 3), they were more balanced in nutrients without the absence or excess of NFC (as was the case for human faeces, cow manure and poultry slaughterhouse waste), protein (poultry slaughterhouse waste) and fibre (cow manure and mill by-products). It should be stated that all of the formulations included at least 50% mill by-products or canteen waste or both, and these were the wastes that supported high BSFL performance when used individually.

Variability in the performance parameters was, nevertheless, higher than expected in the formulations; bioconversion rates were expected to be similar between the formulations as protein and NFC appear to have the greatest influence on larval development (Barragán-Fonseca et al. 2018a, Barragán-Fonseca et al. 2018b; Cammack and Tomberlin, 2017). However, variable bioconversion rates (with a range of 87%) are not practical for BSFL treatment facilities. Such variability between formulations could be due to variable fibre and lipid contents. In the formulation feeding experiments, for example, lipid correlated positively and fibre negatively with waste reduction (R^2 = 0.96, $p < 0.01$ for lipids, R^2 = −0.97 and p = <0.01 for fibre) and the bioconversion rate (R^2 = 0.96, p = <0.01 for lipids, R^2 = −0.95, $p < 0.01$ for fibre). Formulations 1 and 5 resulted in the greatest waste reduction and bioconversion rate. These formulations were highest in lipids and lowest in fibre (see Tables 2 and 4) due to the high proportion of canteen wastes (see Table 1). In contrast, formulations 2 and 3 had the lowest lipid and highest fibre content due to a high proportion of human faeces and cow manure. This suggests that the variability in performance could be further reduced by keeping content of lipids and fibre within narrower limits. However, maintaining all macronutrients within fixed limits is difficult in practice considering that wastes typically have variable amounts of each macronutrient.

In addition to different lipid and fibre contents between the formulations, biowaste microbial numbers and communities could have been contributing to the variable BSFL treatment performance despite a similar protein and NFC content. This was not part of this study but can be expected considering that microbes can influence biowaste decomposition (De Smet et al., 2018; Gold et al., 2018a) and larval growth and typically differ between biowastes (Bruno et al., 2018; Ryckeboer et al., 2003; Wynants et al., 2019).

Similar to the larvae grown on the individual wastes, larval protein content was variable between the formulations (Table 4). Larvae fed on the formulations with a lower bioconversion rate tended to have a higher protein content. Protein efficiency was not significantly different between formulations 1, 4, and 5, and between formulations 2, 3, and 6, with the latter having a lower protein conversion efficiency, overall.

4. Conclusions

Given reliable biowaste compositional data, the formulation of mixed biowaste offers a promising systematic approach for the more efficient and predictable operation of black soldier fly larvae (BSFL) treatment facilities using a range of biowastes. Formulating biowaste mixtures in such a way that similar protein and non-fibre carbohydrate (NFC) contents are achieved can be expected to increase BFSL treatment performance and to reduce performance variability. Performance variability could be further reduced by keeping lipids and fibre within narrower limits. Future research should investigate whether these bench-scale results are transferrable to industry-scale BSFL treatment plants with higher larval densities and feed temperatures. Benefits of biowaste formulations need to be balanced with the additional resources required for biowaste analysis and the needed technologies to produce formulations as part of biowaste pre-treatment (e.g. scales, shredder, dewatering, mixer and tank).

Declaration of Competing Interest

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2019.10.036.

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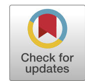

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ABSTRACT

Cities of low and middle-income countries face severe challenges in managing the increasing amount of waste produced, especially the organic fraction. Black Soldier Fly (BSF) biowaste treatment is an attractive treatment option as it offers a solution for waste management while also providing a protein source to help alleviate the rising global demand for animal feed. However, to-date very little information is available on how this technology performs with regard to direct greenhouse gas (GHG) emissions and global warming potential (GWP).

This paper presents a study that uses a life cycle assessment (LCA) approach to assess the GWP of a BSF waste treatment facility in the case of Indonesia and compares it with respective values for an open windrow composting facility. Direct CH_4 and N_2O samples were extracted from BSF treatment units and analyzed by gas chromatography. Results show that direct CO_2eq emissions are 47 times lower the emissions from composting. Regarding the overall GWP, the LCA shows that composting has double the GWP of BSF treatment facility based on the functional unit of 1 ton of biowaste (wet weight). The main GWP contribution from a BSF facility are from: (1) residue post-composting (69%) and (2) electricity needs and source (up to 55%). Fishmeal production substitution by BSF larvae meal can reduce significantly the GWP (up to 30%). Based on this study, we conclude that BSF biowaste treatment offers an environmentally relevant alternative with very low direct GHG emissions and potentially high GWP reduction. Further research should improve residue post-treatment.

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1. Introduction

Cities of low and middle-income countries face tremendous challenges with providing adequate solid waste management (SWM) services to ensure public health and avoid pollution to the environment. Besides rapid urbanization and population growth, limited skilled human resources, unreliable and lacking financial resources, ineffective institutional arrangements and inappropriate technical infrastructure exacerbate the challenge (Guerrero et al., 2013; Scheinberg et al., 2015; Wilson, 2015). In low and middle-income settings, SWM systems are still characterized by low collection rates and inadequate waste disposal: collection rates range between 30 and 80% and of the collected waste often well less than 50% is disposed of in controlled disposal site, and uncontrolled disposal is still quite common in rural areas in many countries (Scheinberg et al., 2015). Uncontrolled disposal may result in the release of methane into the environment – a potent greenhouse gas (GHG). Methane from landfills and wastewater account for ~90% of all global waste sector emissions, or about 18% of the global anthropogenic methane emissions (Bogner et al., 2008). This is especially relevant as one of the main characteristics of municipal solid generated in low and middle-income settings is its high fraction of organic waste, also called biowaste, comprising food and kitchen waste (e.g. from households, restaurants, hotels, schools, hospitals), market waste, yard and park waste, and residues from food and wood processing industries (Hoornweg and Bhada-Tata, 2012). In low and middle-income settings, biowaste reaches around 50–70% of the total waste produced, contrasting the 20–40% obtained in high-income settings (Wilson, 2015). Therefore, if the disposal of biowaste can be decreased by diversion and treatment with lower emissions measures (e.g. composting or other organic waste treatment options) it is possible to reduce considerably the amount of methane emissions.

Under the global warming and climate change debate, addressing the issues of biowaste treatment, and implementing treatment alternatives to disposal, has gained the interest of national and municipal decision-makers as well as researchers worldwide

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(Salomone et al., 2017; Sandec, 2008; Wilson, 2015). Besides the benefits of diversion from landfills, biowaste treatment and valorisation also stimulates waste collection through the creation of a product with economic value (Iacovidou et al., 2017a; Iacovidou et al., 2017b), thus returning resources to the economy using a circular approach – an important feature of sustainable development (Millward-Hopkins et al., 2018). Biowaste treatment and valorisation options can be classified according to the use of the end-products generated. These can be end-products such as fertilizer or soil amendment, energy and fuel or protein for animal feed. A comprehensive overview of potential recycling pathways for biowaste are shown in more detail in Lohri et al. (2017). From this variety of option, this paper focuses on one of the more innovative biowaste treatment options, where waste is fed to insect larvae - in this case the larvae of the Black Soldier Fly (BSF) - and when harvested are used as animal feed. The availability of conventional animal feed, such as soymeal or fishmeal, is increasingly limited and expensive, or associated with high land and water needs (Makkar et al., 2014; Smetana et al., 2016). Using biowaste as substrate for insect rearing as protein source in animal feed is therefore considered a very promising alternative (Salomone et al., 2017).

After its first introduction for waste treatment back in the 1990s, Black Soldier Fly (BSF), *Hermetia illucens,* is gaining more and more interest (Makkar et al., 2014; Smetana et al., 2016; Surendra et al., 2016) as an efficient way to convert biowaste into protein-rich and fat-rich biomass suitable for animal feeding. The approach is to feed fly larvae with biowaste. This reduces the waste amount by 50–80% (wet weight) to a residue and can grow larvae that can be harvested after about 14 days with a waste-to-biomass conversion rate of up to 20% (on a total solid basis) (Lohri et al., 2017). Larvae can be further processed and used as substitute for fishmeal in conventional animal feed (Henry et al., 2015) and residue can be composted and used as soil amendment (Diener et al., 2009).

Given the continuing efforts of nations and local governments towards reducing GHG emissions, and the commitments taken by all states that are parties to the United Nations Framework Convention on Climate Change, decision-makers increasingly evaluate the Global Warming Potential (GWP) of different treatment options when choosing biowaste management approaches (Wilson, 2015). For the more conventional approaches such as composting or anaerobic digestion this information is well established and simplified methodologies are available under the Clean Development Mechanism (CDM) framework. However for innovative technologies, such as BSF waste treatment that is still in an early stage of research (Lohri et al., 2017), to-date such information is scarce. Previous studies using a LCA approach for assessing the GWP of BSF exist, but typically used literature data from other insects to evaluate the potential direct GHG emissions, and focus either on a comparison of feed from BSF versus production of other feed (Smetana et al., 2016) or biowaste conversion using reference data from high-income countries (Komakech et al., 2015; Salomone et al., 2017). The main objective of the present study was therefore to fill this research gap by: (1) evaluating the direct GHG emissions from the BSF treatment process in terms of methane (CH_4) and nitrous oxide (N_2O) emissions and production pathways, (2) using a life cycle approach to assess the GWP of an Indonesian BSF treatment facility, and (3) comparing this to theoretical calculations of GWP using data from an Indonesian composting facility.

2. Methods

2.1. Direct GHG emissions

2.1.1. BSF treatment process

To evaluate CH_4 and N_2O emissions and production pathways from the BSF treatment process, a sampling campaign was conducted at the BSF treatment facility of Puspa Agro (7°22′10.7″S, 112°41′05.4″E) in Sidoarjo, Indonesia in June 2016. CO_2 production was also measured to check its accumulation and to detect leakages.

At Puspa Agro BSF facility, source segregated kitchen waste is treated by BSF larvae. The treatment process takes place in plastic boxes of 40 × 60 × 15 cm and lasts for 13 days. On day one, boxes are each filled with 10,000 5-day old larvae and 5 kg of kitchen waste (23% TS). Subsequently, on day five and eight, another 5 kg of kitchen waste is added to the boxes, thus obtaining an overall treatment capacity of 15 kg per box. These procedures follow the recommendations of Dortmans et al. (2017).

Direct CH_4 and N_2O emissions and production pathways in a BSF treatment process treating 1 ton of biowaste were assessed by a gas sampling campaign conducted at this BSF treatment facility. During the sampling campaign, the kitchen waste fed to the larvae consisted mainly of fruit and vegetable raw peeling as well as cooked food remain such as rice and vegetables. Through a rough calculation, a carbon-to-nitrogen ratio (C/N) of 15–20 was estimated. Households segregated the kitchen waste, which was then collected daily by a separate collection vehicle and delivered directly to the facility.

Gas sampling was done every day in triplicates. To ensure even distribution of gases within the box, a small battery driven fan was inserted into three boxes with larvae and kitchen waste. Each box was covered with a second plastic box (40 × 60 × 10 cm) equipped with a valve outlet. The two boxes were hermetically sealed and left untouched following the static chamber principle (Chan et al., 2011; Nigussie et al., 2017). From the air inside each sealed box, a predefined volume of gas was sampled in duplicate after 90 min. On every second day, an additional duplicate sample was extracted 45 min after sealing. For the extraction of the sample, a 100 ml gas-tight syringe was used. The gas from the syringe was then injected into 120 ml glass vial pre-filled with argon (Ar). Pre-filled vials with Ar were used to ensure pure gas sample for stable storage and transport conditions. For filling each vial, five consecutive extractions from the boxes and injections into the vials were conducted. To avoid over-pressure in the vial, Ar was left to exit through an inlet-outlet valve system.

This sampling process was conducted daily from the same three defined boxes always at the same time of the day. The schedule of sampling was established based on the assumption that a maximum GHG production rate is most likely observed around 1–2 h after adding fresh feedstock to the box. Ambient air samples were also taken every second day. All vials were transported by plane to Switzerland, where they were analyzed within a month using a gas chromatograph (GC) configured for CH_4, CO_2 and N_2O[1]. The residual Ar concentration was assessed using the *miniRuedi* portable mass spectrometric system (Brennwald et al., 2016), which resulted in a gas dilution factor of 4%.

The overall gas production was calculated as follows: firstly, we subtracted the ambient gas concentration from the box's gas sample concentrations using the GHG concentrations obtained with the GC device expressed in ppm by volume. Secondly, we calculated the amount of GHG obtained in the boxes following the ideal gas law. Thirdly, we evaluated daily gas production rates based on the 45 and 90 min measurements. Results showed that 45 min after closure of the boxes, on average 55% of CO_2 was emitted, so a linear production rate over time can be assumed. However, for N_2O and CH_4 emission rates after 45 min were 77% and 86% of the total. Therefore, closing of the boxes has an impact on BSF larvae behavior and feeding which results in lower emissions in the

[1] CH_4 and CO_2: Carbonplot 30 m × 0.53 mm × 30 micron (Ref Supelco 25467), FID (370 °C); N_2O: Carbonplot 30 m × 0.32 mm × 3 μm (Ref Agilent 113-3133), ECD (50 °C)).

second half of closure period. We used the 45 min measurements and assumed this rate as constant gas flux between two consecutive samplings.

The potential GHG production pathways were assessed analyzing the daily average CH_4 and N_2O concentrations and reviewing related scientific literature.

2.1.2. Residue post-composting process

To assess gas production by residue post-composting, we used the default values defined for Clean Development Mechanism (CDM) projects (UNFCC, 2011). Lacking real measured data, these estimates were assumed to best describe the direct GHG emissions from low-tech composting facilities found in low- and middle-income settings.

2.2. Life cycle assessment

To assess the GWP of BSF treatment and compare it with composting treatment, a LCA with SimaPro8 software was performed using Ecoinvent 3.1 database for background data. Energy consumption of BSF treatment relied on the facility in Sidoarjo whereas for composting data from the TEMESI composting facility in Bali (Temesi, 2016) was utilized. The standardized LCA (ISO, 2006) was followed and is detailed hereafter. The recommendations given by Laurent et al. (2014) when performing a LCA on SWM issues were also considered.

2.2.1. Goal, scope and functional unit

The goal of the study was to conduct a LCA of BSF treatment and compare the GWP of treating 1 ton of biowaste with the GWP from composting. Therefore, the functional unit (FU) was to effectively treat 1 ton (wet weight) of biowaste and produce compost. The focus was on treatment options in Indonesia and aims at providing a first qualitative and quantitative GWP assessment of BSF treatment in low and middle-income settings as well as comparing these to composting.

2.2.2. System boundaries

The system boundaries used in the study are shown in Fig. 1 and Fig. 2. The systems, as analyzed, start with segregated household biowaste entering the treatment facility and end with compost production.

All aspects of waste generation, collection and transport were ignored as they are not expected to differ between the two treatment technologies. Also, compost transportation to customers and subsequent use with its respective impacts was not considered in the LCA. Given the low market demand in Indonesia for soil improver or fertilizer (Verstappen et al., 2016), compost was assumed to be used as landfill cover whereby neither negative (leachability) nor positive impacts (methane oxidation) (Laurent et al., 2014) were taken into account.

To consider the added value of produced larvae meal, the substitution method (JRC-IES, 2010) was used. We assumed that the produced larvae meal substitutes conventional Peruvian fishmeal (production and transport), using available statistical data from

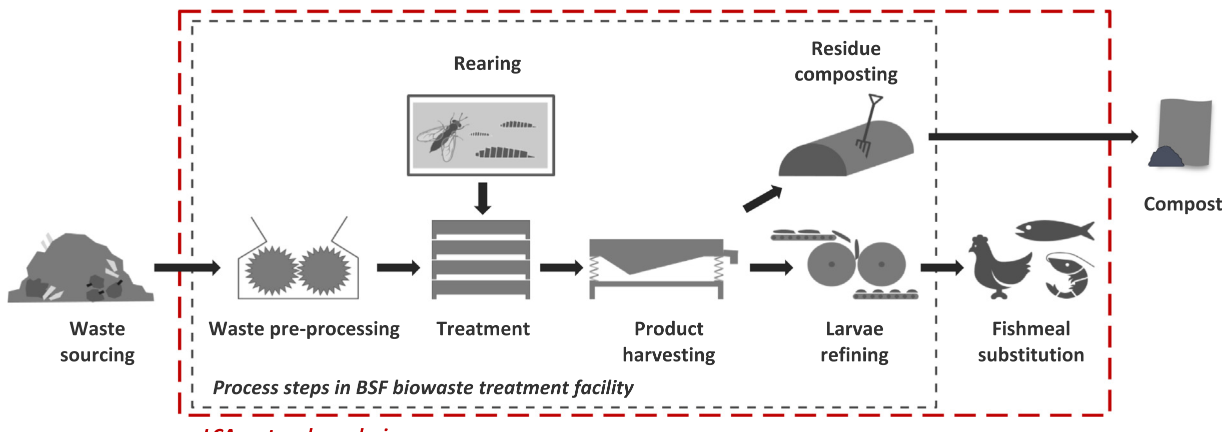

Fig. 1. Schematic diagram of the BSF treatment system considered.

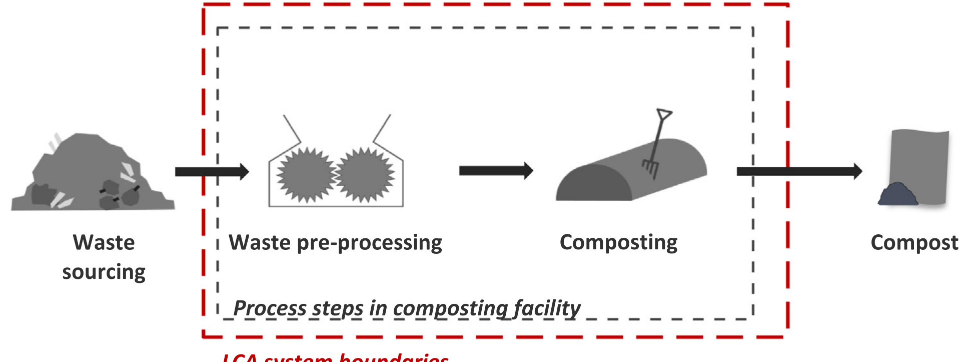

Fig. 2. Schematic diagram of the composting system considered.

Indonesia regarding fishmeal imports. In a second scenario we also considered only the substitution of locally produced Indonesian fishmeal. Potential benefits from BSF-derived oil were not considered in the present study as limited primary data is available (Surendra et al., 2016).

The indirect emissions related to infrastructure, equipment and machinery were neglected. The study of Salomone et al. (2017) shows little influence of the BSF facility equipment on GWP. As both treatment approaches (BSF and composting) use a similar level of infrastructure and machinery we hypothesize that this impact can be neglected.

Direct CO_2 emissions from biowaste during the treatment processes were regarded as biogenic and not considered in the inventory analysis due to the scope of the current LCA study (Christensen et al., 2009).

2.2.3. Life cycle Inventory (LCI)

All systems analyzed are briefly described below and the assumptions with relevant references are summarized in Table 1 and Table 2. For background data such as electricity, water and energy supply, Ecoinvent database 3.1 was used.

2.2.3.1. Black Soldier Fly treatment. Energy and water consumption for the different processes was assessed directly at the BSF treatment facility of Sidoarjo, adapted to a treatment capacity of 1 ton of household biowaste per day. Detailed explanations on the treatment processes can be found in Dortmans et al. (2017).

Rearing. Rearing of the flies takes place in the nursery and is distinctly separated from the waste treatment process. The rearing process consists of 4 phases: (1) fly mating and egg laying phase in cages, (2) egg hatching phase and growth of larvae until 5 day old larvae (5-DOL) – where at the end of this phase a large part of 5-DOL are moved from the rearing section to the waste treatment process, (3) the larvae remaining in the rearing section are then fed further until their prepupae stage, and finally (4) a pupation and fly emerging phase in dark cages.

Most of the electricity consumption in the rearing process is used for ventilation and lighting. Ventilation is required to ensure

Table 1
Life Cycle Inventory data for the BSF treatment facility.

BSF treatment facility	BSF treatment facility of Sidoarjo in use in June 2016		
	Assumptions for 1 ton/day	Details	Source
Rearing	Ventilation: 1.76 kWh	2 ventilators 220 W; 12 h/day for 3 tons	*Sidoarjo*
	Lighting: 1.10 kWh	1 lamp 300 W; 5 h/day for 3 tons 12 lights 25 W; 6 h/day for 3 tons	
	Mixing: 0.02 kWh	1 mixer 5500 W; 5 min/week for 21 tons	
	Compost: 3.57 kg	75 kg/week for 21 tons	
	Chicken feed: 3.1 kg	1.8 mg/larvae from hatching to 5-DOL 240 mg/larvae from hatching to prepupae	Zeist et al. (2012)
	Water: 1.19 l	25 l/week for 21 tons	
Waste pre-processing	Manually done		*Sidoarjo*
	No electricity consumption	No shredder use	
Treatment	Natural air flow simulation		
	No electricity consumption		
	CH_4: 0.4 g	Direct emissions	Sampling campaign
	N_2O: 8.6 g	Direct emissions	
Product harvesting	Pressurized water: 2.93 kWh	2.2 kW; 80 min/day	*Sidoarjo*
	Water: 664 l	8.3 l/min; 80 min/day	
Larvae processing	Boiling: 3 kg LPG	1.5 LPG/day for 0.5 ton	*Sidoarjo*
	Sun drying		
Residue composting	Electricity: 0.56 kWh		Temesi (2016), UNFCC (2011)
	Diesel: 0.22 kg		
	CH_4: 630 g	Direct emissions	
	N_2O: 63.3 g	Direct emissions	
Cleaning	Cleaning: 1.46 kWh	Cleaning: 2.2 kWh; 40 min/day	*Sidoarjo*
	Washmachine: 0.96 kWh	Washmachine: 350 kWh/365 use; 1 use/day	
	Water: 381 l	Cleaning: 8.3 l/min; 40 min/day Washmachine: 49 L/wash	
Avoided emissions	100% fishmeal substitution		
Fishmeal production	Peruvian fishery		
	Diesel: 2.08 kg	Peruvian fishmeal production	Zeist et al. (2012)
	Light fuel oil: 1.98 kg		
	Electricity: 8.178 MJ		
Transport	Cargo: 1047 tkm	Boat from Callao-Surabaya	SeaRates (2005–2017)
	Lorry: 1.873 tkm	Lorry from Surabaya Sidoarjo	

Table 2
Life Cycle Inventory data for the composting facility.

Composting facility	Yayasan Pemilahan Sampah Temesi Facility Bali – Annual electricity and diesel consumption		
	Assumptions	Details	Source
Waste pre-processing	*No electricity consumption*	Manual (no shredder used)	
Composting	Electricity: 1.77 kWh	Forced aeration + Sieve	Temesi (2016)
	Diesel: 0.7 kg	Excavator	
	CH_4: 2000 g	Direct emissions composting	UNFCC (2011)
	N_2O: 200 g	Direct emissions composting	

fresh air circulation inside the rearing room. Electric lights are used to attract emerged flies from the dark cages into the mating cages and to light the whole rearing room. Water, compost and an electric blender are necessary to prepare the attractant material placed in the mating cages. Chicken feed is used to feed larvae for the first 5 days and for larvae kept in the rearing facility until they reach their prepupae stage. The compiled data presented in Table 1 are derived from a larvae production facility treating 3 tons of biowaste per day.

Pre-processing. Household biowaste arriving at the BSF treatment plant is already segregated and therefore free of inorganics, so no further sorting was required. Although for other biowaste types shredding may be required, in this particular case no shredder was used.

Treatment. Waste treatment takes place in plastic boxes piled on top of each other in stacks located in the treatment hall. The treatment process takes 13 days. A specified number of 5-DOL from the rearing facility are added to a certain amount of waste in each plastic box. The larvae consume the organic waste and grow. Each plastic box contains 10,000 larvae and 15 kg of biowaste is fed manually in three feeding events. Direct gas emissions from plastic boxes containing larvae and waste were sampled during the campaign conducted in Sidoarjo in June 2016 and analyzed for CH_4 and N_2O in fall 2016.

Product harvesting. After 13 days, larvae are manually separated from residue using a sieve. Hereby the mixture of larvae and residue is spread out onto the sieve, where then larvae crawl through sieve's holes to a recipient below to avoid sunlight. A pressurized water system is used to clean the recipient and flush larvae to a harvesting system.

Larvae processing. Harvested larvae are dipped into boiling water. Water is brought to boil with liquefied petroleum gas (LPG). Thereafter larvae are sun dried.

Residue composting. After larvae harvesting, the remaining residue is composted using the same approach as a typical biowaste composting process and as presented in Section 2.2.3.2. The emission values from composting were adjusted for treating 320 kg of residue (this represents the average amount after treatment of 1000 kg of incoming waste) following a linear extrapolation.

The emissions from composting of the residue were not measured directly, as explained in Section 2.1.2. Our assumption was that the residue shows similar emissions during composting as fresh biowaste composting. As the residue is partly degraded from the BSF digestion process, we expect less emissions as compared to fresh biowaste composting, thus our assumption may be overestimating the direct emissions of residue composting.

Additional processes. The facility requires regular cleaning of all equipment. Energy (washing machine and high pressure cleaner) and water consumption were hereby considered.

Fishmeal substitution. Harvested larvae were assumed to substitute current conventional Peruvian fishmeal by a ratio 2:1. This ratio is justified as fishmeal contains on average twice the protein content of larvae meal on a wet weight basis (adapted from Diener et al. (2009)).

Energy consumption in production process of fishmeal was based on the data provided by FeedPrint 2015.03 database (Zeist et al., 2012). Furthermore, transport by cargo ship from El Callao-Peru to Surabaya, Indonesia, and by lorry from Surabaya to Sidoarjo was also considered. The transport distances were used from SeaRates (2005–2017).

2.2.3.2. Composting. Composting facility of Yayasan Pemilahan Sampah Temesi – Bali (Temesi, 2016) was used as source of data for energy consumption during composting process. This composting facility operates as an open forced aeration composting system, with the capacity to treat 60 tons of biowaste per day. Based on the energy consumption at this capacity, the equivalent energy consumption at 1 ton of waste capacity was estimated using a linear extrapolation.

Waste handling. The handling of waste input is manual without consideration of machinery (no waste shredder is used).

Composting process. During composting blowers powered by electric fans ensured forced aeration. Composting heaps are also turned using a diesel fueled wheel loader. After 3–4 months of composting duration, compost product is sieved using an electrical powered compost sieve. The sieved product is then left to further mature for 1–2 months before it is sold (Zurbrügg et al., 2012).

Values for direct emissions of CH_4 and N_2O from composting are taken from UNFCC (2011) (default values of non-monitored data used).

2.2.4. Impact assessment and interpretation

The study presented here focuses on GHG emissions and GWP, calculated using IPCC 2013 100a method (IPCC, 2013).

ReCiPe Midpoint Hierarchist (H) methodology was also used to assess all impact categories. These results are not presented here. The impacts show high correlation with the Indonesian coal power plant electricity production.

A sensitivity analysis was carried out following the recommendations given by Clavreul et al. (2012) to evaluate which variables have a dominant influence on the overall results. A contribution analysis was conducted to show the critical processes in terms of kg CO_2eq emissions. Also a perturbation analysis was conducted to highlight the critical variables using the sensitivity ratio (SR) with an increase of 10% of each variable separately. Finally, also alternative scenarios were developed and analyzed. A combined sensitivity analysis was however not carried out as we considered only one main impact category.

3. Results and discussion

3.1. Direct GHG emissions

The results obtained from the three boxes confirmed the reliability of the method of emission measurement used. The three boxes showed similar process efficiency, with a biowaste reduction of 50% and larvae biomass growth of 20–25% (wet weight). An average variation of GHG emissions of 20–30% between boxes was observed. We considered this as an acceptable range of variation, considering it being a biological treatment process with non-homogeneous feedstock.

Results show an average CH_4 production of 0.4 g and N_2O production of 8.6 g per ton of organic household waste treated. The results were compared with GHG emissions from open composting as described in the literature (see Table 3). The wide range of composting GHG emissions mentioned in literature results from a large range of different feedstocks, and/or treatment parameters measured (Boldrin et al., 2009; Chan et al., 2011). In general, the assumption is however valid that lower technical complexity of the composting system results in higher direct GHG emissions. The default values defined by UNFCC (2011) for Clean Development Mechanism (CDM) projects thus represent the direct GHG

Table 3
CH_4 and N_2O direct emission production from BSF treatment and composting.

	CH_4 [g/ton ww]	N_2O [g/ton ww]	References
BSF	0.4	8.6	Present study
Composting	30–6′800	7.5–252	Boldrin et al. (2009)
	2′000	200	UNFCC (2011)

emissions from low-tech composting facilities quite well. Such facilities would typically be found in Indonesia and other low- and middle-income settings.

The BSF direct emissions are also closely linked with feedstock characteristics and treatment parameters (ratio of larvae and amount of waste). The comparison of BSF treatment versus composting with regard to direct GHG emissions (Table 3) nevertheless highlights the potential of BSF treatment as a low GHG emissions option.

Besides evaluating how much GHG emissions the BSF biowaste treatment emits, this study also assessed the potential production pathways through an analysis of the daily production rates over the whole treatment period. When considering GHG production from BSF biological treatment process two pathways can be assumed: 1) metabolic GHG production from the larvae 2) waste decomposition itself enhanced or hindered by larvae's activity. The potential CH_4 and N_2O production pathways are detailed in Sections 3.1.1 and 3.1.2.

3.1.1. CH_4 production pathways

Fig. 3 shows that over the whole treatment period, CH_4 concentrations were low (1.4 to 2.7 ppm on average) and similar to the one observed in air samples. No pattern can be detected. This indicates that metabolic CH_4 production from the larvae is low or absent and that larvae movement and related aeration of the waste hinders anaerobic conditions. This finding goes in line with the study of Hackstein and Stumm (1994) and Čičková et al. (2015) showing no methanogenic bacteria present in the hindgut of House Fly larvae and stating that fly larvae's main contribution on biodegradation seems to be the mechanical aeration. This was also confirmed more recently with the study of Perednia et al. (2017) that state that BSF larvae grown under aerobic condition do not generate significant quantities of CH_4.

3.1.2. N_2O production pathways

As can be seen in Fig. 4, the N_2O concentrations in the treatment boxes do not differ significantly from the ambient air sample (0.3 ppm), except after the two feeding event (day 5 and 8). The increase on day 5 and 8 is proportional to larvae weight, which indicates an impact of larvae activity on N_2O production.

To the knowledge of the authors, even though direct N_2O emissions from insects have been studied before (Oonincx et al., 2010), none of these focused on BSF larvae specifically. According to the

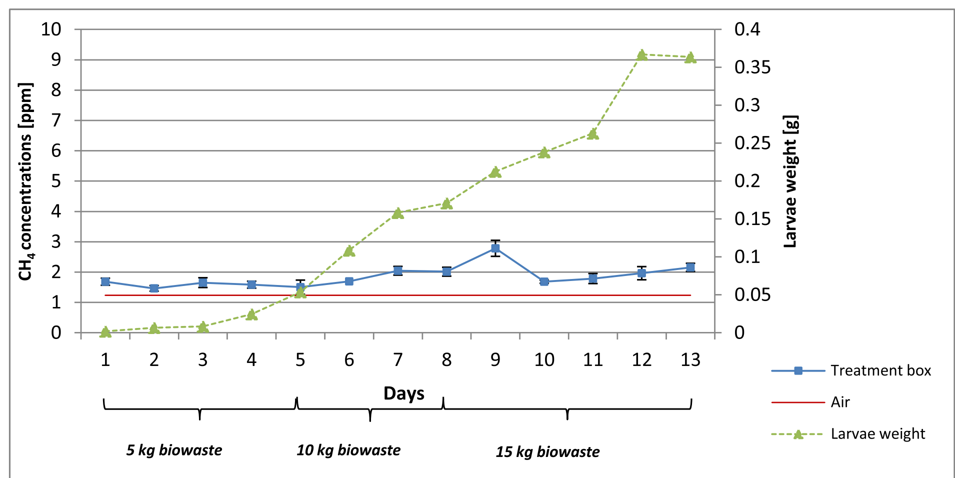

Fig. 3. Daily average direct CH_4 emission concentrations in BSF treatment boxes (solid line with square) and related standard deviation (error bars) observed after a 90 min closing time, as well as average CH_4 concentrations in air (solid line) expressed in ppm in correlation with larvae size expressed in g of average wet weight per larvae (dashed line with triangle).

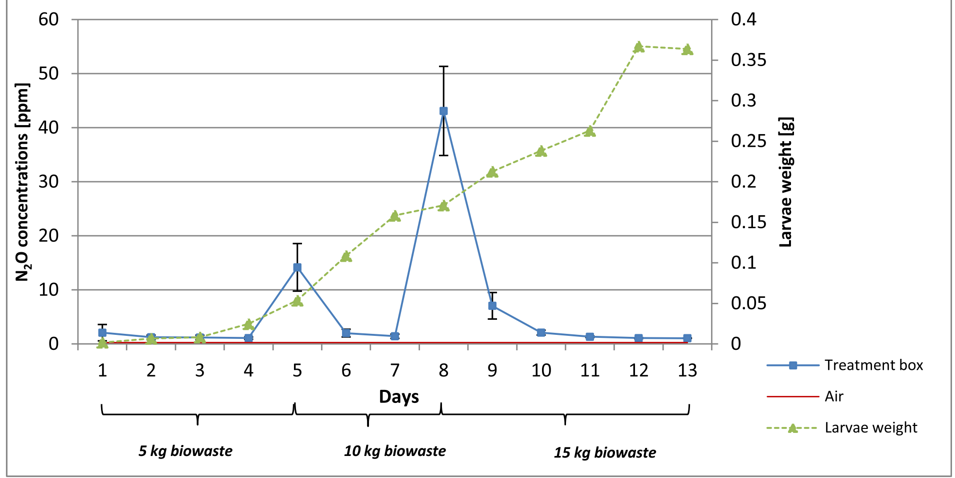

Fig. 4. Daily average direct N_2O emission concentrations in BSF treatment boxes (solid line with square) and related standard deviation (error bars) observed after a 90 min closing time, as well as average N_2O concentrations in air (solid line) expressed in ppm in correlation with larvae size expressed in g of average wet weight per larvae (dashed line with triangle).

study of Iwasa et al. (2015) on GHG emissions from fly larvae on cattle dung, metabolic N_2O emissions from fly larvae are unlikely as the presence of fly larvae show lower overall N_2O emissions when compared to cattle dung decomposition without larvae. Based on the results of our study it is not possible to distinguish between metabolic or bacterial N_2O production. We estimated that mechanical aeration occurring in the waste could be responsible of general low N_2O emissions.

The complexity of evaluating the impact of saprophages on N_2O emissions was already highlighted by the study of Nigussie et al. (2017) on earthworms and garden waste decomposition. Results of Nigussie et al. (2017) showed that a high feeding rate increases N_2O emissions. This is also seen in our results, where after a feeding event, N_2O increases.

3.2. Global warming potential assessment

Based on the considered systems and assumptions, the overall GWP per ton of biowaste is 35 kg CO_2eq for BSF treatment and 111 kg CO_2eq for composting (Fig. 5).

3.2.1. Direct emissions

Direct emissions from a BSF treatment facility (i.e. BSF treatment itself and residue composting) are lower than from a composting facility. For BSF treatment they represent 72% of the overall related GWP while for composting they represent 98%. The calculation of the sensitivity ratio (SR) shows that N_2O direct emissions is the most critical parameter with a SR of 278 for the emissions from biowaste composting, 88 for residue composting and 12 for the BSF treatment process. CH_4 direct emissions is the second most important parameter, having a SR of 30 and 9.5 for the emissions from biowaste composting and residue composting respectively. This highlights the importance of optimizing the biological processes for ensuring low GHG emissions. All other SR values are below 0.3.

In the BSF system considered, the direct emissions from residue composting is responsible for 68% of the total GWP of BSF treatment. These high values are a consequence of the default value used, regarding emissions from composting according to UNFCC (2011) and our conservative assumption that emissions from residue composting are the same as those when composting fresh biowaste. Boldrin et al. (2009) present a wide range of values for open composting emissions. Compared to the default value of UNFCC (2011) these range from 3 to 235% of the default value and are explained by the wide range of operational parameters that influences emissions, such as system design, aeration rate, turning frequency, and/or feedstock used. Based on these literature values, it is likely that we overestimate emission from residue composting especially considering the composition of the "feedstock". Residue after BSF treatment is lower in nutrients and carbon (as these have been consumed by the larvae) when compared to fresh biowaste. The resulting emissions based on feedstock characteristics would therefore also go towards the lower end of the values given by Boldrin et al. (2009). To confirm this, a detailed analysis on residue composition and carbon and nitrogen mass balance would be necessary. This was unfortunately not achievable in this study due to technical on-site limitations and lack of secondary data. Therefore, we feel the use of the default value from CDM is justifiable and allows an approximation. Even if residue composting emissions are overestimated the results presented in Fig. 5 nevertheless emphasize the importance of residue post-treatment options.

3.2.2. Indirect emissions

When considering the indirect emissions, it is the electricity consumption for BSF treatment that plays a major role. We considered a direct electricity consumption of 8 kWh for BSF treatment and 1.8 kWh for composting. This contributes to 19% of the overall GWP of BSF treatment and 2% for composting. The rearing and harvesting phases in BSF treatment are those consuming most of the electricity needs in the current BSF system and scale (around 35% each). The sensitivity ratio obtained for electricity consumption (0.22) shows that a slight reduction of electricity consumption with the current system will not considerably affect the overall GWP. However, introducing new processing steps and equipment such as a shredder for the feedstock, active ventilation systems or a shaking sieve for larvae harvesting, could increase electricity consumption significantly. With such equipment we estimated that the electricity consumption could amount to 35 kWh per ton of waste treated. This would increase the overall GWP by a factor of 1.8. However, this factor is strongly influenced by the energy source. In the system presented, we used the Ecoinvent 3.1 data on Indonesian electricity supply at grid coming from coal power facilities. The associated GWP per kWh consumed is therefore high (1.17 kg CO_2 eq/kWh) in comparison with the average

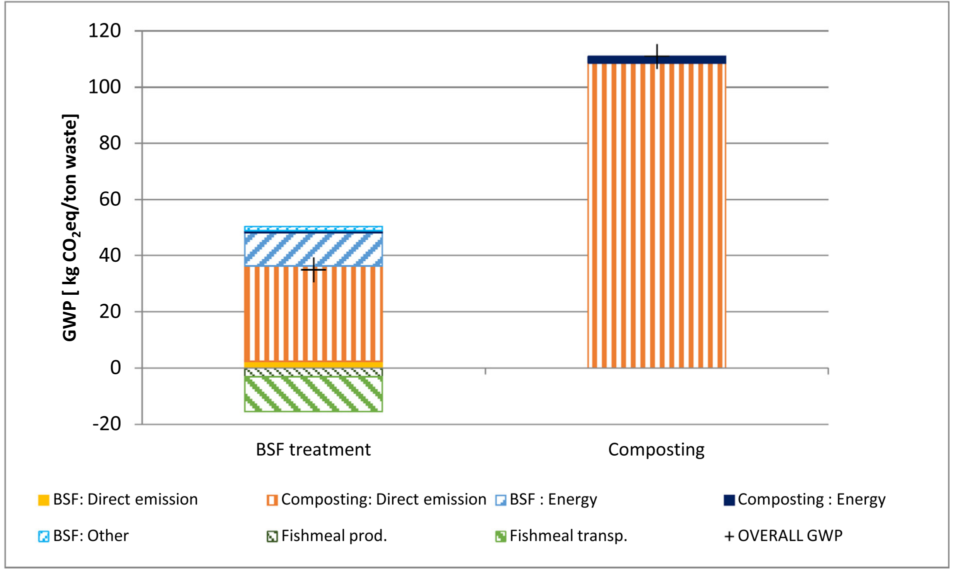

Fig. 5. GWP per ton biowaste (ww) expressed in kg CO_2eq from direct, indirect and avoided emissions of BSF treatment and composting.

non-European electricity production (0.72 kg CO_2 eq/kWh) or photovoltaic electricity production (0.07 kg CO_2 eq/kWh). When considering a 35 kWh/ton of electricity consumption, this would reduce the GWP from the electricity consumption and the overall GWP by 17 to 48%. Increasing the scale of operation will also affect the electricity consumption. With larger scale we expect more automation and an increase in power consumption, although economies of scale could also reduce the unit consumption per ton. As data on BSF treatment at different scales is not yet accessible, this could not be taken into account.

3.2.3. Avoided emissions

Avoided emissions from fishmeal substitution can amount to 31% when considering a 100% substitution of fishmeal produced in Peru and transported to Sidoarjo (Fig. 5). The major avoidance relates to avoiding cargo transport from Peru to Indonesia (25% of the total GWP avoided). Under the assumption that BSF larvae would substitute a locally produced fishmeal, this would only impact by 0–6% on avoided emissions, depending on the level of mechanization of the fishmeal production process. It is important to note that the larvae meal quality and its effect on how much fishmeal can be substituted was not considered in the scope of the present study.

In the analyzed system, the potential avoided emissions from compost transport and use were neglected. Residue composting in BSF generates half of the amount of compost as compared to fresh biowaste composting. When considering the fertilizer substitution value, Salomone et al. (2017) in their LCA showed that accounting for nitrogen fertilizer replacement could allow a significant negative contribution to GWP (−456 kg CO_2 eq/ton of waste treated). The higher compost product amounts would therefore favour the fertilizer substitution benefits from composting as compared to BSF treatment. However these estimated benefits depend on composition which relates to feedstock characteristics and on regional climate, soil and crop parameters (Laurent et al., 2014). Therefore the current information available does not allow a reliable estimate. This highlights the importance of further investigating the residue composition, possible post-treatment options and related end-product market demand when analyzing the overall GWP.

4. Conclusion and outlook

The main findings of this study show that: (1) when considering direct GHG emissions, BSF treatment shows lower emissions when compared to composting, (2) results from the LCA show that the overall GWP for BSF treatment mainly depends on the type of residue post-processing and the electricity consumption and energy source used, (3) More data on larvae meal quality are still required to better assess the potential of substitution of fishmeal and respective emission savings.

GHG emissions by the larvae feeding on the waste is very low, when compared to the microbial emissions in the open composting process. This can be explained given that the larvae continuously move the waste when feeding and thus ensure aeration and aerobic conditions. Unfortunately, we observed an influence of our experimental setup on the behavior of the larvae where sealing the boxes started to impact on larvae's behavior after at least 45 min. Thus, further experiments should be conducted using a continuous flow chamber sampling method.

Electricity consumption in the BSF treatment facility is a crucial element when considering overall GWP. With increased mechanization, electricity consumption per ton of waste will increase (shredder, mechanical sieve, etc.). Also, maintaining stable environmental conditions in indoor systems will significantly increase electricity requirements thus increasing GWP. In low and middle-income settings where electricity production is based on fossil fuel origin, this accounts for high GWP. Here reducing and optimizing the electricity need is therefore of crucial interest. Our results show that consideration of renewable energy source such as solar panels is an eligible option as it could decrease the environmental impact associated with local electricity consumption.

Composting of the residue shows relatively high level of emissions given the default assumptions related to composting emissions. On one hand, better values about material-specific composting emissions would be helpful for more detailed assessments, while on the other hand further research on the characteristics of residues and best practice alternatives on how to process these residues considering emissions and end-product markets value is required. Among the post-treatment options, anaerobic digestion looks promising as it could tackle two problems at the same time: the residue management and the provision of an energy source to operate the facility. A recent study conducted by Lalander et al. (2018) shows promising results on biogas potential of BSF residue from food waste from a Swedish campus canteen (417 NmL g VS^{-1}). Further research is therefore needed to evaluate the biomethane potential of the household residue.

Finally, evaluating and quantifying avoided emissions from BSF biowaste treatment depends on emissions of the products which are substituted and how well the products from BSF-treatment are requested and accepted in the market. In our study, we considered larvae meal as an ideal substitute of conventionally produced fishmeal. Yet we know waste characteristics fed to the larvae influence larvae yield as well as larvae protein content (Smetana et al., 2016) and other quality parameters of the larvae meal obtained. Further research on suitable BSF diets, operational effects on product quality and impacts of feeding BSF larvae meal on farmed animals are necessary.

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PERPUSTAKAAN SULTANAH NUR ZAHIRAH

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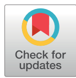
Black soldier fly larvae meal can replace fish meal in diets of sea-water phase Atlantic salmon (*Salmo salar*)



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ABSTRACT

A feeding trial was conducted to test the growth potential, nutritional utilization, liver health and fillet sensory parameters of sea-water Atlantic salmon (*Salmo salar* L.) fed diets with increasing substitution of fish meal with insect meal. The insect meal was produced from black soldier fly larvae (*Hermetia illucens*, L.). Triplicate sea-cages of salmon were fed one of four isonitrogenous and isolipidic diets for 16 weeks. The control diet (IM_0) contained 100 g kg^{-1} fish meal, which was replaced up to 100% with insect meal (33% (IM_{33}), 66% (IM_{66}) and 100% (IM_{100})), corresponding to dietary insect meal inclusion levels at 50 g kg^{-1}, 100 g kg^{-1} and 150 g kg^{-1}, respectively. Replacing the dietary fish meal with insect meal did not affect the apparent digestibility coefficients (ADC) of protein, lipid, amino acids and fatty acids, or the digestive enzyme activities. Feed intake, daily growth increase, and feed conversion ratio were also unaffected by the inclusion of insect meal in the diets. Whole body protein, lipid and amino acid composition were not affected by dietary substitution of fish meal with insect meal, while the whole body fatty acid composition generally reflected that of the diets. Liver lipid accumulation was not affected by replacing the fishmeal with insect meal, as assessed by both histological examinations and chemical analyses. The sensory testing of the fillet revealed only small changes in the fillet sensory quality. In general, this study showed that a total replacement of fish meal with black soldier fly larvae meal in the diets of sea-water Atlantic salmon was possible without negative effects on growth performance, feed utilization, nutrient digestibility, liver traits or the sensory qualities of the fillet.

1. Introduction

The choice of ingredients and formulation of the fish diets can greatly influence the environmental impact of the aquaculture industry (Boyd and McNevin, 2015). Therefore, continuous improvement in this sector is crucial. Finding nutritionally appropriate and sustainable alternatives to fishmeal (FM) and –oil is an area of intense research, with possible alternative sources of ingredients coming from terrestrial plants, animal by-products, microalgae, macroalgae or insects, to mention some (Barroso et al., 2014; Boyd and McNevin, 2015; Gatlin et al., 2007; Olsen and Hasan, 2012; Wan et al., 2018). The interest in insects as feed ingredients for terrestrial and aquatic animals continues to grow every year, with increasing numbers of new scientific articles being published on the subject (Vargas-Abúndez et al., 2018; Barroso et al., 2014; Belghit et al., 2018a; Borgogno et al., 2017; Dumas et al., 2018; Lock et al., 2016; Magalhães et al., 2017; Van Huis, 2013; Veldkamp et al., 2012, Nogales-Mérida et al., 2018). The black soldier fly (BSF) (*Hermetia illucens*) larvae is considered an important candidate species to be used for animal feeds (Cammack and Tomberlin, 2017; Van Huis, 2013). Since the 1970s, this species has been used as a protein source in animal feed, mainly due to its ability to convert food waste (vegetable, fruit, factory waste, and animal tissues) into high-quality protein (Hale, 1973; Newton et al., 1977). The research and industrial-scale production of BSF larvae as feed ingredients have been intensified the last few years (FAO, 2013; Wang and Shelomi, 2017).

Available documentation of the nutritional composition and value of different insect species considered as candidates for use in animal feeds has become substantial (Alegbeleye et al., 2012; Barroso et al., 2014; Henry et al., 2015; Makkar et al., 2014). BSF larvae contain high amounts of protein (≈ 40% of dry weight (DW)) and have a well-balanced profile of essential amino acids (AA) (Henry et al., 2015; Liland et al., 2017; Wang and Shelomi, 2017). The larvae of BSF are also a

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good source of lipids, reaching up to 30% lipids (on DW basis) if grown on optimal growth media. The lipid is dominated by saturated fatty acids (FA), being especially rich in the medium-chained FA 12:0 lauric acid (Liland et al., 2017; Sealey et al., 2011; St-Hilaire et al., 2007). Replacement of FM with BSF larvae meal in aquafeeds without negative effects on growth or performance has successfully been demonstrated in some fish feeding trials (Belghit et al., 2018a; Cummins et al., 2017; Dumas et al., 2018; Elia et al., 2018; Lock et al., 2016; Magalhães et al., 2017; Renna et al., 2017), but not in others (Gasco et al., 2016; Kroeckel et al., 2012; St-Hilaire et al., 2007). Replacement of dietary FM with BSF meal reduced the growth of juvenile turbot (165–756 g insect meal (IM) kg^{-1} diet, *Psetta maxima*) and rainbow trout (300 g IM kg^{-1} diet, *Oncorhynchus mykiss*) (Kroeckel et al., 2012; St-Hilaire et al., 2007). We previously demonstrated that it is possible to include as much as 600 g kg^{-1} of IM in combination with insect oil in the diets of fresh-water Atlantic salmon without affecting the growth performance or the feed utilization (Belghit et al., 2018a). In the current trial, we aimed to study the effects of partial or total dietary replacement of FM with IM on Atlantic salmon in the sea-water stage. By rearing the fish up to a typical commercial slaughter size (~4 kg) we could gain consumer-relevant knowledge on how such a dietary change would affect both the nutritional and sensory quality of the fish fillet. To our knowledge, this is the first trial using dietary insect ingredients to grow Atlantic salmon up to slaughter-size.

2. Materials and methods

2.1. Experimental diets and feeding trial

2.1.1. Diets

The IM used in this study was produced from BSF larvae by Protix Biosystems BV (Dongen, The Netherlands). The larvae were grown on media partially containing seaweed (ground seaweed (*Ascophyllum nodosum*) mixed with organic plant-derived waste (60:40)). At the end of an eight-day growth period, the larvae were mechanically separated from the feeding media, washed and partially defatted before being dried and ground to make the IM. The nutritional composition of the IM is given in the supplementary Table 1. The diets were produced by Cargill (Dirdal, Norway), and supplemented with 1% yttrium oxide as an inert digestibility marker. Four experimental diets were formulated to be isonitrogenous (39% crude protein), isolipidic (29% crude lipid) and isoenergetic (25 MJ/kg DM gross energy) (Table 1). The control diet (IM_0) contained the protein sources FM and plant-based protein (20:80, *w*/w). The main lipid sources in all the diets were fish oil and vegetable oil (33:66, w/w). Three experimental diets were formulated, in which 33% (IM_{33}), 66% (IM_{66}) and 100% (IM_{100}) of the FM was replaced with IM, corresponding to dietary IM inclusion levels of 50 g kg^{-1}, 100 g kg^{-1} and 150 g kg^{-1}, respectively (Table 1). The diets were balanced to provide for the requirements of essential AA (methionine and lysine were added). Additional fish oil was included in the diets with less fishmeal (IM_{33}, IM_{66} and IM_{100}) to ensure sufficient dietary long-chained highly unsaturated FAs (LC-HUFAs).

2.1.2. Feeding trial and facilities

The feeding trial was conducted at Gildeskål Research Station (GIFAS) in Langholmen, Inndyr, Norway (67°N, Northern Norway) during August–December 2017, following the institutional and national guidelines for the care and use of animal, and approved by the National Animal Research Authority in Norway. Post-smolt Atlantic salmon were randomly distributed among 12 sea-cages (*n* = 3) (5x5x5m; 125 m^3; 90 fish per cage), with a water temperature ranging between 7 °C (December) and 13 °C (August). Prior to the start of the feeding trial, fish were acclimated to the environmental conditions for two weeks. The fish were fed one of the four diets (Table 1) during 114 days. Each diet was distributed by hand until visual satiation. Two daily meals (or 1 meal, due to the light conditions) were provided with a minimum of four hours between the meals. Uneaten feed was collected and pellets weighed and subtracted from the total daily feeding.

Table 1
Formulation, proximate composition and amino acid composition (all analyses on wet-weight basis) of the four experimental diets fed to Atlantic salmon (*Salmo salar*).

	IM_0	IM_{33}	IM_{66}	IM_{100}
Ingredients (%)				
Fishmeal LT94	10	6.67	3.33	0.0
Insect meal	0.0	4.91	9.84	14.75
Soy protein concentrate	25	25	25	25
Corn gluten meal	7.5	7.5	7.5	7.5
Wheat gluten meal	3.35	4.51	5.7	6.88
Pea protein concentrate 55	8.8	6.8	4.8	2.84
Fish oil	10.18	11.70	13.23	14.76
Rapeseed oil	20.95	18.86	16.79	14.73
Binder	12.32	12.08	11.72	11.24
Additives	1.89	1.96	2.1	2.29
Yttrium	1.0	1.0	1.0	1.0
Proximate analysis				
DM (%)	93	93	94	95
Crude Protein (%)	38	38	39	39
Crude Lipid (%)	29	29	29	29
Ash (%)	4.6	4.6	4.5	4.5
Carbohydrates (%)	11.6	11.5	11.5	11.4
Gross energy (MJ/kg)	24.6	24.9	24.8	25.0
TBARS (nmol/g)	3.0	3.4	4.2	4.9
Amino acid composition (g kg^{-1} diet)				
Essential amino acids				
His	8.5	8.6	8.0	8.8
Ile	14.0	15.0	14.0	15.0
Leu	33.5	34.0	32.7	34.0
Lys	20.5	20.0	19.5	20.0
Met	10.0	10.0	10.0	10.0
Phe	20.0	20.5	19.0	20.0
Thr	14.5	14.5	14.0	14.0
Val	16.5	17.5	16.5	18.0
Arg	22.5	22.0	20.0	20.6
Non-essential amino acids				
Ala	19.0	19.5	19.0	19.7
Asp	36.0	35.6	34.6	34.6
Glu	73.0	75.0	75.0	79.0
Gly	16.5	16.0	15.0	15.6
Hyp	1.0	0.8	0.5	0.3
Pro	23.5	25.0	25.0	27.5
Ser	20.0	20.0	19.5	20.3
Tau	0.5	0.3	0.2	0.0
Tyr	13.5	14.5	14.5	16.0

IM_0 = diet without insect meal (IM) inclusion; IM_{33}, IM_{66} and IM_{100} = 33, 66 and 100% replacement level of FM with IM, respectively. DM = dry matter; TBARS = Thiobarbituric acid-reactive substances.

2.2. Sampling

Fish were sampled at the start of the trial and at the end of the trial (day 114). At all samplings the fish were anaesthetized with Tricaine methane-sulfonate (MS-222), individually weighed and body length measured. The fish were examined externally to check for possible abnormalities. Liver and viscera were removed and weighed for calculation of organosomatic indices. At the final sampling (day 114), faeces were collected by manual stripping from 30 fish per sea-cage, pooled per sea-cage and frozen on dry ice for digestibility measurements (*n* = 3). From additional 6 fish per tank, the whole digestive tract was dissected, cleaned of attached adipose tissue and divided into proximal (PI), mid (MI) and distal (DI) intestine. Digesta from the PI1 (the proximal half of PI), PI2 (the distal half of PI), MI, DI1 (the proximal half of DI) and DI2 (the distal half of DI) of fish from the same tank was pooled and snap-frozen in liquid N_2 for the analysis of trypsin activity and total bile acids level (*n* = 3). The empty intestinal segments (PI, MI, and DI) were frozen for the brush border enzyme activity

analysis ($n = 18$). Blood was collected from the caudal vein by means of heparinized medical syringes from six fish per sea-cage and haemoglobin was measured in each individual sample (n = 18). Plasma was separated from the red blood cells by centrifugation (3000 *g* for 15 min at 4 °C), pooled per sea-cage and frozen in liquid N_2 ($n = 3$). For analysis of proximate composition, six whole fish per cage were pooled, homogenized and samples frozen on dry ice (n = 3). Four salmon from each sea-cage were gutted, dissected and filleted at GIFAS; the fillets were vacuum-packaged in plastic bags, packed with wet ice in polystyrene boxes and shipped over-night to NOFIMA (Ås, Norway) for sensory testing. Individual liver samples were taken from six fish per cage (only the fish fed the IM_0 and IM_{100} diets) for histological assessment of lipid droplets as well as for lipid class analyses ($n = 18$). For histological analyses, samples of ~0.5 × 0-5 × 1.0 cm were cut from the midsection of each liver, put in a tissue processing/embedding cassette (Simport, Quebec, Canada) and fixed in 4% formaldehyde in 1xPBS for 24 h. The samples were then infiltrated with sucrose in increasing concentrations (10%, 20% and 30% *w/v* sucrose in 1xPBS solution, ~24 h in each solution). Tissue was cut out from the lower-mid section of the liver for lipid class analysis and flash-frozen in liquid N_2. All frozen samples were stored at −80 °C.

2.3. Analysis of chemical composition

Total nitrogen was analyzed on freeze-dried, ground samples (feed, whole fish and faeces) using a CHNS elemental analyser (Vario Macro Cube, Elementar Analysensysteme GmbH, Langenselbold, Germany) and quantified according to Dumas (1831). The instrument was calibrated with ethylene diamine tetra acetic acid (EDTA) (Leco Corporation, Saint Joseph, MI, USA). Sulfanilamide (Alfa Aesar GmbH & Co, Karlsruhe, Germany) and a standard meat reference material (SMRD 2000, LGC Standards, Teddington, UK) was used as the control sample.

Analysis of amino acids (not including cysteine and tryptophan) of the feed, whole fish and faeces was carried out by ultra-performance liquid chromatography (UPLC, Waters Acquity UPLC system) coupled with a UV detector (Espe et al., 2014). Wet, ground samples equivalent of 30–40 mg of protein were hydrolysed in 6 M HCl at 110 °C, the residue was diluted in MilliQ-Plus water and filtered through a syringe-driven filter. Prior to the instrumental analysis, a derivatisation agent (AccQ.Tag™, Waters, Milford, MA, USA) was added to each sample. Finally, amino acids were separated by UPLC (column: Aquity UPLC BEH C18 1.7 μM, Waters, flowrate 0.7 ml min^{-1}) and results integrated by Empower 3 (Waters). Amino acids were quantified using standards from Sigma (St. Louis, MO, USA).

Starch in the feeds was quantified using an enzymatic method according to Hemre et al. (1989). Starch in 0.5 g freeze-dried, ground material was hydrolyzed with the heat-stable enzymes amylase (Termamyl-120 L; Novo-Industries, Bagsværd, Denmark) for 30 min at 80 °C and amyloglucosidase (EC 3.2.1.3.; Boehringer, Ingelheim, Germany) for 30 min at 60 °C. Glucose was subsequently measured spectrophotometrically as nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) at 340 nm after a hexokinase/glucose-6-phosphate dehydrogenase reaction using a Maxmat PL multianalyser (Montpellier, France). Starch concentration was calculated as the difference in glucose concentration before and after enzymatic breakdown. Dextrin was used as reference material.

Neutral lipid (storage fat) content of the whole fish homogenates was determined gravimetrically after ethyl-acetate extraction, while the fat in feed and faeces after acid hydrolysis and extraction with diethyl ether. Energy density (kJ g^{-1}, on wet-weight basis) was calculated by assuming caloric values of 39.7 J mg^{-1} for lipids, 18.2 J mg^{-1} for starch and 17.1 J mg^{-1} for proteins.

FA analysis was performed on feed, whole homogenized fish and faeces by gas-chromatography (GC) as previously described by Jordal et al. (2007), modified after Lie and Lambertsen (1991). Briefly, lipids from the samples were extracted using chloroform/methanol (2:1, *v*/v). The extracted lipids were filtered and the remaining samples were saponified and methylated using 12% BF3 in methanol. The FAs were detected by a flame ionization detector (FID) and identified by retention time using a standard mixture of methyl-esters (Nu-Chek Prep, Elyain, MN, USA) to determine the FA composition (area %). All samples were integrated using the software Chromeleon® version 7 (Thermo Scientific, Waltham, MA, USA). Amount of FA per gram sample was calculated using 19:0 methylester as an internal standard.

Yttrium oxide concentrations in freeze-dried feed and faeces was analyzed according to Otterå et al. (2003). Briefly, yttrium oxide was quantified by ICP-MS after wet digestion in a microwave oven (Otterå et al., 2003).

Thiobarbituric acid-reactive substances (TBARS) were determined in the feed by a method modified from Schmedes and Hølmer (1989). Homogenized samples (0.2 g) were weighed into screw-capped glass tubes and added 4.0 ml of chloroform: methanol (2:1, v:v) and 0.2 ml butylated hydroxytoluene. Samples were purged with N_2, and tubes were closed and incubated with constant shaking for 30 min at room temperature. Thereafter, 2.0 ml of a saturated EDTA solution was added and the tubes were centrifuged for 20 min at 1500 x *g*. A 2.0 ml aliquot of the methanol:water layer was transferred to clean screw-capped glass tubes, mixed with 2.0 ml TBA reagent (1% thiobarbituric acid in 5% trichloroacetic acid) and heated for 30 min at 100 °C. Absorption was measured at 532 nm and TBARS quantified by reference to an external standard (Malondialdehyde (MDA)).

2.4. Digestive enzyme activity and total bile acids level determination

Freeze-dried digesta from PI1, PI2, MI, DI1 and DI2 was mixed thoroughly with cold distilled H_2O (1:10, *w/v*) on a rotating shaker at 4 °C for 10 min. After centrifugation (13,000 *g*, 4 °C, 10 min), the supernatants were collected into 2 ml Eppendorf tubes, frozen in liquid N_2 and stored at −80 °C. Prior to fast freezing, supernatants for total bile acids determination were subjected to sonication for 60 s at 4 °C. Trypsin activity was measured using benzoyl arginine *p*-nitroanilide (Sigma no. B-4875, Sigma Chemical Co., St. Louis, MO, USA) as substrate modified from (Kakade et al., 1973). As bovine trypsin shows a very different activity than that of salmon, the standard curve was not used for the calculation but to check if the assay worked. The trypsin activity is expressed as the difference in absorbance between the test and blank tube per mg dry matter (ΔOD/mg dry matter). Total bile acids were determined using the Enzabile test kit (catalog no. 550101, BioStat Diagnostic Systems, Cheshire, U.K.) and a curve derived from standardized taurocholic acid solution.

The PI, MI, and DI tissue was homogenized in cold tris-mannitol buffer (1:20 *w/v*) containing the serine protease inhibitor (24 μg/ml), 4-(2-aminoethyl)benzenesulfonyl fluoride HCl (Pefabloc® SC; Pentapharm Limited, Basel, Switzerland), using an Ultra Turrax® homogenizer (IKA, Staufen, Germany) followed by sonication at 4 °C for 15 s. The homogenates were frozen in liquid N_2 in aliquots and stored at −80 °C awaiting analysis. The leucine aminopeptidase (LAP) activity was determined using L-leucine-β-naphthylamide as substrate (Krogdahl et al., 2003). The enzyme activity is expressed as specific activity, normalized by the tissue protein. The protein concentration of homogenates was determined using the BioRad® Protein Assay kit based on the Bradford dye-binding method (BioRad Laboratories, Munich, Germany).

2.5. Haemoglobin and plasma metabolite assays

Haemoglobin was measured using a Cell-Dyn 400 (Sequoia-Turner, Santa Clara, CA, USA) according to the manufacturer's instructions, using Para 12 control blood (Streck) for calibration. Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose (Glu), free fatty acids (FFA), total protein (Tprot) and sodium (Na^+)

were analyzed using a clinical bioanalyser (Maxmat PL analyser, Montpellier, France) and controls to determine the fraction cholesterol and triacylglycerol (TAG) concentrations.

2.6. Histology

2.6.1. Preparation of histological slides

The fixed liver samples were washed in PELCO® Cryo-Embedding Compound (Ted Pella, Altadena, USA) before being frozen in 22x22x20 mm Peel-a-way embedding molds (Ted Pella, Altadena, CA, USA) using the same medium. An ice bath made of dry ice mixed with 70% ethanol was used for the initial freezing. The frozen blocks were then put in a freezer at −22 °C for a minimum of 24 h and cut in 10 µm slices with a Cryostat Leica CM19500 (Leica, Wetzlar, Germany) set at −25 °C. The slides were then air-dried at room temperature in a protected environment for 24 h before being stained with Oil red O (Sigma-Aldrich) and hematoxylin (Sigma-Aldrich).

2.6.2. Quantification of lipid droplets

Visiopharm Integrator System (Version 3.6.5.0, Visiopharm, Haersholm, Denmark) was used to randomly select 20 frames covering the area of interest. An Axioskop microscope (Zeiss, Oberkochen, Germany) equipped with an Olympus DP72 camera (Olympus, Tokyo, Japan) was used for the microscopy. The pictures were taken at 20× magnification and processed with the java-based image processing and analysis tool ImageJ (Version: 1.50i). By actively adjusting the threshold colors on a red-, green-, blue (RGB) scale, one could select a certain range of hues, which in this case were the lipid drops colored red (Fig. 1). The “analyze particles”-function was used to obtain data on the size and number of lipid droplets as well as the percentage of the area of interest covered by lipids. Settings were adjusted to only count particles with a minimum of 15 pixels to avoid irrelevant/non-lipid selections.

2.7. Lipid class analyses

Liver lipid was extracted in chloroform/methanol 2:1 (Merck, Darmstadt, Germany) with 1% BHT (2,6-di-tert-butyl-4-methylphenol; Sigma–Aldrich). The samples were analyzed for relative and absolute amounts of lipid classes by high performance thin layer chromatography (HPTLC) as previously described by Torstensen and Frøyland (2004).

2.8. Sensory testing

The center part of the fillet was divided into 3 × 3 cm skin- and boneless sections that were vacuum-packed and frozen at −40 °C for two months. Thawing was performed at 0–1 °C before sample preparation the next day. Fillet sections from salmon from each sea-cage were pooled and evaluated as raw and baked in triplicate by each assessor (n = 3). Baking was performed in a combi-oven (Electrolux Air-o-steam, Model AOS061EANQ) at 75 °C (50% steam/50% heat) until a core temperature of 59 °C was reached. Samples were cooled at 0–1 °C.

The sensory panel consisted of ten trained assessors with an average of 15 years of experience in sensory analysis (see Ådland Hansen et al. (2012) for details regarding the sensory panel and laboratory design). The raw and heated samples were served in white plastic containers with a lid at a temperature of 20 °C. The panelists recorded their results on a 15-cm non-structured continuous scale with the left side of the scale corresponding to the lowest intensity, and the right side corresponding to the highest intensity. The responses were transformed into numbers between 1 (low intensity) and 9 (high intensity). In a pre-test session, the assessors were calibrated on samples that were considered the most different (IM_0 versus IM_{100}) on the selected sensory attributes as shown in the supplementary Table 2. Tap water and unsalted crackers were available for palate cleansing.

2.9. Calculations

Growth and nutritional indices were calculated as followed:

Condition factor (CF) = body weight (g)/Length^3 (cm)*100.
Daily growth index (DGI) = 100 x ((final body weight) $^{1/3}$ - (initial body weight)$^{1/3}$) day^{-1}.
Hepatic Somatic Index (HSI) = liver weight (g)/body weight (g) * 100.
Visceral Somatic Index (VSI) = viscera weight (g)/body weight (g) * 100.
Feed conversion ratio (FCR) = feed intake (g)/fish weight gain (g).
Protein production value (PPV) = (final protein content – initial protein content)*protein fed $^{-1}$.
Lipid production value (LPV) = (final lipid content – initial lipid content)*lipid fed $^{-1}$.
Apparent digestibility (AD) = 100 − (Y_d*CX_f)*(Y_f*CX_d)$^{-1}$*100, where d is diet, f is faeces, Y is yttrium concentration, and CX is nutrient concentration.

Fatty acid productive value (FAPV):

$$FAPV = \frac{\text{g FA per tank at end of trial–g FA per tank at start of trial}}{\text{g FA eaten in total per tank during 16 week feeding trial}}$$

2.10. Statistical analysis

All statistical analyses were performed using the free software environment R (R Development Core Team, 2011). All data except for results from analyses of lipid droplet size were statistically evaluated by a regression design using a linear model (lm) and one-way ANOVA (Tukey test) to find differences due to dietary treatments. For data from the histological assessment of liver area covered by lipid (random factors: tank and picture). For lipid droplet size, generalized linear models (*glmer*) were used due to the gamma distribution of the data (random factors: tank and picture). All data were analyzed for homogeneity of variance using a Levene's test and for normality using a Shapiro Wilk's

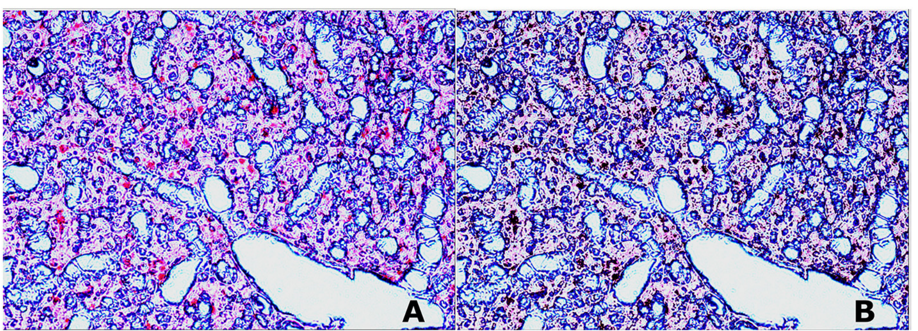

Fig. 1. The storage of neutral lipids in the cryo-cut liver tissue was stained red with Oil Red O (A) and image analyses tools were used to assess size and number of lipid droplets per cell. Hepatocytes selected by grid selection for lipid measurements were manually color adjusted to select the dyed lipid (selected area marked in black in B) and compared to the original image (A). The size of each individual lipid droplet was then measured in pixels using automatic quantification tools. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

test, as well as being evaluated graphically by QQ-plots before utilizing parametric tests. Differences were regarded as significant when $P \leq 0.05$. All data are presented as means and pooled standard error (SE), if not otherwise stated.

3. Results

3.1. Dietary composition

Analyzed proximate compositions of the experimental diets were similar to calculated compositions. All diets were similar in dry matter, protein, fat, energy and carbohydrates (Table 1). The level of peroxidation product (TBARS) increased slightly with increasing replacement of dietary FM with IM (Table 1). The diets had close to identical concentrations of the essential amino acids (Table 1). Dietary replacement of FM with IM resulted in lower concentrations of the non-essential AAs hydroxoproline and taurine, while leading to higher tyrosine, proline and valine concentrations (Table 1). Surprisingly, the concentration of the essential AA was lower in the diet where FM was replaced with IM at 66% than IM_{33} and IM_{100} diets (Table 1). This difference in AA composition might be due to the various protein sources included at different levels in the three experimental diets (IM_{33}, IM_{66} and IM_{100}) (Table1).

An increased dietary IM inclusion also led to some changes in dietary fatty acid composition (Table 2), such as an increase in the content of the medium-chain fatty acid 12:0 (lauric acid). The IM-containing diets were also slightly higher in the LC-HUFAs eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA). An increase in the total n-3 fatty acids combined with a reduced n-6 fatty acid content led to an increased dietary n-3/n-6 ratio.

3.2. Growth performance and somatic index

The fish almost tripled their weight during the 114 day feeding trial (initial weight: 1398 g, final weight: 3686 g) ($n = 3$). There were no significant effects of IM inclusion on final weight, weight gain or any of the growth or feed intake parameters (DGI, SGR, HSI, VSI, FI, FCR, CF, PPV and LPV) (Table 3).

Table 2
Fatty acid composition (g/100 g) and total fatty acids (FA, mg/g of wet weight) of the four experimental diets fed to Atlantic salmon.

	IM_0	IM_{33}	IM_{66}	IM_{100}
12:0	< LOQ	0.5	1.2	2.3
14:0	2.2	2.7	3.2	3.6
16:0	8.5	8.8	9.0	9.0
18:0	3.0	3.0	3.0	3.0
18:1n-9	40.0	36.0	33.0	30.0
18:1n-7	2.5	2.3	2.2	2.0
18:2n-6	14.0	13.0	12.0	11.0
18:3n-3	6.5	5.7	5.6	5.0
18:4n-3	1.4	1.6	2.0	2.0
20:4n-6 ARA	0.2	0.2	0.2	0.3
20:5n-3 EPA	3.0	3.5	4.0	4.4
22:5n-3 DPA	0.3	0.3	0.4	0.4
22:6n-3 DHA	2.9	3.4	4.0	4.0
Sum saturated FA	15.0	16.0	17.0	19.0
Sum MUFA	55.0	53.0	52.0	50.0
Sum EPA + DHA	6.0	7.0	8.0	8.5
Sum n-3	15.0	15.0	16.5	17.0
Sum n-6	14.0	13.0	12.3	11.6
Sum PUFA	29.0	28.0	29.0	29.0
n-3/n-6	1.1	1.2	1.3	1.4
Total FA (mg/g)	265	265	248	277

IM_0 = diet without insect meal (IM) inclusion; IM_{33}, IM_{66} and IM_{100} = 33, 66 and 100% replacement level of FM with IM, respectively. LOQ: limit of quantification (0.01 mg/kg sample). ARA = arachidonic acid; EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acid.

3.3. Apparent nutrient digestibility

Digestibility of crude protein, crude lipid, amino acids and fatty acids was not affected by dietary IM inclusion ($n = 3$) (Table 4).

3.4. Digestive enzyme activity and total bile acids level

No diet effect was observed for the activity of brush boarder enzyme leucine aminopeptidase ($n = 18$), nor for the trypsin activity (n = 3) or total bile acids level (n = 3) in the digesta (Table 5).

3.5. Whole fish composition

Replacing FM with IM had no significant effects on whole fish dry matter (39.4 ± 0.6), crude protein (17.1 ± 0.2), crude lipid (20.7 ± 0.8), ash (1.7 ± 0.0) (all shown as % of wet weight) or amino acid composition ($n = 3$) (Table 6). The whole body FA composition of the salmon was, however, significantly affected by IM inclusion (n = 3) (Table 7). By including IM in the diets, the concentration of lauric acid (12:0) increased (0.5–1.6% of the whole body total FA in the IM fed fish), while this FA was below the quantification limit in the whole fish fed diets without IM (IM_0) (Table 7). The concentration of EPA and DHA as well as the n-3/n-6 ratio increased significantly in a linear manner with increasing inclusion of IM at 33 (IM_{33}), 66% (IM_{66}) and 100% (IM_{100}) in the diets (Table 7). The concentrations of ARA, PUFA and stearidonic acid (18:4n-3) was not affected by dietary inclusion of IM (Table 7).

3.6. Fatty acid productive values

The fatty acid productive values (FAPV) calculated for the fish fed the different experimental diets, showed few dietary effects (supplementary Table 3) ($n = 3$). The FAPVs of the LC-HUFAs EPA and DHA decreased, however, significantly in the fish fed with increasing inclusion of dietary IM (as an example, FAPV for EPA decreased significantly from 0.44 in the IM_0 fed fish to 0.34 in the IM_{100} fed fish (supplementary Table 3).

3.7. Haemoglobin and plasma clinical chemistry

Dietary inclusion of IM did not affect the concentration of Hg ($n = 3$), ALT (n = 3), FFA ($n = 18$), TAG ($n = 18$), Chol (n = 3), Tprot (n = 3) or Na^+ (n = 18) in the plasma (Table 3). AST ($n = 18$) values significantly decreased due to increasing substitution of FM with IM in the diets, whereas a higher Glu (n = 18) level was found in fish fed the IM_{66} diet (Table 3).

3.8. Assessment of liver lipid storage

A total of 80′720 individual lipid droplets were measured in the liver of the IM_0 and IM_{100} fed fish by analyzing the cryo-cut liver slides (n = 18). The size of the lipid droplets ranged from the 15px cutoff in the bottom to the largest ones measuring 5863 and 6647 px in the IM_0 and IM_{100} fed fish, respectively. Most of the lipid droplets were, however, in the lower size range with 99.3% of the lipid droplets measuring < 300px and 88.2% < 50px. The median size of liver lipid droplets were 21 px for IM_0 and 22 px for IM_{100} and there was no significant difference in the size distribution of the droplets. The area of liver covered with stained neutral lipid was also measured in all photos taken of the histological slides (351 and 333 photos of IM_0 and IM_{100}, respectively). The percent of liver area covered by lipid in the IM_{100} fed fish was not affected by dietary treatment (0.63 ± 0.90% and 0.22 ± 0.48% of liver area covered by lipid in the IM_0 and IM_{100} fed

Table 3
Growth parameters and haemoglobin (Hg) and plasma clinical chemistry of Atlantic salmon fed diets with increasing replacement of fish meal with insect meal.

	Diets				Linear regression		*ANOVA*	
	IM_0	IM_{33}	IM_{66}	IM_{100}	R^2	*P*	Pooled SE	*P*
Growth parameters								
IW (g)	1398	1400	1386	1409	< 0.001	0.82	9.35	NS
FW (g)	3702	3650	3721	3668	< 0.001	0.91	36.51	NS
DGI	3.8	3.7	3.8	3.7	< 0.001	0.80	0.04	NS
SGR	0.9	0.8	0.9	0.8	< 0.001	0.80	0.008	NS
FI	1.9	1.9	1.9	1.9	< 0.001	0.55	0.01	NS
FCR	1.1	1.1	1.1	1.1	< 0.001	0.55	0.006	NS
CF	1.5	1.5	1.4	1.5	< 0.001	0.34	0.008	NS
HSI	1.1	1.1	1.1	1.1	< 0.001	0.33	0.28	NS
VSI	12.0	11.5	11.5	11.8	< 0.001	0.70	0.14	NS
PPV	0.3	0.3	0.3	0.3	< 0.001	0.67	0.003	NS
LPV	0.7	0.7	0.6	0.7	< 0.001	0.88	0.02	NS
Hg and plasma clinical chemistry								
Hg (g/100 ml)	9.1	9.7	10.0	9.0	< 0.001	0.95	0.16	NS
ALT (IU/l)	9.1	13.3	8.8	8.7	< 0.001	0.71	1.85	NS
AST (IU/l)	765	694	631	587	*0.2*	*0.05*	35.50	NS
Glu (mmol/l)	6.4^a	6.5^{ab}	7.4^b	6.1^a	< 0.001	0.96	0.20	*0.04*
FFA (mmol/l)	0.7	0.6	0.8	0.7	< 0.001	0.64	0.14	NS
TAG (mmol/l)	3.1	2.1	2.5	2.6	< 0.001	0.48	0.23	NS
Chol (mmol/l)	6.6	7.1	7.0	6.6	< 0.001	0.93	0.18	NS
T_{prot} (g/l)	41.4	44.8	45.6	42.6	< 0.001	0.66	0.89	NS
Na^+ (mmol/l)	178	176	177	174	0.18	0.10	1.2	NS

IM_0 = diet without insect meal (IM) inclusion; IM_{33}, IM_{66} and IM_{100} = 33, 66 and 100% replacement level of FM with IM, respectively. IW = initial weight; FW = final weight; DGI = daily growth increase (%/fish/day); SGR = specific growth rate; FI = feed intake (g/fish/day); FCR = food conversion ratio; CF = condition factor; HSI = hepatosomatic index; VSI = viscerosomatic index; PPV = protein productive value; LPV = lipid productive value; Hg = haemoglobin; ALT = alanine aminotransferase; AST = aspartate aminotransferase; Glu = glucose; FFA = free fatty acids; TAG = triacylglycerol; Chol = cholesterol; T_{prot} = total protein; Na^+ = sodium. Values are means and pooled standard error (SE). Significant differences $P \leq 0.05$; linear regression (R^2 = Adjusted R-squared) and one-way ANOVA (mean values in the same row with different superscript) were recorded among the dietary groups.

fish, respectively) (Fig. 1). There were no significant effects of the IM inclusion on liver lipid class composition (Table 8).

3.9. Sensory testing

Sensory scores for pleasant odor (fresh and sea-water, see supplementary Table 4) showed no significant difference between the dietary groups (Fig. 2) ($n = 3$). However, a 100% replacement of FM with IM resulted in a higher score for rancid odor of the baked salmon compared with the control group (IM_0) (supplementary Table 4). Rancid odor of the raw salmon and off-odor of the baked salmon showed the same tendencies. There were no significant flavor differences between the dietary groups, but the numerical scores for rancid flavor of the baked fillet increased with increasing inclusion level of dietary IM. No significant differences were recorded for the color scores of raw salmon between the dietary groups, but the color intensity of the cooked salmon was significantly lower in the IM_{66} group compared with the control group (IM_0) (supplementary Table 4). The raw salmon fed the IM_{100} were softer compared with the IM_{33}, while an opposite trend was observed for the baked salmon, where the salmon fed higher inclusion of IM tended to be harder. Higher moisture release was detected from the raw salmon fed the IM_{66} diet compared with the control group (IM_0) (supplementary Table 4). Overall regression analyses revealed no significant relationship between dietary inclusion level of insect meal and any of the sensory properties.

4. Discussion

In the current study, a partial or complete substitution of FM protein with IM in the diet did not negatively affect feed intake, growth performance or nutrient utilization of sea-water phase Atlantic salmon. These results are in agreement with previous studies on inclusion of BSF larvae ingredients in salmonid diets (BSF larvae meal included at 150–600 g kg $^{-1}$ diet), where no differences on the growth parameters were reported (Belghit et al., 2018a; Dumas et al., 2018; Lock et al., 2016; Renna et al., 2017). Similarly, a partial or total dietary replacement of FM with yellow mealworm (*Tenebrio molitor*) or housefly maggot (*Musca domestica*) meal did not led to negative effects on the growth of blackspot sea bream (*Pagellus bogaraveo*) or barramundi (*Lates calcarifer*), respectively (Iaconisi et al., 2017; Lin and Mui, 2017). Interestingly, a total replacement of dietary FM with cricket (*Gryllus bimaculatus*) meal (at an inclusion level of 350 g kg $^{-1}$ diet) increased body weight gain and specific growth rate of African catfish (*Clarias gariepinus*) (Taufek et al., 2016). However, reduced growth and feed utilization has been reported in juvenile turbot and rainbow trout when FM was replaced with BSF meal (Kroeckel et al., 2012; St-Hilaire et al., 2007). The authors of these papers speculate whether these negative effects on growth could be due to the presence of chitin in the BSF meal, which could affect the digestibility of the nutrients and therefore resulting in reduced fish growth. Kroeckel et al. (2012) also concluded that the diets with BSF were less palatable and that this had led to a reduced feed intake of the fish. Other feeding trials found that salmonids fed diets containing BSF larvae raised on fish offal or seaweeds grew better than fish fed dietary BSF larvae raised without marine nutrients (Sealey et al., 2011; Belghit et al., 2018a). The current feeding trial used an IM made from BSF grown on seaweeds. This means that the selection of substrate used to grow the insect is of potential importance for the success of the use of insect ingredients in aquafeeds. Overall, these varied results in growth performances between different trials might be due to differences in tolerance level of insect ingredients between different fish species, but also due to the various life stages of fish used for the trials.

In line with absence of diet effect on the proteinase activity (trypsin and leucine aminopeptidase) and total bile acids level in the digesta, no significant effects of including IM were found on the digestibility of crude protein, crude lipid, or AAs. The obtained values for AA apparent digestibility were comparable to those observed in other studies with European seabass (*Dicentrarchus labrax)*, rainbow trout and fresh-water

Table 4
Apparent digestibility coefficients (ADC %) of crude protein, crude lipid, amino acids and fatty acids in Atlantic salmon fed diets with increasing replacement of fish meal with insect meal.

	Diets				Linear regression		*ANOVA*	
	IM_0	IM_{33}	IM_{66}	IM_{100}	R^2	*P*	Pooled SE	*P*
CP	84	83	83	82	0.006	0.21	0.66	NS
CL	85	84	88	86	0.01	0.37	0.91	NS
Amino acid								
Ala	87	83	85	86	< 0.001	0.96	0.83	NS
Arg	93	90	91	91	< 0.001	0.68	0.66	NS
Asp	78	74	75	77	< 0.001	0.87	1.10	NS
Glu	90	87	90	91	< 0.001	0.58	0.81	NS
Gly	82	78	78	78	0.005	0.23	0.94	NS
His	87	83	84	84	< 0.001	0.57	0.92	NS
Hyp	67	n.c.	n.c.	n.c.	–	–	–	–
Ile	86	82	84	84	< 0.001	0.92	0.98	NS
Leu	89	85	87	88	< 0.001	0.81	0.82	NS
Lys	88	83	84	85	< 0.001	0.48	0.86	NS
Met	91	88	90	90	< 0.001	0.88	0.58	NS
Phe	90	87	88	88	< 0.001	0.85	0.74	NS
Pro	88	85	86	88	< 0.001	0.64	0.77	NS
Ser	86	82	84	85	< 0.001	0.85	0.80	NS
Tau	n.c.	n.c.	n.c.	n.c.	–	–	–	–
Thr	80	76	77	78	< 0.001	0.85	1.04	NS
Tyr	88	84	86	87	< 0.001	0.90	0.89	NS
Val	86	82	84	85	< 0.001	0.95	0.92	NS
Fatty acid								
12:0	n.c.	n.c.	n.c.	n.c.	–	–	–	–
14:0	92	85	86	85	0.11	0.16	1.39	NS
16:0	82	77	78	79	< 0.001	0.59	1.22	NS
18:1n-9	97	93	95	92	< 0.001	0.59	1.20	NS
18:1n-7	92	86	89	85	0.02	0.28	1.63	NS
18:2n-6	97	93	95	91	0.005	0.33	1.38	NS
18:3n-3	98	95	97	94	0.001	0.34	1.18	NS
20:1n-9	95	91	93	91	0.01	0.31	1.13	NS
18:4n-3	99	97	99	96	< 0.001	0.47	1.02	NS
20:4n-6 ARA	77	65	83	80	0.06	0.22	2.68	NS
22:1n-11	98	93	93	90	0.21	0.08	1.39	NS
20:5n-3 EPA	98	95	98	94	< 0.001	0.48	1.14	NS
22:5n-3 DPA	93	90	94	90	< 0.001	0.72	1.17	NS
22:6n-3 DHA	95	92	96	92	< 0.001	0.68	1.22	NS
Saturated FA	93	89	91	88	0.03	0.27	1.17	NS
Sum MUFA	97	93	94	91	0.05	0.23	1.20	NS
Sum EPA + DHA	97	94	97	93	< 0.001	0.58	1.20	NS
Sum n-3	98	94	97	93	< 0.001	0.43	1.21	NS
Sum n-6	97	92	95	91	< 0.001	0.34	1.39	NS
Sum PUFA	97	93	96	92	< 0.001	0.41	1.27	NS

IM_0 = diet without insect meal (IM) inclusion; IM_{33}, IM_{66} and IM_{100} = 33, 66 and 100% replacement level of FM with IM, respectively. n.c. = not calculated due to very low concentrations in either feed or faeces; ARA = arachidonic acid; EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acid. Values are means and pooled standard error (SE). Significant differences $P \leq .05$; linear regression (R^2 = Adjusted R-squared) and one-way ANOVA were recorded among the dietary groups.

Atlantic salmon fed dietary insect ingredients (Magalhães et al., 2017; Dumas et al., 2018; Belghit et al., 2018a). BSF larvae have a profile of both the essential and non-essential AAs close to that of FM, except for the content of the essential AAs methionine and lysine (supplementary Table 1) (Henry et al., 2015; Liland et al., 2017; Makkar et al., 2014). In the current study, these two essential amino acids were added to all diets in order to fulfil the requirement of Atlantic salmon (NCR, 2011; Espe et al., 2014). The addition of these essential AA to the diets could also be a reason why no changes in growth were seen in the current trial. Similarly, no differences were found in the growth of rainbow trout or Atlantic salmon fed with dietary IM supplemented with methionine and lysine (Belghit et al., 2018a; Dumas et al., 2018; Lock et al., 2016). On the other hand, feeding European seabass with IM (195 g kg^{-1} diet) did not lead to a negative effect on the growth performance, in spite of the low level of lysine in the insect-based diet (Magalhães et al., 2017). Thus, some fish species might tolerate higher FM replacement with insect ingredients without the need for additional essential AA, due to different AA requirements. Additionally, Magalhães et al. (2017) reported that the ADC of arginine, although not different in the diets, increased when fish were fed with dietary IM compared to seabass fed with plant ingredients and FM as protein sources. The high ADC of arginine in this study was probably due to the high bio-availability of this essential AA in BSF larvae (Magalhães et al., 2017). Our own studies confirmed the high levels of arginine in the IM (supplementary Table 1) and we also saw a high ADC of this essential AA (91%). Therefore, compared to plant protein ingredients, where arginine is a limiting AA (Andersen et al., 2013), the larvae of BSF are a valuable sources of this essential AA.

Non-essential AAs are not strictly needed in the diet because the fish can synthesize them themselves. They can, however, have beneficial effects on fish health and performance if present in the right concentrations (Wu et al., 2013). The IM used in the current trial contained low levels of the non-essential AAs hydropxoproline and taurine compared to a typical FM. In a previous study, we found that the hepatosomatic index increased in fresh-water salmon fed insect-based diets compared to fish fed with diets devoid of IM (Belghit et al., 2018a). We

Table 5
Digestive enzyme activity and total bile acids level in the intestine of Atlantic salmon fed diets with increasing replacement of fish meal with insect meal.

	Diets				Linear regression		*ANOVA*	
	IM_0	IM_{33}	IM_{66}	IM_{100}	R^2	*P*	Pooled SE	*P*
Trypsin (ΔOD/mg DM)								
PI1	353	423	470	356	< 0.001	0.89	90.5	NS
PI2	300	352	348	381	< 0.001	0.45	75.0	NS
MI	107	177	139	165	< 0.001	0.38	34.1	NS
DI1	92	109	111	125	0.023	0.29	22.1	NS
DI2	78	62	47	82	< 0.001	0.99	19.9	NS
Bile acids (μmol/g DM)								
PI1	209	234	228	217	< 0.001	0.91	35.5	NS
PI2	176	214	172	179	< 0.001	0.75	23.6	NS
MI	91	114	103	90	< 0.001	0.79	10.0	NS
DI1	38	39	46	39	< 0.001	0.78	7.2	NS
DI2	12	15	18	13	< 0.001	0.79	3.8	NS
Leucine aminopeptidase (μmol/h/mg protein)								
PI	460	407	391	375	0.098	0.17	30.3	NS
MI	180	187	205	175	< 0.001	0.94	11.4	NS
DI	368	331	399	336	< 0.001	0.89	38.6	NS

IM_0 = diet without insect meal (IM) inclusion; IM_{33}, IM_{66} and IM_{100} = 33, 66 and 100% replacement level of FM with IM, respectively. DM = dry matter; PI = proximal intestine; MI = mid intestine; DI = distal intestine. Values are means and pooled standard error. Values are means and pooled standard error (SE). Significant differences $P \le 0.05$; linear regression (R^2 = Adjusted R-squared) and one-way ANOVA were recorded among the dietary groups.

Table 6
Whole-fish amino acid composition (mg/ g) of Atlantic salmon fed diets with increasing replacement of fish meal with insect meal.

	Diets				Linear regression		*ANOVA*	
	IM_0	IM_{33}	IM_{66}	IM_{100}	R^2	*P*	Pooled SE	*P*
Ala	9.9	9.8	10.1	10.0	0.16	0.10	0.05	NS
Arg	9.1	9.1	9.4	8.9	< 0.001	0.88	0.11	NS
Asp	16.3	16.0	16.4	16.7	0.22	0.06	0.09	NS
Glu	20.6	19.9	20.9	20.8	0.05	0.22	0.14	NS
Gly	9.8	9.7	10.4	9.4	< 0.001	0.72	0.18	NS
His	4.0	4.0	4.1	4.1	0.03	0.26	0.04	NS
Hyp	1.1	1.0	1.2	0.9	< 0.001	0.39	0.05	NS
Ile	6.7	6.7	6.8	6.9	0.11	0.14	0.04	NS
Leu	12.0	11.9	12.1	12.1	0.06	0.22	0.05	NS
Lys	14.8	14.6	14.8	15.2	0.23	0.06	0.09	NS
Met	4.8	4.8	4.9	4.8	< 0.001	0.98	0.03	NS
Phe	6.4	6.5	6.6	6.4	< 0.001	0.94	0.08	NS
Pro	6.6	6.5	6.9	6.6	< 0.001	0.76	0.07	NS
Ser	6.7	6.7	6.9	6.7	< 0.001	0.95	0.05	NS
Tau	0.8	0.8	0.9	0.8	< 0.001	0.77	0.01	NS
Thr	7.5	7.4	7.6	7.5	< 0.001	0.43	0.03	NS
Tyr	5.1	5.2	5.3	5.1	< 0.001	0.94	0.05	NS
Val	7.8	7.7	7.9	8.0	0.08	0.17	0.04	NS

IM_0 = diet without insect meal (IM) inclusion; IM_{33}, IM_{66} and IM_{100} = 33, 66 and 100% replacement level of FM with IM, respectively. Values are means and pooled standard error (SE). Significant differences $P \le 0.05$; linear regression (R^2 = Adjusted R-squared) and one-way ANOVA were recorded among the dietary groups.

speculated that the low level of taurine in the IM might have affected the fat content of the liver, as the addition of taurine to a high-plant diet (low in taurine) had a positive effect on lipid metabolism and reduced liver lipid depositions in juvenile Atlantic salmon (Espe et al., 2012). In the current trial, however, a replacement of FM with IM did not affect the hepatosomatic index of the fish. Insect meal was included at a maximum level of 150 g kg^{-1} diet in the current trial, while in the previous study, 600 g kg^{-1} of IM in combination with insect oil were included in the diets of fresh-water stage salmon (Belghit et al., 2018a), which could explain the different results obtained between these two trials. Hydroxoproline is an abundant AA in animal tissues, predominantly found in collagens. Insect collagen does, however, not contain hydroxoproline. The work of Dumas et al. (2018) showed an increased digestibility of hydroxoproline when rainbow trout were fed dietary BSF meal and oil, compared to when fed diets devoid of insect ingredients. The authors also reported that the whole body content of this non-essential AA increased in rainbow trout fed with increasing level of BSF ingredients, even though the level of hydroxoproline was below detection limits in insect-based ingredients (Dumas et al., 2018). In the case of low dietary supply of hydroxoproline, animals can produce it by a post-translational hydroxylation of proline. Dumas et al. (2018) hypothesized that insect ingredients might contain some component able to promote the hydrolysis and absorption of proline and hydroxoproline in the small intestine.

An ~8% decrease in the whole body total FA was seen when replacing all the FM with IM. This was, however, not accompanied by a reduction in growth or performance, nor was it associated with any general reduction in the digestibility or retention of lipids (assessed by ADC and FAPVs of total lipid and individual FAs). Medium-chained FAs, like lauric acid (12:0), are known to be easily oxidized and to reduce lipid storage in both mammals and fish (Nordrum et al., 2003; St-Onge and Jones, 2002; St-Onge et al., 2008; Williams et al., 2006; Smith et al., 2005; Belghit et al., 2018b). Lauric acid (12:0) is the dominant fatty acid in BSF larvae, representing between 21% and 50% of total FAs (Liland et al., 2017; Oonincx et al., 2015), and increased when IM was included in the current experimental diets. The dietary lauric acid content was, however, quite low in the current trial, even in the diets where 100% of the FM was replaced with IM. This is due to the low FM content of the diets as well as the use of a partially defatted BSF meal (dietary lauric acid reaching a maximum of 6.5 g kg^{-1} diet, compared to 65 g kg^{-1} in Williams et al. (2006). The dietary lauric acid could therefore explain some of the reduced lipid storage in the IM fed fish, but is probably not the sole responsible for this.

The whole body content of EPA and DHA increased in the salmon fed with increasing inclusion of dietary IM. Additional fish oil was added to all the IM diets to replace the EPA and DHA removed by replacing the FM with IM. A slightly higher than estimated EPA and DHA content of the fish oil used in the diets led to a higher content of these FAs in the IM diets, finally resulting in higher whole body content of the same FAs. A higher dietary supply of long-chained n-3 FAs also likely reduced the need for biosynthesis of these FAs from the shorter n-3 FAs by the fish, reflected as reduced FAPV for many n-3 FAs in the IM fed fish.

Elevated activities of serum AST or ALT, are an indication of damage to liver cells. In the current trial, serum AST activity decreased by almost 24% in salmon fed with inclusion of IM in the diets. These results demonstrated that a partial or complete replacement of FM with IM in the diets did not lead to negative effects and suggest that dietary IM might have a protective effects in the liver of Atlantic salmon. To look for more changes in hepatic health, a histological evaluation of lipid accumulations was conducted on the two extreme dietary groups (IM_0 and IM_{100}). The size distribution of hepatic lipid droplets was not affected by dietary IM inclusion, neither were the hepatic TAG concentrations, as measured by lipid class analyses. Conversely, Li et al. (2017) reported histological changes in the liver of Jian carp fed defatted BSF larvae meal. In Li's study, the lipid content of hepatocytes decreased in fish fed with defatted BSF larvae meal compared to fish fed dietary FM (Li et al., 2017). The authors suggested that those observed effect might be related to the content of chitin and its derivatives found in the insect exoskeleton. These polymers have been shown to decrease the FA synthesis and to increase TAG hydrolysis in rat liver (Zhang et al., 2008). The difference in liver traits between Li et al. (2017) (IM inclusion at 10 g kg^{-1} diet) and the current findings (IM inclusion at 150 g kg^{-1} diet) could be due to differing content of chitin in the IMs used.

Modifications in fish feed ingredients can affect the color, flavor and

Table 7
Fatty acid (FA) composition (% of total FA) and total FAs (mg/g of wet weight) of the whole body of Atlantic salmon fed diets with increasing replacement of fish meal with insect meal.

	Diets				Linear regression		*ANOVA*	
	IM_0	IM_{33}	IM_{66}	IM_{100}	R^2	*P*	Pooled SE	*P*
12:0	< LOQ	0.5	1.0	1.6	–	–	0.17	–
14:0	2.0 [b]	2.4 [a]	2.7 [a]	3.0 [a]	0.97	*< 0.001*	0.11	*< 0.001*
16:0	9.1 [b]	9.4 [b]	9.5 [ab]	9.8 [a]	0.77	*< 0.001*	0.08	*0.002*
18:1n-9	38.3 [a]	36.5 [b]	34.1 [c]	32.6 [d]	0.86	*< 0.001*	0.02	*< 0.001*
18:1n-7	2.7 [a]	2.7 [a]	2.6 [b]	2.5 [b]	0.97	*< 0.001*	0.14	*< 0.001*
18:2n-6	13.2 [a]	12.7 [b]	12.3 [c]	11.9 [d]	0.95	*< 0.001*	0.06	*< 0.001*
18:3n-3	4.7 [a]	4.6 [a]	4.3 [b]	4.1 [b]	0.64	*< 0.001*	0.01	*0.01*
18:4n-3	0.8	0.8	0.9	0.9	0.32	*0.05*	0.01	NS
20:4n-6 ARA	0.8	0.8	0.8	0.8	0.34	*0.05*	0.02	NS
20:5n-3 EPA	2.2 [b]	2.3 [b]	2.5 [a]	2.6 [a]	0.71	*< 0.001*	0.02	*< 0.001*
22:5n-3 DPA	1.1 [b]	1.1 [b]	1.2 [a]	1.2 [a]	0.96	*< 0.001*	0.10	*< 0.001*
22:6n-3 DHA	4.2 [d]	4.5 [c]	4.8 [b]	5.2 [a]	0.95	*< 0.001*	0.37	*< 0.001*
Sum SFA	14.1 [d]	15.3 [c]	16.2 [b]	17.5 [a]	0.89	*< 0.001*	0.42	*< 0.001*
Sum MUFA	53.6 [a]	52.8 [a]	51.0 [b]	50.2 [b]	0.89	*< 0.001*	0.15	*< 0.001*
Sum EPA + DHA	6.4 [d]	6.8 [c]	7.3 [b]	7.7 [a]	0.92	*< 0.001*	0.15	*< 0.001*
Sum n-3	14.3 [b]	14.7 [b]	15.3 [a]	15.7 [a]	0.93	*< 0.001*	0.19	*< 0.001*
Sum n-6	15.6 [a]	15.1 [b]	14.4 [c]	14.0 [c]	< 0.001	0.36	0.07	*< 0.001*
Sum PUFA	30.2	30.1	30.0	30.0	0.98	*< 0.001*	0.02	NS
n-3/n-6	0.9 [b]	1.0 [b]	1.1 [a]	1.1 [a]	0.50	*0.005*	2.44	*< 0.001*
Total FA (mg/g)	206 [a]	196 [ab]	189 [b]	190 [b]	0.86	*< 0.001*	0.02	*0.02*

IM_0 = diet without insect meal (IM) inclusion; IM_{33}, IM_{66} and IM_{100} = 33, 66 and 100% replacement level of FM with IM, respectively. LOQ: limit of quantification (0.01 mg/kg sample). ARA = arachidonic acid; EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids. Values are means and pooled standard error (SE). Significant differences P ≤ 0.05; linear regression (R^2 = Adjusted R-squared) and one-way ANOVA (mean values in the same row with different superscript) were recorded among the dietary groups.

Table 8
Lipid class composition of liver (mg/g wet weight) of Atlantic salmon fed a diet without insect meal or a diet with 100% of the fish meal replaced with insect meal.

	Diets		Linear regression		*ANOVA*	
	IM_0	IM_{100}	R^2	*P*	Pooled SE	*P*
SM	4.2	3.7	< 0.001	0.60	0.22	NS
PC	19.7	16.4	0.06	0.07	1.10	NS
PS	2.1	2.0	< 0.001	0.67	0.10	NS
PI	3.1	4.4	0.02	0.16	0.43	NS
CL	0.8	0.8	< 0.001	0.31	0.01	NS
PE	4.3	5.0	< 0.001	0.32	0.55	NS
Total polar lipid	34.1	32.3	< 0.001	0.52	1.79	NS
DAG	0.8	0.7	*0.10*	*0.02*	0.01	NS
CHOL	3.3	3.3	< 0.001	0.84	0.10	NS
FFA	1.8	1.4	0.04	0.10	0.10	NS
TAG	29.4	22.4	< 0.001	0.37	3.22	NS
Total neutral lipid	35.3	27.8	< 0.001	0.33	3.23	NS
Total lipid	69.5	60.1	0.10	0.25	3.98	NS

IM_0 = diet without insect meal (IM) inclusion; IM_{100} = 100% replacement level of FM with IM. SM: sphingomyelin, PC: phosphatidylcholine, PS: phosphatidylserine, PI: phosphatidylinositol, CL: cardiolipin, PE: phosphatidylethanolamine, DAG: diacylglycerols, CHOL: cholesterol, FFA: free fatty acids, TAG: triacylglycerols Values are means and pooled standard error (SE). Significant differences P ≤ 0.05; linear regression (R^2 = Adjusted R-squared) and one-way were recorded among the dietary groups.

the aroma of fish fillet (Olsson et al., 2003; Turchini et al., 2009), which might affect the perceived fillet quality and consequently the consumers' acceptance. In the present trial, rancid odor increased in the baked fillet obtained from salmon fed with increasing IM inclusion in the diet. The values for rancid odor, however, remained low for all dietary groups, and were similar to the values obtained for Atlantic salmon fed with other alternatives diets (Ådland Hansen et al., 2012; Rødbotten et al., 2009). The rancidity odor scores of cooked salmon were higher than in the raw salmon. An increase in fillet HUFA, like in the IM fed fish in the current trial, renders fish flesh more susceptible to oxidation during thermal treatment (Medina, et al., 1998). The elevated temperatures during the cooking process might have further stimulated the degradation of lipid components in the fillet. The initial quality of the feed ingredients might also influence the sensory profile. By measuring sensory attributes and physico-chemical parameters, Borgogno et al. (2017) found differences in perceived intensity of aroma, flavor and texture in the fillet of rainbow trout fed with dietary IM. The authors reported an increase in metallic flavor in the fillet of fish fed with increasing inclusion of IM in the diet compared to fish fed with FM (Borgogno et al., 2017). Other feeding trials, however, did not find any sensory differences in the fillet of fish fed with insect-based diets (Lock et al., 2016; Sealey et al., 2011). Based on the current trial and earlier published results on sensory attributes of the fillet of insect-fed fish, we can conclude that using insect ingredients in fish feeds leads to only marginal changes in fillet sensory quality.

5. Conclusion

In this study, we evaluated the effects of graded inclusion level of a partially defatted black solider fly larvae meal on growth performances, digestibility, nutrient utilization, liver health and fillet sensory qualities of Atlantic salmon of a commercially relevant size. Only minor effects were detected of replacing up to 100% of the fishmeal with the insect meal. Therefore, our conclusion is that the insect meal made from BSF is a nutritionally appropriate source of protein for sea-water stage Atlantic salmon.

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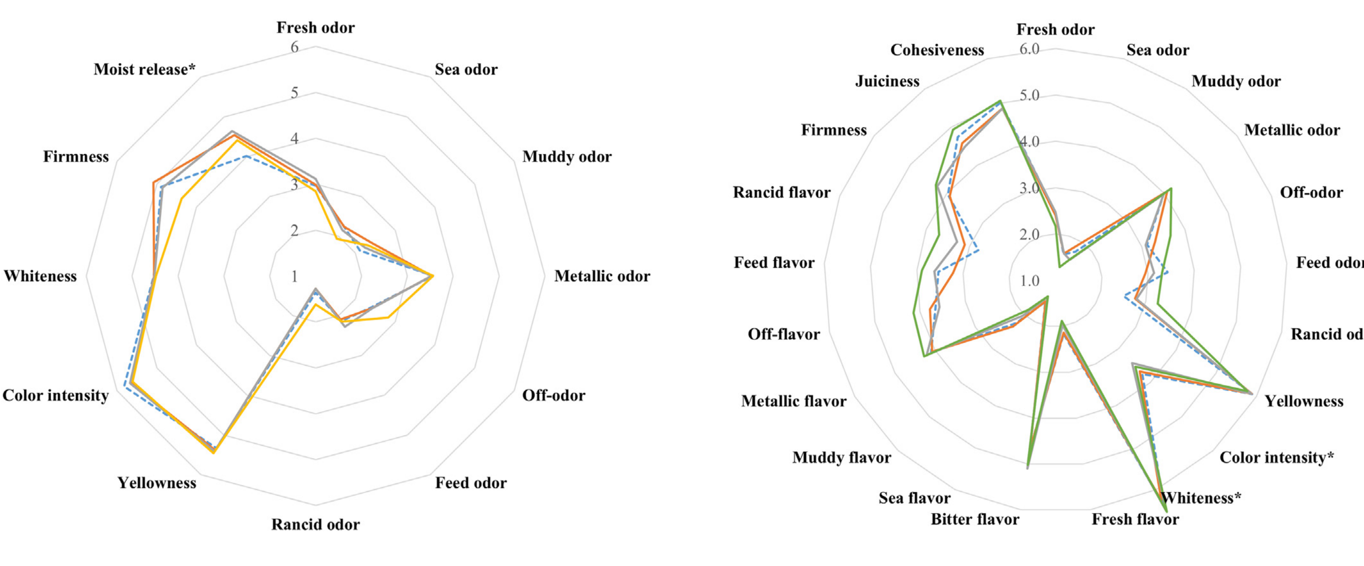

Fig. 2. Spider web diagram of the sensory evaluation of (A) raw and (B) baked fillets from IM_0, IM_{33}, IM_{66} and IM_{100} fed Atlantic salmon. A 100% substitution of fishmeal with insect meal (IM_{100}) resulted in a softer texture of the raw fillets compared with the fillets of fish fed with only 33% fishmeal replacement (IM_{33}). The rancidity odor of baked fillet from the IM_{100} group was significantly higher than in the control group without insect meal (IM_0). * Significant differences $P \leq 0.05$; (one-way ANOVA). IM_0 = diet without insect meal (IM) inclusion; IM_{33}, IM_{66} and IM_{100} = 33, 66 and 100% replacement level of FM with IM, respectively.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2018.12.032.

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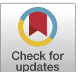

Dietary fishmeal replacement by black soldier fly larvae meals affected red drum (*Sciaenops ocellatus*) production performance and intestinal microbiota depending on what feed substrate the insect larvae were offered

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ABSTRACT

This study evaluated the production of two black soldier fly larval meals (BSFLMs) when rearing the larvae on brewers' spent grains (BSFLM-B) or a commercially available substrate (BSFLM-C, Gainesville house fly diet). The resulting nutrient composition of the BSFLMs were significantly impacted by the feeding substrates, and they were evaluated as a protein ingredient for red drum, where BSFLM-B and BSLFM-C replaced 65% of the fishmeal protein from the control diet. For a comparative feeding trial, three experimental diets were formulated to be isonitrogenous and isoenergetic. Groups of 12 red drum (~ 5 g) were stocked in each of 15, 38-L aquaria, and the three experimental diets were distributed in a completely randomized block design. The experimental diets were offered for 8 weeks at fixed percentage of the body weight that was weekly adjusted. Red drum fed BSFLM-B diets presented impaired weight gain and feed efficiency when compared to the control group. However, red drum fed BSFLM-C diets had growth performance numerically lower, but it was not statistically different than that of fish fed the control diet. Dietary inclusion of BSFLMs significantly affected the whole-body saturated fatty acid composition, total omega-3 and omega-6, and reduced the mineral profile of red drum. The intestinal microbiota was also affected by the dietary BSFLMs, with possible reduction of the relative

Abbreviations: ANOSIM, Analysis of similarities; BSF, Black soldier fly; BSFL, Black soldier fly larvae; BSFLM, Black soldier fly larvae meal; CMC, Carboxymethyl cellulose; FE, Feed efficiency; HSI, Hepatosomatic index; IPF, Intraperitoneal fat; LDA, Linear discriminant analysis; LEfSe, Linear discriminant analysis effect size; PCE, Protein conversion efficiency; PD, Faith's phylogenetic distance; PSE, Pooled standard error; PWG, Percentage of weight gain; OTU, Operational taxonomic units; %SC, Percentage similarity coefficient.

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abundance of a prominent pathogenic bacterial *Flavobacterium* spp. The findings of the present study highlight the importance of the nutrition of BSFL prior ingredient manufacturing to be a suitable sustainable replacement of forage fishmeal for carnivorous fish, and the potential nutraceutical properties of BSLFMs to possibly combat enteric pathogenic in industrial conditions.

1. Introduction

In the past two decades, the rapid growth of fed-aquaculture worldwide, along with the downward trend in the capture of forage fish, has led to increased use and demand of alternative protein ingredients to manufacture aquafeeds (Hua et al., 2019; Naylor et al., 2021). Among the group of novel protein ingredients, insect meals are the most promising replacement for forage fishmeal in aquaculture diets (Cottrell et al., 2020). The commercial production of insects as ingredients has been gaining traction not only to supply protein and lipid for aquafeeds, but also as sustainable waste management tool to recycle essential nutrients from several organic waste streams (Lock et al., 2018).

The black soldier fly (BSF, *Hermetia illucens*) is the most investigated insect species as an alternative protein ingredient for aquaculture diets (Hua, 2021). This insect thrives in temperate and tropical climates and is widely spread geographically (Barragan-Fonseca et al., 2017). Another positive aspect of farming BSF, is that this creature is not considered a pest (Barragan-Fonseca et al., 2017); thus, no precautionary measures are required for culturing BSF. The meals from BSF are usually manufactured from the larval stage, and their nutrient composition is another desirable characteristic that makes this insect a promising and potentially prominent aquaculture feed ingredient. Moreover, the black soldier fly larvae meal (BSFLM) contain bioactive molecules with nutraceutical properties that can improve the health of the fish, such as chitin, lauric acid, and antimicrobial peptides (Surendra et al., 2020). These molecules have been reported to have either prebiotic property, *i.e.*, being fermented by beneficial commensal bacteria in the intestine and creating metabolites that will ameliorate the health of the host (Lauzon et al., 2014; Ringø et al., 2012), or bacteriostatic/bactericidal effects (Surendra et al., 2020). It is well established that the interaction between the intestinal microbiome and immunity dictates the nourishment and health of the host, whether by aiding the development of the digestive system, maintaining the intestinal mucosal barriers, or conferring increased disease resistance (Merrifield et al., 2011; Romero et al., 2014). Thus, the deleterious effects of replacing fishmeal in the diets of carnivorous fish could be diminished by the nutraceutical compounds found in the BSFLM, that can be beneficial to the intestinal microbiome and improve the intestinal health of the fish.

Given the volatility of the commodity markets and the increase in the demand and prices of high-quality feed ingredients, aquaculture feed millers need to diversify their protein feedstuff portfolio to keep manufacturing aquafeeds in a sustainably and economically manner. The inclusion of BSFLM in animal feeds can be a sustainable approach since their production can recycle and reutilize nutrients from several organic waste streams (Fischer and Romano, 2021; Gasco et al., 2020). Therefore, including the BSFLM as an aquafeed ingredient not only brings value for underutilized waste and promotes a circular economy approach, but can also align with the target 12.3 of the 17 Sustainable Development Goals established in 2015 by the United Nations, which aims to cut food waste by half by 2030 (UN, 2015). One prospective waste stream that is readily available and can be explored at an industrial scale is spent grains from the brewing industry. After fermentation and ethanol extraction, the remaining protein-fiber rich fraction has a short shelf life due to the high moisture content and microbial load, and is often underutilized, with an estimated 20% of total world production being wasted and dumped in landfills (Scala et al., 2020).

Red drum (*Sciaenops ocellatus*) is a marine teleost fish species that is naturally found from the Gulf of Mexico to the northern Atlantic Ocean (Matlock, 1987). In the early 1980's, efforts to restock the depleted red drum wild population prompted the development of technologies for spawning and rearing this species in captivity. In its natural habitat, this species is considered a high-trophic-level fish that mainly preys on other fish, mollusks, and arthropods (Matlock, 1987). However, in farming conditions, red drum can easily transition to manufactured diets during the juvenile stages and will consume high inclusion of plant protein ingredients and by-products derived from animal rendering (Davis et al., 1995; Kureshy et al., 2000; Minjarez-Osorio et al., 2016; Rossi et al., 2017b). Nevertheless, identifying and evaluating alternative protein sources that can be sustainably produced to potentially replace fishmeal in carnivorous marine fish is paramount. The objectives of this study were two-fold: firstly, to evaluate the BSFLM nutrient composition when the BSFL were cultured on either brewers' spent grains or a commercial insect feeding substrate. And secondly, to evaluate the resultant BSFLMs as protein ingredients in the diets of red drum when replacing menhaden fishmeal on an equal-protein basis. This assessment included the evaluation of dietary BSFLM on red drum production performance, whole-body nutrient composition, intestinal microbiota using next-generation sequencing, and the predicted functions of the bacterial communities.

2. Materials and methods

2.1. Black soldier fly larvae rearing conditions and nutrient composition

An individual container (PopWorms! LIVE™, Popworms, College Station, TX) consisting of approximately 10,000 small BSFL was placed on 7 kg of either of the two substrates: 1) spent grains from brewers' production at 70% moisture or 2) Gainesville House Fly Diet, which mainly consisted of 300 g kg^{-1} of alfalfa meal, 500 g kg^{-1} of wheat bran, 200 g kg^{-1} of cornmeal, supplemented with peanut hulls, (Hogsette, 1992), also at 70% moisture. Spent grains from the brewery industry was selected as an organic waste due to their

constant supply, and due to the larvae better production performance when compared to other vegetable and fruit wastes (Scala et al., 2020). Substrates and BSFL were placed in 27-L polypropylene trays (Sterilite Corporation, Townsend, MA) and stored in an environmental room at 27.0 ± 1.0 °C, with 70.0% relative humidity, and controlled photoperiod of 14 h light:10 h dark. Larvae were allowed to feed on either of the rearing substrates and then harvested at optimum weight at the 7th day. The resulting larvae were separated from the rearing substrate, placed in a clean tray containing dry Gainesville diet, and allowed to sit in the environmental chamber overnight to purge their intestinal contents for 24 h. Fasted larvae were then sifted from the dry residue and dried using an industrial microwave (MAX-6B, MAX Industrial Microwave, Shandong, China). The dried larvae were then ground using a mechanical blender and stored at − 20 °C prior to manufacturing the experimental diets. These procedures were performed twice and considered as replicates for measuring the nutritional composition of the meals.

BSFLMs were analyzed for dry-matter gravimetrically by drying the ground samples overnight at 105 °C; crude protein by using the Dumas method (Nitrogen × 6.25) (AOAC, 2005); crude lipid was also measured gravimetrically after cold solvent extraction with chloroform-methanol (4:1) (Folch et al., 1957); and ash was measured by weighing samples after combustion at 650 °C for 5 h (AOAC, 2005). The gross energy of the samples was determined in a bomb calorimeter (Parr 6200; Parr Instrument Company, Moline, IL). A lipid droplet subsample was isolated from these ingredients and preserved in N_2, at − 80 °C prior to characterization of fatty acid profile by flame ionized gas chromatography (Smith et al., 2012). The amino acid profile of the two BSFLMs was determined by

Table 1
Proximate composition, energy content, essential amino acid and fatty acid profile of the black soldier fly (*Hermetia illucens*) larvae meals reared in two different substrates. Data is presented as g 1000 g^{-1} of dry sample, unless otherwise stated.

	BSFLM-B	BSFLM-C	PSE	P-value
Proximate composition (g/1000 g of sample)				
Dry matter	949.9	949.4	3.9	0.93
Crude protein	433.1	448.0	10.3	0.41
Crude lipid	337.9^{A}	269.1^{B}	5.7	0.01
Ash	61.6^{B}	93.3^{A}	3.3	0.02
Gross energy (MJ kg^{-1})	26.4^{A}	24.1^{B}	0.2	0.01
Amino acid profile (g/1000 g of sample)				
Histidine	14.7	14.3	0.2	0.24
Taurine	0.0	0.2	0.0	0.13
Arginine	22.7	22.8	0.3	0.83
Threonine	18.1	17.6	0.2	0.26
Lysine	24.3	23.6	1.1	0.67
Met + Cys	7.1^{A}	6.6^{B}	0.0	0.0004
Valine	24.1	23.3	0.3	0.15
Isoleucine	17.6	17.2	0.3	0.33
Leucine	28.7	27.7	0.4	0.14
Phenylalanine	18.3	17.4	0.5	0.26
Fatty acid profile (g/1000 g of fatty acid methyl esters)				
C12:0	297.5^{A}	232.7^{B}	10.9	0.05
C14:0	103.9	97.2	7.3	0.58
C16:0	175.7^{A}	148.8^{B}	4.1	0.04
C18:0	33.1	35.6	3.6	0.66
C20:0	1.4^{B}	14.2^{A}	0.2	0.0004
SFA[a]	612.2^{A}	529.1^{B}	12.7	0.04
C14:1	4.0^{B}	7.5^{A}	0.4	0.02
C16:1	26.2	35.9	3.7	0.2
C18:1 n-9	127.4	140.0	9.4	0.44
C20:1	1.7	1.6	0.2	0.6
MUFA[b]	159.2	185.2	11	0.23
C18:2	209.8	142.3	12.7	0.06
ω-6[c]	210.6	142.9	12.4	0.06
C18:3	21.3	13.3	3.4	0.24
C22:6	2.5^{B}	6.0^{A}	0.5	0.04
ω-3[d]	24.0	19.3	3	0.39
PUFA[e]	258.5	181.4	14.4	0.06
ω-3:ω-6[f]	1.1	1.4	0.2	0.53

Values are expressed as means of two replicates.
Different superscript letters are significantly different ($P < 0.05$).
Abbreviations: BSFLM-B: Black soldier fly larvae meal reared in spent grains; BSFLM-C: Black soldier fly larvae meal reared in the Gainesville commercial fly diet; Met + Cys: Methionine + Cysteine; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; PSE: Pooled standard error; SFA; Saturated fatty acids.
[a] Sum of all fatty acids without double bonds.
[b] Sum of all fatty acids with a single double bond.
[c] Sum of all ω-6 fatty acids.
[d] Sum of all ω-3 fatty acids.
[e] Sum of all fatty acids with ≥ 2 double bonds.
[f] Ratio between the sum of all ω-3 and the sum of all ω-6 fatty acids.

high-pressure liquid chromatography (Acquity UPLC, Waters Corporation, Milford, MA) by derivatizing the samples after acid hydrolysis. Results from the proximate analysis of the BSFLMs including their fatty acid and indispensable amino acid profile are reported in Table 1.

2.2. Experimental diets, fish, and culturing conditions

Experimental diets were formulated to be isonitrogenous, isolipidic, and isoenergetic, containing 420 g kg^{-1} of crude protein, 120 g kg^{-1} of crude lipid, and an estimated 14.2 MJ of digestible energy per kg of diet. The control diet was formulated on a dry-matter basis to have crude protein mainly provided by menhaden fishmeal (25%), poultry by-product meal (10%), soy protein concentrate (27.5%), and dehulled soybean meal (37.5%) (Table 2). The BSFLMs were included in each of the experimental diets at 150 g kg^{-1}, replacing 16% (out of the 25%) of the total protein provided by menhaden fishmeal, or 65% of fishmeal protein. The ingredients for each experimental diet were individually weighed, mixed for 30 min using in a V-mixer machine (Blend Master, Buflovak, NY), and then gradually blended for 15 min with the lipid source and water in an industrial mixer (A-200, Hobart, Hillsboro, OH). The resultant mixture was cold-pelleted through a 3-mm die plate and dried for 48 h at room temperature with forced ventilation. The diets were ground and sieved throughout the trial to appropriate size for the fish. Ingredients prior to diet formulation and experimental diets were analyzed for proximate composition (Table 2), fatty acid, and indispensable amino acids (Supplementary Table 1), using the aforementioned methods for the BSFLMs analysis.

Red drum fingerlings were provided by the Texas Parks and Wildlife Department (TPWD, Lake Jackson, TX) and transported to the Aquacultural Research and Teaching Facility (ARTF, College Station, TX) of the Texas A&M University System. The fingerlings were pellet-trained using a commercial diet for marine carnivore fish (Otohime C1, Marubeni Nisshin Feed Co., Tokyo, Japan) and raised to adequate size prior to the comparative feeding trial. This study was carried out in compliance with the Institutional Animal Care and Use Committee at Texas A&M University (IACUC 2019-0448).

One hundred and eighty red drum juveniles were equally distributed in 15, 38-L aquaria, operating as a recirculating aquaculture system, which included a common settling chamber and biological and mechanical filtration. Aeration was provided continuously through air stones connected to a regenerative air blower. The water temperature was conditioned by the ambient air, and salinity, hardness, and alkalinity were maintained at minimum levels tolerated by red drum (Gatlin et al., 1992) through the addition of synthetic marine salt (Red Sea Salt, Red Sea, Houston, TX). The three experimental diets were randomly distributed to the 15 aquaria

Table 2
Formulation and analyzed proximate composition of the experimental diets fed to the red drum (*Sciaenops ocellatus*) for 8 weeks in the comparative feeding trial. Values are expressed as g kg^{-1} on a dry matter basis.

Ingredients	Control	BSFL-B	BSFL-C
Menhaden fishmeal[a]	154.5	55	55
Poultry by-product meal[b]	60.5	60.5	60.5
Soy protein concentrate[c]	163	163	163
Dehulled soybean meal[d]	301	301	301
BSFLM Brewers' spent grains[e]	0	150	0
BSFLM Commercial diet[e]	0	0	150
Dextrinized corn starch[f]	50	50	50
Fish oil[a]	84	48	58.5
Glycine[f]	10	10	10
Lysine[g]	10	10	10
Taurine[f]	10	10	10
Methionine[h]	7.5	7.5	7.5
CMC[f]	20	20	20
Cellufill[f]	59.5	45	34.5
Mineral premix[f]	40	40	40
Vitamin premix[i]	30	30	30
Analyzed proximate composition			
Dry matter	903.3	903.5	905.9
Crude protein	471.1	473.1	470.7
Lipid	137	137.2	129.1
Ash	107.9	92.3	98.1

Values are expressed as means of 3 analyzed replicates
Abbreviations: BSFLM: Black soldier fly larvae meal; CMC: Carboxymethyl cellulose.
[a] Omega Protein Corporation, Abbeville, LO.
[b] Tyson Foods, Springdale, AR.
[c] ProFine F. DuPont Nutrition & Biosciences, New Century, KS.
[d] Producers Cooperative Association, Bryan, TX.
[e] Black soldier fly larvae meal reared in the Entomology department at Texas A&M University.
[f] MP Biomedicals, Solon, OH.
[g] ADM Animal Nutrition, Quincy, IL.
[h] Ajinomoto North America Inc., Itasca, IL.
[i] Same as in Moon and Gatlin III (1991).

(n = 5), that were set up side by side, in a fashion that all dietary treatments were equally spaced and could be statistically blocked by their position. Fish were acclimated with the control diet for 1 week before beginning the experiment. Before the start of the feeding trial, a subset of 10 fish from the same cohort was euthanized with an overdose of tricaine methanesulfonate (250 mg L^{-1}, MS-222, Western Chemical, Ferndale, WA) (Topic Popovic et al., 2012), to analyze the initial whole-body composition. At the beginning of the feeding trial, fish were group weighed and counted, and feed rations were offered according to a percentage of the biomass. This procedure was repeated weekly to monitor weight gain, and feed rations were adjusted to the biomass but close to apparent satiation (initially starting at 6% and progressively reducing to 2.75%). Fish rations were offered twice daily (9:00 AM and 4:00 PM) for 8 weeks.

Water quality parameters were measured three times a week, and parameters were kept within a suitable range for red drum culture (Neill, 1987). Water temperature and dissolved oxygen were measured using an optical dissolved oxygen meter (ProODO, YSI Inc., Yellow Springs, OH), pH was measured using a portable pH meter (Pocket Pro pH tester, Hach Company, Loveland, CO), salinity was measured with a portable salinity meter (Pocket Pro Salinity Tester, Hach Company), total alkalinity was measured by the sodium hydroxide-phenolphthalein titration and bromocresol green-methyl red indicator (Hach Company), total hardness was measured by EDTA titration and a calcium indicator (Hach Company), and total ammonia-, and total nitrite-nitrogen were measured photometrically with test reagents and the DR2000 spectrophotometer (Hach Company). Average water quality parameters throughout the trial were as follows (average ± standard deviation): Temperature: 26.7 ± 0.7 °C; dissolved oxygen: 6.95 ± 0.95 mg L^{-1}; total ammonia-nitrogen 0.11 ± 0.12 mg L^{-1}; total nitrite-nitrogen 0.058 ± 0.18 mg L^{-1}; pH 8.2 ± 0.3; salinity 2.54 ± 0.88 mg L^{-1}; total hardness 364.7 ± 146.8 mg $CaCO_3$ L^{-1}, total alkalinity 143.6 ± 27.1 mg $CaCO_3$ L^{-1}. The photoperiod was set for 12:12 h dark:light, using fluorescent lights controlled by timers.

2.3. Sampling procedures

At the end of the week 8 of feeding experimental diets, fish were netted out, counted, and group-weighed to compute weight gain. Three fish per aquarium were euthanized using an overdose of MS-222 (250 mg L^{-1}) to determine the proximate composition of the whole-body, and fatty acid profile using the methods previously described. An additional set of three fish were also euthanized, individually weighed, and liver and intraperitoneal fat were dissected to measure the condition indices [hepatosomatic index (HSI) and intraperitoneal fat (IPF) ratio]. Furthermore, one side of each fish was filleted and skinned to obtain the muscle yield. Production performance parameters and condition indices were computed as follows:

$$\text{Percentage of weight gain (\% of initial)} = 100 \times [(\text{Average weight at the 8th week (g)} - \text{average initial weight (g)})/\text{average initial weight (g)}]$$

$$\text{Feed efficiency (FE)} = \text{weight gain (g)} / \text{dry feed intake (g)}$$

$$\text{Protein conversion efficiency (PCE) (\%)} = \{[(\text{Final body weight (g)} \times \text{final body protein (\%)}) - (\text{initial weight (g)} \times \text{initial body protein (\%)})] \div \text{protein intake (g)}\} \times 100$$

$$\text{Fillet yield (\%)} = [\text{fillet weight (g)} \times 2 / \text{body weight (g)}] \times 100$$

Table 3
Production performance and condition indexes of red drum (*Sciaenops ocellatus*) fed the experimental diets for 8 weeks.

	Initial weight (g)	PWG[a] (%)	FE[a]	Fillet yield[b] (%)	HSI[b] (%)	IPF[b] (%)	Survival[1] (%)
Control	5.6	681.5^{A}	0.82^{A}	29.9	1.57	0.67	95.0
BSFL-B	5.6	521.6^{B}	0.70^{C}	29.6	1.69	0.69	91.6
BSFL-C	5.5	597.2^{AB}	0.77^{B}	29.2	1.80	0.62	93.3
PSE	0.04	21.3	0.01	0.7	0.08	0.10	3.3
P-value		0.002	0.0002	0.78	0.19	0.89	0.78
Block P value		0.10	0.05	0.16	0.30	0.33	0.38

Abbreviations: BSFL-B: Black soldier fly larvae reared in brewers' spent grains; BSFL-C: Black soldier fly larvae reared in Gainesville feeding substrate; FE: Feed efficiency; HSI: Hepatosomatic index; IPF: Intraperitoneal fat; PSE: Pooled standard error; PWG: Percentage of weight gain.
[a] Values are means of five replicates. Different superscript letters designate significant ($P < 0.05$) differences.
[b] Values are means of three fish from each of five replicate groups.
[1] The significance is $P<0.05$, and it should be attributed for PWG and FE only, as those were the only significant parameters found in this table.

Table 4
Proximate composition of whole-body tissues from red drum (*Sciaenops ocellatus*) fed the experimental diets for 8 weeks. Results are expressed as g kg^{-1} in wet-basis, unless otherwise stated.

	Moisture	Protein	Lipid	Ash	PCE (%)
Control	716.5	179.2	52.3^{B}	39.4^{A}	34.0^{A}
BSFL-B	723.1	177.1	57.9^{A}	36.3^{B}	25.1^{B}
BSFL-C	716.5	181.5	57.0^{AB}	36.5^{B}	28.4^{B}
PSE	5.9	2.8	1.3	0.5	1.5
P-value	0.68	0.55	0.03	0.004	0.005
Block P value	0.56	0.61	0.42	0.42	0.03

Values are means of composite samples of three fish from each of five replicate groups. Different superscript letters designate significant ($P < 0.05$) differences.
Abbreviations: BSFL-B: Black soldier fly larvae reared in brewers' spent grains; BSFL-C: Black soldier fly larvae reared in Gainesville feeding substrate; PCE: Protein conversion efficiency; PSE: Pooled standard error.

Table 5
Fatty acid composition of red drum (*Sciaenops ocellatus*) whole-body tissues expressed as g/1000 g of fatty acid methyl esters (FAME).

Fatty acids	Control	BSFL-B	BSFL-C	PSE	P-value	Block P-value
C12:0	0.9^{C}	46.2^{A}	40.0^{B}	0.7	< 0.0001	0.82
C14:0	51.8^{B}	72.2^{A}	71.4^{A}	0.7	< 0.0001	0.71
C16:0	199.5	201.6	196.7	2.4	0.41	0.49
C18:0	45.3	45.8	48.3	0.1	0.10	0.66
C20:0	15.3^{A}	8.9^{B}	10.2^{B}	0.3	< 0.0001	0.78
C22:0	13.9^{A}	10.0^{B}	10.9^{B}	0.2	< 0.0001	0.42
SFA[a]	328.6^{B}	386^{A}	379.3^{A}	3.2	< 0.0001	0.45
C16:1	121.9^{A}	77.2^{B}	87.5^{B}	10.4	0.04	0.44
C18:1 n-9	95.5^{B}	122.8^{A}	122.9^{A}	0.9	< 0.0001	0.87
C18:1 n-11	39.5^{A}	30.9^{C}	34.5^{B}	0.5	< 0.0001	0.09
C24:1	1.6^{A}	1.1^{B}	1.3^{B}	0.07	0.006	0.09
MUFA[b]	270.6	244.0	258.3	10.7	0.27	0.52
C18:2	65.1^{C}	131.0^{A}	99.1^{B}	1.3	< 0.0001	0.26
C20:2	2.0^{B}	3.0^{A}	2.8^{A}	0.2	0.007	0.90
C20:4	13.7^{A}	9.2^{B}	9.7^{B}	0.7	0.003	0.45
ϖ-6[c]	81.3^{C}	143.9^{A}	112.5^{B}	1.7	< 0.0001	0.35
C18:3	16.2	18.0	16.5	0.4	0.054	0.80
C20:5	92.5^{A}	50.4^{B}	57.2^{B}	1.8	< 0.0001	0.91
C22:6	102.8^{A}	56.9^{B}	64.4^{B}	2.3	< 0.0001	0.81
ϖ-3[d]	211.6^{A}	125.3^{B}	138.2^{B}	4.3	< 0.0001	0.86
PUFA[e]	292.9^{A}	269.3^{B}	250.7^{B}	5.5	0.002	0.76
ϖ-3:ϖ-6[f]	2.6^{A}	0.9^{C}	1.2^{B}	0.04	< 0.0001	0.89

Values are means of composite samples of three fish from each of five replicate groups.
Different superscript letters designate significant ($P < 0.05$) differences.
Abbreviations: BSFL-B: Black soldier fly larvae reared in brewers' spent grains; BSFL-C: Black soldier fly larvae reared in Gainesville feeding substrate; MUFA; Monounsaturated fatty acid; PUFA: Polyunsaturated fatty acid; PSE: Pooled standard error; SFA: Saturated fatty acids.
[a] Sum of all fatty acids without double bonds.
[b] Sum of all fatty acids with a single double bond.
[c] Sum of all ϖ-6 fatty acids.
[d] Sum of all ϖ-3 fatty acids.
[e] Sum of all fatty acids with ≥ 2 double bonds.
[f] Ratio between the sum of all ϖ-3 and the sum of all ϖ-6 fatty acids.

$$\text{Viscerosomatic indices}\quad (\text{HSI or IPF ratio})(\%) = [\text{liver or IPF}\quad (g)/\text{body weight}\quad (g)] \times 100$$

$$\text{Survival } (\%) = 100 \times (\text{number of surviving fish/initial number of fish})$$

The remaining fish were fed the experimental diets for an additional four days until the 60th day of feeding to collect digesta samples for intestinal microbiome analysis. On collection day, red drum were fed to apparent satiation, staggered in a 10-min interval between tanks to ensure that the transit in the intestinal digesta would be similar during the collection time. Three fish were euthanized as previously mentioned, and their intestines were aseptically dissected with sterilized tweezers and scissors. Transient digesta was squeezed and pooled into 2 mL DNAse- and RNAse-free screw caps microtubes, and flash-frozen in liquid nitrogen. Pooled digesta samples represented each aquaria and were transported in liquid nitrogen and stored at − 80 °C until being further processed.

Fig. 1. Dendrogram of red drum (*Sciaenops ocellatus*) digesta microbiota after 60 days of feeding the experimental diets; showing similar populations for fish fed the BSFL diets but not similar when compared to the control (< 79%). Percentage similarity coefficient (%SC; bar) 79 ≤% = not similar populations; %SC = 80–84% somewhat similar; %SC = 85–89% similar; SC% = 90–94% very similar; and %SC ≥ 95% likely the same or identical.

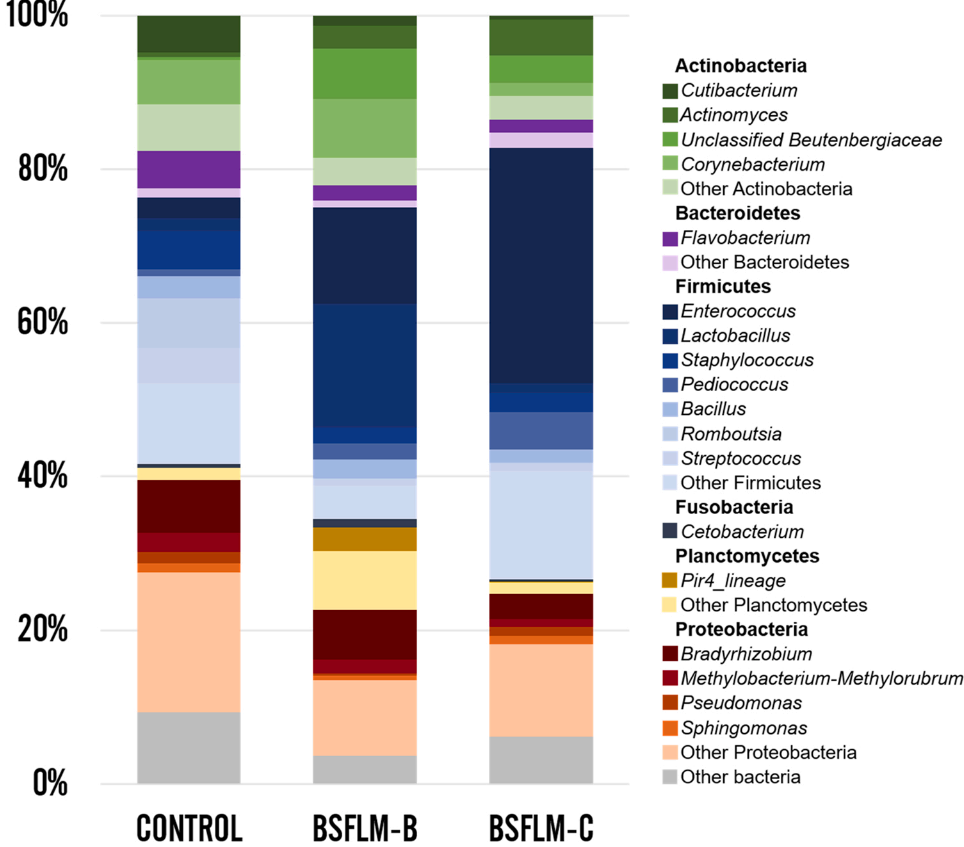

Fig. 2. Average relative abundance of bacterial genera present in the red drum (*Sciaenops ocellatus*) digesta after feeding the experimental diets for 60 days.

2.4. DNA isolation from transient digesta, PCR and denaturing gradient gel electrophoresis

Transient digesta samples were thawed and immediately centrifuged at 5000 × *g* for 10 min. The supernatant was discarded, and 0.2 g of the resulting pellet was digested using 180 μL of lysing buffer (20 mg mL^{-1} lysozyme; 20 mM Tris-HCl; pH 8.0; 2 mM EDTA; 1.2% Triton-X), and incubated in a water bath for 30 min at 37 °C. Samples were further processed for DNA extraction using the QIAmp DNA Mini Kit following the manufacturer's protocol (cat# 51306, Qiagen, Germany). At the last incubation step, the digested samples were incubated with 4 μL of RNAse A (100 mg mL^{-1}) for 2 min at room temperature. Genomic DNA was isolated by the silica-membrane-based nucleic acid purification method (QIAamp DNA Mini Kit membrane filter tubes) and quantified using a NanoDrop One (ThermoFisher, Madinson, WI), and if necessary, DNA samples were diluted to a final concentration of 50 ng of DNA μL^{-1}. Samples were aliquoted, and a subset of the DNA samples were sent to the University of Minnesota Genomics Center for next-generation sequencing.

The remainder DNA samples were subjected to PCR using universal bacterial primers, flanking the V3 region of 16S rRNA gene (Hume et al., 2003). Primers were mixed with Jump Start RED-Taq Ready Mix (cat# P0982, Sigma Aldrich, St Louis, MO) following the manufacturer's protocol, and PCR was set up according to Hume et al. (2003). PCR products were verified using 4% agarose gels (cat# G800804, Invitrogen, Carlsbad, CA), and replicates were pooled by treatment and mixed with an equal volume of 2 × loading buffer (bromophenol 0.05%; xylene cyanol 0.05%, glycerol 70%). The resultant mixture was aliquoted, and 7 μL were loaded into each well on a polyacrylamide gel (8% v/v acrylamide-bisacrylamide ratio of 37.5:1). Electrophoresis was performed for 17 h at 60 V using a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA), and the DNA gel fingerprints were stained for 15 min using SYBR Green I (1:10,000 dilution). Gel images were digitalized under UV fluorescence using a MultiImage Light Cabinet (Protein Simple, San Jose, CA). Band patterns were compared using the Dice percentage similarity coefficient (%SC), and dendrograms were constructed using the unweighted pair group with arithmetic averages (UPGMA) method, using the Gel Compare II 6.6 software (Applied Maths Inc., Austin, TX).

2.5. Next generation sequencing (NGS)

DNA samples extracted from the digesta were sequenced using an Illumina MiSeq (Illumina, San Diego, CA), at the University of

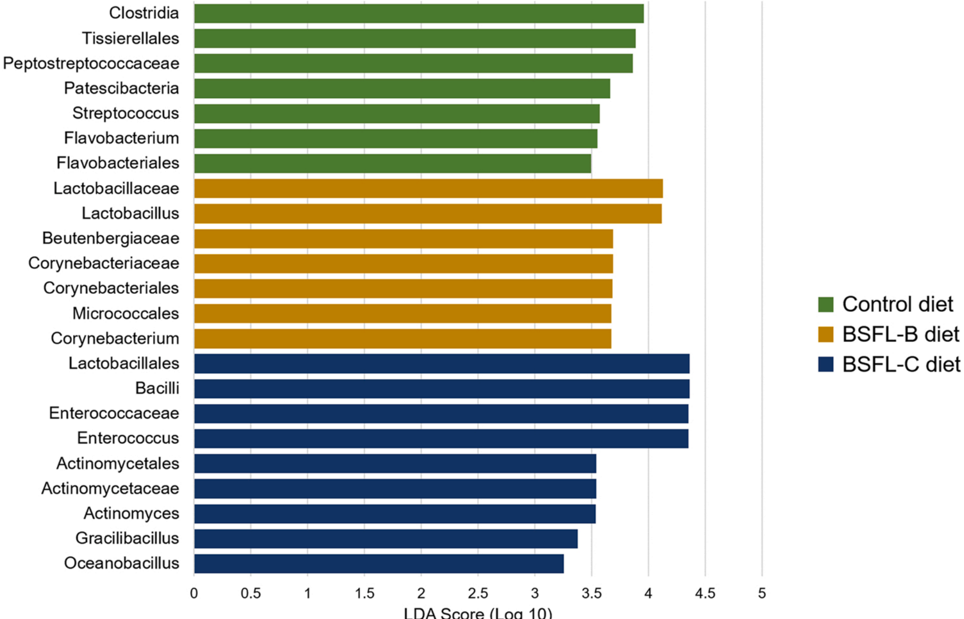

Fig. 3. Intestinal bacteria of red drum (*Sciaenops ocellatus*) found to be significantly differentially abundant ($P < 0.01$) among dietary treatments, determined by the linear discriminant analysis effect size (LEfSe).

Minnesota Genomics Center (UMGC). The V4 region of the 16 S rRNA gene was targeted using primers 515F: GTGCCAGCMGCCGCGGTAA and 806 R: GGACTACHVGGTWTCTAAT as described by Gohl et al. (2016), but with 30 cycles for amplification. Raw fastq files are available under the BioProject ID PRJNA736987 in the NCBI Sequence read archive. From the resulting sequencing data, adapters were removed using Trim-Galore! version 0.6.1 (Krueger F. Trim-Galore!, accessible at http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and primers were removed using cutadapt. A total of 122,295 sequences were obtained after quality filtering, with an average of 8153 sequences per sample. Data were further processed using QIIME2 (version 2020.6) (Bolyen et al., 2019), where denoising was performed with DADA2 (Callahan et al., 2016), and taxonomic classification was performed using the scikit-learner classifier (Abraham et al., 2014) trained on the 99% operational taxonomic unit (OTU) data set from the SILVA database release 132 (Quast et al., 2013), trimmed to the 515 F/806 R sequencing region (Bokulich et al., 2018). To analyze the alpha- and beta-diversity, samples were rarefied to 3020 sequences per sample. The functions of the red drum intestinal microbiota were predicted using PICRUSt2 (Douglas et al., 2019).

2.6. Statistical analysis

The data generated from the proximate composition and amino acid profile of the BSFL meals were subjected to one-way ANOVA using JMP software (v 14.0, SAS Institute, Cary, NC). Significant differences ($P < 0.05$) detected for the nutrient composition and energy of the different BSFLMs were analyzed using a Student's *t*-test. Data for the feeding trial, data were analyzed as a completely randomized block design, having the disposition of the aquaria as a statistical block. The homogeneity of variance for both sets of data were validated by the Brown-Forsythe test (Brown and Forsythe, 1974). The data generated from the feeding trial and the whole-body proximate composition were subjected to a comparison of means using Tukey-HSD test.

The alpha diversity of the data generated for the intestinal microbiome using NGS was calculated using Shannon diversity index, Chao1, observed features, Faith's phylogenetic distance (PD), and Pielou's evenness metrics. Alpha diversity data was analyzed using the non-parametric test Kruskal-Wallis, followed by pairwise Wilcoxon tests when significances were detected. Distance matrices from the beta-diversity output were visualized using Emperor (Vázquez-Baeza et al., 2013), and statistically analyzed using the analysis of similarities (ANOSIM) test, available in the vegan package (Oksanen et al., 2013) for R software (R Core Development Team, 2019). Testing for differential relative abundance of bacteria and predicted metabolic pathways were performed using linear discriminant analysis effect size algorithm (LEfSe) (Segata et al., 2011) available at the Huttenhower Lab Galaxy (accessed at: https://huttenhower.sph.harvard.edu/galaxy/). For LEfSe results, a linear discriminant analysis (LDA) score $Log_{10} > 3.0$, and $P < 0.05$ were considered significant for the relative abundance of intestinal bacteria, and $Log_{10} > 2.5$, and $P < 0.01$ were considered significant for the abundance of predicted functional pathways.

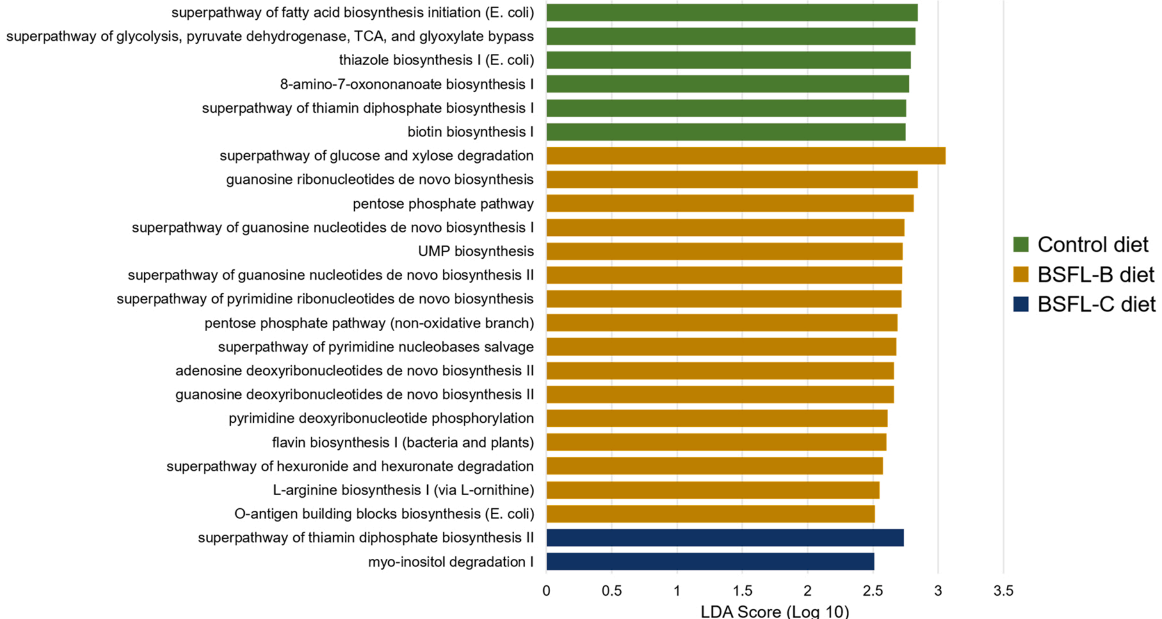

Fig. 4. Predicted functions of the digesta microbiota from the red drum (*Sciaenops ocellatus*) fed the experimental diets. Data were analyzed with the linear discriminant analysis effect size (LEfSe) to show significant differences in the abundance of pathways ($P < 0.01$) between dietary treatments.

Table 6
Alpha and beta diversity results of red drum (*Sciaenops ocellatus*) intestinal microbiota after 60 days of feeding. Alpha diversity was compared between the dietary treatment groups using Kruskal-Wallis tests, and beta diversity with analysis of similarities (ANOSIM) tests.

	α-diversity	β-diversity		
	(Prob > ChiSq)		R	P-value
Faith PD	0.16	Bray Curtis	0.54	0.001
Shannon	0.31	Jaccard	0.45	0.001
Observed features	0.21	Unweighted UniFrac	0.23	0.01
Pielou's evenness	0.046	Weighted UniFrac	0.36	0.001
Chao1	0.22			

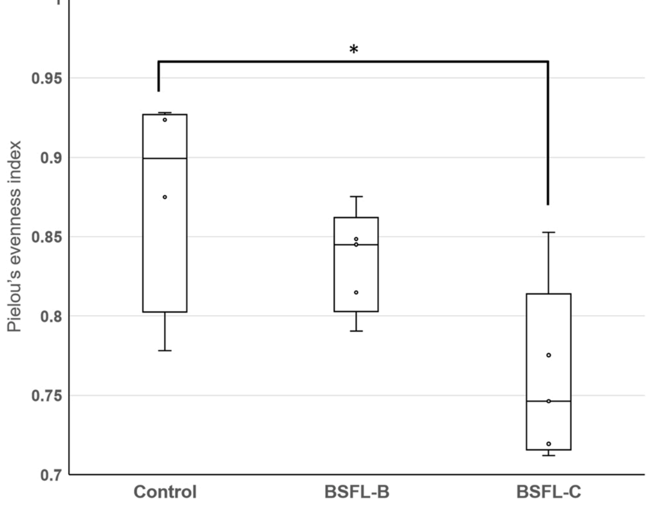

Fig. 5. Comparing the alpha diversity (using Pielou's evenness index) of the intestinal microbiota of red drum (*Sciaenops ocellatus*) fed the three experimental diets for 60 days. The bar and asterisk represent significant differences ($P < 0.05$) detected between two dietary treatments, control and BSFL-C, using a pairwise Wilcoxon test.

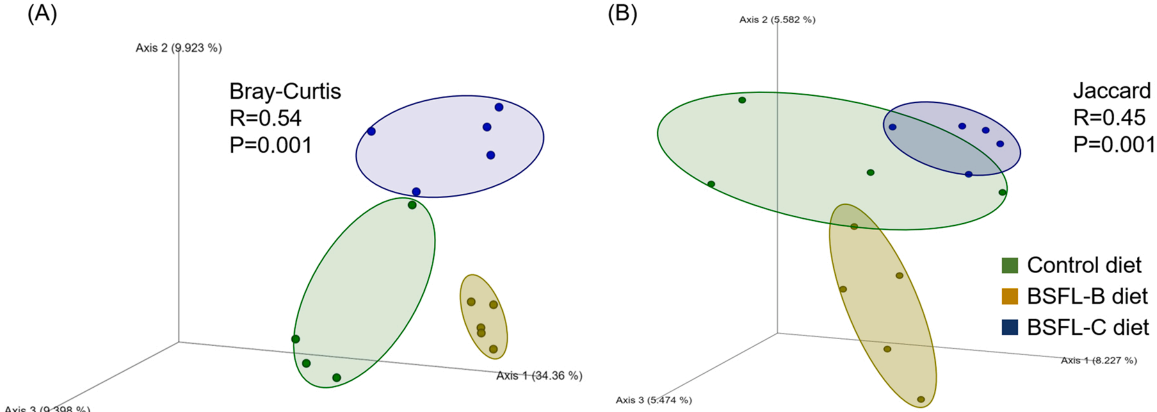

Fig. 6. Principal coordinate analysis (PCoA) plot comparing the intestinal microbiome population from the different dietary treatments with Bray-Curtis (A) and Jaccard (B). R and P-values from the analysis of similarities (ANOSIM) test.

3. Results

3.1. Proximate composition, fatty acid, and essential amino acid profiles of the BSFL meals

Significant differences were observed for the crude lipid ($P = 0.01$) and ash ($P = 0.02$) of BSFLM due to substrate, with the BSFL reared in brewers' spent grains having higher lipid and energy, but lower mineral contents (Table 1). Higher levels of methionine and cysteine (Met + Cys) ($P = 0.0004$) in the BSFLM-B meals were detected when compared to the commercial substrate. Only a few fatty acids were significantly affected by the rearing substrate offered to the BSFL. Larvae fed the brewers' spent grains had higher lauric acid (12:0), palmitic acid (16:0), and total saturated fatty acids (SFA), but lower arachidic acid (20:0), myristoleic acid (14:1), and docosahexaenoic acid (22:6 n-3). Marginal significance (P = 0.06) was observed for linoleic acid, total omega-6 (ɷ-6), and total polyunsaturated fatty acids (PUFA).

3.2. Production performance, whole-body proximate composition, and fatty acid profile

Percentage weight gain of the red drum was significantly ($P = 0.002$) affected by the dietary treatments (Table 3), where fish fed the control diet had the best growth performance but not statistically different from that of fish fed BSFL-C diet. Feed efficiency was also significantly ($P = 0.0002$) affected by the dietary treatments, where fish fed the control diet had the best feed efficiency, followed by the BSFL-C diet, and those fed the BSFL-B diet had the worst feed efficiency. No differences were detected for fillet yield, HSI, IPF, and survival. The moisture and protein content of red drum whole-body tissues were also not statistically affected by the dietary treatments (Table 4). However, the lipid and ash content of whole-body tissues were significantly impacted by the diets ($P = 0.03$ and $P = 0.004$, respectively). Red drum fed BSFL-B, and BSFL-C diets had a higher lipid content when compared to those fed the control diet. The mineral content of whole-body tissues of red drum fed the control diet also was significantly higher than the fish fed the two BSFL diets. The same pattern was observed for protein conversion efficiency, where red drum fed the control diet were significantly ($P = 0.005$) more efficient in converting dietary protein into whole-body protein than fish fed the BSFL diets.

The fatty acid profile of red drum whole-body was significantly impacted by the dietary treatments (Table 5). Fish fed the control diet had higher levels of eicosanoic (20:0), docosanoic (22:0), palmitoleic (16:1), nervonic (24:1), eicosapentaenoic (20:5), docosahexaenoic (22:6), total saturated fatty acids (SFA), total omega-3 (ɷ-3), and total polyunsaturated fatty acids (PUFA), when compared to fish fed the BSFLMs diets. The control diet also yielded higher omega-3:omega-6 (ɷ-3: ɷ-6) ratio and higher levels of vaccenic acid (18:1 n-11), followed by fish fed the BSFL-C diet with intermediate levels, and fish fed the BSFL-B with the lowest ratio. Fish fed the BSFLMs diets also had elevated contents of lauric (12:0), myristic (14:0), oleic (18:1 n-9), and eicosadienoic (20:2) acid, when compared to fish fed the control diet.

3.3. Intestinal microbiota

The DGGE dendrograms (Fig. 1) from the digesta samples of red drum indicated that fish fed the different BSFL meals were similar to each other but not similar when compared to the fish fed the control diet. NGS data showed that the microbial digesta of red drum fed the experimental diets were mainly composed of Firmicutes (average relative abundance = 43%), followed by Proteobacteria (22%), Actinobacteria (17%), Planctomycetes (4.6%), Bacteroidetes (4.3%), and Fusobacteria (0.6%) (Fig. 2). The intestinal microbiota of fish fed the control diet had higher relative abundances of the phyla Patescibacteria, the class Clostridia, orders Tissierellales and Flavobacteriales, the family Peptostreptococcaceae, and the genera *Streptococcus.* and *Flavobacterium* (Fig. 3). A higher relative abundance of the families Corynebacteriaceae, Lactobacillaceae, and Beutenbergiaceae, orders Corynebacteriales and Micrococcales, and the genera *Lactobacillus* and *Corynebacterium* were found in the microbial digesta of fish fed the BSFL-B diet. And lastly, fish fed

BSFL-C diets had a higher relative abundance of the class Bacilli, the families Actinomycetaceae and Enterococcaceae, the order Actinomycetales and Lactobacillales, and the genera *Actinomyces*, *Enterococcus*, *Gracilibacillus*, *Oceanobacillus*.

A total of 24 pathways associated with the presumptive functions of the intestinal microbiome were found to be differentially abundant among the three dietary groups (Fig. 4). Red drum fed the control diet had six pathways with higher relative abundance which were related to fatty acid synthesis, biosynthesis of vitamin precursors and vitamins, and components for energy metabolism. The intestinal microbiota of fish fed the BSFL-B diet had 16 enriched functional pathways, which were primarily related to nucleotide biosynthesis, production of vitamin precursors, and arginine biosynthesis. Finally, fish fed the BSFL-C diet had bacterial communities with higher relative abundance of major pathways related to thiamin biosynthesis and myoinositol degradation.

No significant differences were observed for alpha diversity, except for Pielou's evenness ($P = 0.046$) (Table 6 and Fig. 5), where the control diet had significantly higher diversity than the BSFL-C group ($P = 0.02$), but was not different from the BSFL-B group. Clustering of samples by dietary treatment could be observed with beta diversity results (Fig. 6 and Supplemental Fig. 1). Statistical analysis of beta diversity based on dietary treatment resulted in significant P-values regardless of the metric used (Table 5), with the highest R values produced by the Bray-Curtis (Fig. 6A, $P = 0.001$, R = 0.54) and Jaccard (Fig. 6B, $P = 0.001$, R = 0.45) metrics, Weighted Unifrac (Supplementary Fig. 1A, P = 0.001, R = 0.36), and Unweighted Unifrac (Supplementary Fig. 1B, P = 0.01, R = 0.23).

4. Discussion

The nutritional composition of the BSFLM can present wide variations and will depend on what feeding substrate is offered to the larvae and what processing methods are used to manufacture the meal (Hua, 2021). Previous studies reported successful production performance of BSFL when brewers' spent grains were offered as substrate, having either adequate performance or outperforming agro-industry waste products or produce-derived wastes (Bava et al., 2019; Meneguz et al., 2018; Scala et al., 2020). However, when comparing the nutrient composition of substrates from the aforementioned reports with the present study, the BSFLM-B did have numerically lower values for protein (when the protein content was considered as 16% of the nitrogen sample) and higher lipid content. This divergence in the numerical results with the literature may be due to the instrumentation and the analytical procedures applied to quantify these nutrients and the environmental rearing conditions of the BSFL, as well as the drying process of the larvae (Barragan-Fonseca et al., 2017). The BSFLM-C, which was reared with a commercial formulation (Gainesville diet) originally developed for house flies (*Musca domestica*) and stable flies (*Stomoxys calcitrans*) (Hogsette, 1992), had lower lipid and energy composition compared to the BSFLM-B. This finding agrees with other studies, where BSFL reared in this commercial substrate had better production performance and nutrient quality, compared to BSFL offered other sources of waste (Cammack and Tomberlin, 2017; Gligorescu et al., 2018; Miranda et al., 2019). This similarity suggests that this commercial substrate is more nutritionally adequate for this species; hence, the lower lipid deposition and less saturated fatty acids.

Interestingly, for the present study, BSFL reared in brewers' spent grains presented a higher concentration of methionine and cysteine when compared to the larvae offered the control diet (Gainesville). These sulfur-containing amino acids are usually the most critical for meals manufactured from the *Hermetia illucens* larvae, with low values reported (6.5–11.6 g/1000 g) that would not meet the usual requirement ranges for carnivorous fish species (Barragan-Fonseca et al., 2017; Nogales-Mérida et al., 2019). Higher concentration of methionine was also observed for BSFL offered a mixture of vegetables (carrots, broccoli and green beans) when compared to other substrates (Fischer and Romano, 2021). It is of importance to have a better understanding of this phenomenon and to develop strategies that can improve the capability to bio-convert and store these amino acids in the BSFLMs.

The discussion of the nutrient composition of resulting BSFLMs can be cumbersome when a plethora of feeding substrates and their combinations have been tested, and different angles have been taken to analyze the nutrition make up of the BFSL. For instance, the fatty acid profile of the BSFLM-B is somewhat similar of what has been reported by Meneguz et al. (2018), when they also fed brewery by-product to the BSFL; however, the difference on the concentration of linolenic acid (18:3 n-3) is fairly wide, which can indicate that there were initial differences on the fatty acid profile from the same feeding substrate. It is unlikely that the BSFL can elongate and synthesize PUFAs such as the DHA (Ewald et al., 2020), and it is questionable if the residual concentrations of DHA found in the BSFLMs of the present study came from the feeding substrate, when the ingredients from the Gainesville commercial feed should be devoid of DHA. Nevertheless, in agreement with the present findings, minute concentrations of DHA were also observed for the basal BSFLMs fed cow manure (St-Hilaire et al., 2007) and laying hen feed (Barroso et al., 2017).

Although red drum production performance was significantly impaired by the replacement of fishmeal by the BSFLMs, growth performance of fish fed diets containing BSFLM-C was not statistically different from that of fish fed the control diet. The type of substrate offered to the larvae not only impacted weight gain but also the feed efficiency of red drum, where the BSFLM derived from larvae offered the Gainesville diet was more efficient than the meal derived from brewers' spent grains. One may argue that, even though the experimental diets of the present study were formulated to be isonitrogenous, the diets containing BSFLMs may have had underestimated protein values, mainly because protein for all ingredients was considered to be 16% of total nitrogen. This estimate is often employed for most feed ingredients, but it dismisses the input of some non-protein nitrogen sources (*i.e.*, chitin, nucleic acids, phospholipids, ammonia and urea), which can be elevated in insect larvae meals (Janssen et al., 2017) and can have a steadily increase of urea and ammonia as nitrogenous metabolites when BSFL are subjected to purge (Egnew et al., 2021). This hypothesis was substantiated by the lower protein conversion efficiency observed for red drum fed the BSFLMs diets compared to the control. On the other hand, it should be highlighted that the sum of amino acids for the BSFL-B diet was higher than the control and BSFL-C diets, with the indispensable amino acids having similar concentrations for all the treatments. It is suggested that for future studies investigating BSFLM or when formulating commercial diets using insect-based ingredients, more attention should be focused on the amino acid

profile instead of the protein estimation from total nitrogen.

Previous studies successfully achieved partial or total replacement of fishmeal by BSFLM for Atlantic salmon (*Salmo salar*) (at 600 g kg^{-1} and 147.5 g kg^{-1} inclusion, respectively) (Belghit et al., 2019, 2018) and partial replacement for European seabass (*Dicentrarchus labrax*) (from 65 to 195 g kg^{-1} inclusion) (Magalhães et al., 2017), without impairing their production performance. However, for the evaluation with salmonids, the BSFL were offered a mixture of organic wastes with ground seaweed (*Ascophyllum nodosum*) provided at a 50:50 ratio, which could have yielded a nutritionally superior BSFLM. Two separate studies evaluating graded inclusion levels of BSLFM in diets for Atlantic salmon only observed negative effects on growth performance when dietary inclusion levels surpassed 200 and 161.3 g BSFLM kg^{-1}, respectively (Fisher et al., 2020; Weththasinghe et al., 2021). It was rather unexpected to observe the production performance of red drum hampered by the inclusion of 150 g of BSFLMs per kg of diet when all the aforementioned reports attained successful replacements. In addition, feeding trials conducted by our research group reported otherwise, where optimum levels of BSFLM inclusion were determined for omnivorous species such as tilapia (*Oreochromis niloticus*), channel catfish (*Ictalurus punctatus*), and the carnivorous hybrid striped bass (*Morone chrysops* × *M. saxatilis*) to range between 150 and 200 g of BSFLM kg^{-1} of diet (G. Bake, 2017, *personal communication*). Furthermore, when compared to 300 g kg^{-1} inclusion level of BSFLM that was considered relatively safe in a meta-analysis (Hua, 2021), the inclusion of 150 g of BSFLM kg^{-1} in the current study was considered relatively conservative; thus, it was not foreseen as a potential negative impact on the red drum growth performance.

In agreement with our findings, the growth performance of Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) also were reported to be not statistically different but numerically lower than the control diet when BSFL partially replaced fishmeal (at 100 g kg^{-1} and 131.9 g k^{-1} inclusion, respectively), with a lower feed conversion ratio and protein conversion efficiency for rainbow trout (Dumas et al., 2018; Lock et al., 2016). Inclusions of as low as 119 and 165 g of BSFLM kg^{-1} also impaired the production performance of largemouth bass (*Micropterus salmoides*), and the turbot (*Psetta maxima*), respectively (Fischer et al., 2022; Kroeckel et al., 2012). It was rather intriguing to observe that the rearing substrate of the BSFL could have a major influence on the production performance of fish, but this was also observed by Sealey et al. (2011), when diets containing BSFLM, derived from larvae offered fish offal, yielded better productivity for rainbow trout. Nevertheless, the hypothesis raised by these authors and Lock et al. (2016), suggested that the high concentrations of lauric acid could have benefited the performance of these salmonids by serving as an energy substrate during β-oxidation, which was not translated to the present study where red drum had reduced growth performance and feed efficiency when offered the experimental diet richest in lauric acid (12:0) (BSFL-B).

Even though the fatty acid profile drastically changed with the inclusion of BSFLMs, the established requirements for ω-3 long-chain PUFA by red drum were met (Lochmann and Gatlin, 1993); therefore, the possibility of growth impairment being caused by a deficiency of eicosapentaenoic or docosahexaenoic acids was discarded. The composition of the fatty acids in whole-body tissues typically reflects the fatty acid profile of the diet, and indeed, this influence can be observed in the present study, where most of the fatty acids from the whole-body mirrored the fatty acid composition of the experimental diets. However, in contrast to this mirroring trend, myristic acid (14:0) was found in whole-body tissues to be greater than what was provided in the diet. As such, perhaps lauric acid (12:0) content of the diet contributed as a substrate for elongation and stored as myristic acid. Several fish species also exhibited similar responses of fatty acid profiles in whole-body and fillets when fed diets with increasing levels of BSFLMs, in which there was a higher accumulation of saturated fatty acids (SFA) and reduced concentration of long-chain PUFA (Agbohessou et al., 2021; Guerreiro et al., 2020; Renna et al., 2017). Differently than these studies and the present results, the fatty acid profile of Atlantic salmon post-smolts (Lock et al., 2016) was not altered by the increasing levels of BSLFM, and to a lesser extent, in rainbow trout juveniles (Sealey et al., 2011). It is unclear whether the variables playing a role in fatty acid uptake and lipid metabolism are related to the better aptitude of salmonid species to utilize SFA as an energy source, or if these differences could be attributed to the nutritional enrichment of the BSFLM by the different rearing substrates the larvae were offered. In the present study, the rich linoleic (18:2) profile of fish treated with the BSFL-B diet was likely transferred to the BSFL from the malt and other grains, which can present elevated levels of this fatty acid (Fărcaş et al., 2015). The same elongation and storage phenomena, as previously discussed for lauric and myristic acids, has been noticed for linoleic acid and eicosadienoic acid (20:2), where red drum fed BSFLMs accumulated a higher concentration of this fatty acid (20:2) in their whole-body.

The inclusion of BSF in the diets of farmed fish has been shown to positively influence the intestinal health and the gut microbial communities of farmed fish regardless of insect life stage (Zarantoniello et al., 2020). A study from Kumar et al. (2021) observed that the inclusion of BSFLMs in the diets of rainbow trout alleviate soybean meal induced enteric inflammation by decreasing the expression of proinflammatory genes. Other studies presented a consistent shift on the intestinal microbial community of fish fed BSFMs, which can be associated with the prebiotic-like molecules present in this ingredient, such as chitin, that may serve as an energy substrate for the intestinal microbiota and have bacteriostatic effects (Zarantoniello et al., 2020). In addition, lauric acid and glycerol monolaurate, which are known to have antimicrobial properties (Borrelli et al., 2021) could have also played role influencing the gut microbiota of red drum fed BSFLM. In the present study, the results from DGGE dendrogram and 16 S rRNA gene sequencing revealed that the intestinal microbiota of red drum was heavily affected by the inclusion of the BSFLMs in the experimental diets. Alpha diversity seemed relatively stable regardless of dietary treatment, with only one diversity metric, Pielou's evenness, found to be significantly different. The beta diversity results revealed distinct clustering by dietary treatments, regardless of beta diversity metric used. These results are in accordance with what has been previously reported for the intestinal microbial population of rainbow trout and Atlantic salmon fed diets containing BSFLMs based on the various beta-diversity parameters (Gaudioso et al., 2021; Huyben et al., 2019; Li et al., 2021; Rimoldi et al., 2021; Terova et al., 2019). Interestingly, more distinct clustering and higher dissimilarity could be observed with the two non-phylogenetic metrics (Bray-Curtis and Jaccard) relative to the phylogenetic metrics (weighted and unweighted UniFrac). These results may suggest that while community structure is affected by the dietary treatment, the changes in composition that occur are primarily at lower taxonomic levels; for example, BSFLMs may result in the reduction of one bacterial

species with a different but closely related species flourishing in its absence. The stable presence of higher taxonomic levels (*e.g.*, phyla or order) may indicate the importance of these groups to host homeostasis. Considering species even of the same genus can have drastically different roles in host health, identifying the particular species that may be changing in abundance and their relationship within the red drum may be useful in developing these diets with the microbiome in mind.

The characterization of the red drum intestinal microbiota by NGS showed that the bacterial phyla were mainly comprised of Firmicutes, Proteobacteria, and Actinobacteria, which is in agreement with previous reports on the phyla of the intestinal bacteria present for this species (Givens et al., 2015; Rossi et al., 2017a). In the present study, fish fed the control diet presented a higher relative abundance of *Flavobacterium*, a genus of Gram-negative bacteria commonly found in freshwater bodies, with several species known to be potential etiological agents of diseases for farmed fish (Bernardet and Bowman, 2006). Considering that this feeding trial was conducted in a brackish environment with low salinity, it is not surprising that this bacterium genus could be present in the red drum intestine. Similarly, two separate studies observed in the intestinal microbiota of rainbow trout fed the control diets a higher relative abundance of *Aeromonas*, a genus that also encompasses several pathogenic species for fish, when compared to groups fed diets containing BSFLMs (Rimoldi et al., 2019, 2021). These reports, along with our findings, substantiate the hypothesis that the inclusion of BSFLM may benefit the host either by inhibiting the proliferation of these potential pathogenic bacteria or by reducing their relative abundance when other bacteria taxa are benefitting from the BSFLM and proliferating in the intestine. However, it is necessary to perform other molecular or standard bacteriology assays to better define if these bacteria genera are in fact pathogenic and in fact in higher abundances for the control fish.

It is interesting to observe in the published literature the consistent modulation and shifts in the intestinal bacterial populations caused by dietary BSFLMs despite the model fish species tested, the primer pairs selected to amplify the bacterial DNA, and what the sample material for metagenome sequencing was collected (*i.e.*, the whole digesta, intestinal mucosa, cecal or fecal material). For instance, it was also previously reported on samples representing the intestinal microbiota a higher relative abundances of *Actinomyces*, *Bacillus*, *Corynebacterium*, *Enterococcus*, *Lactobacillus,* and *Oceanobacillus* for Atlantic salmon, rainbow trout, and Siberian sturgeon (*Acipenser baerii*) fed diets containing BSFLMs (Gaudioso et al., 2021; Huyben et al., 2019; Józefiak et al., 2019; Li et al., 2021; Rimoldi et al., 2021; Terova et al., 2019). The prevalence of *Actinomyces*, *Bacillus, Corynebacterium,* and *Lactobacillus*, in the fish intestine can be attributed to their ability to produce chitinolytic enzymes and nourish from the chitin present in the BSFLMs (Gaudioso et al., 2021; Huyben et al., 2019; Terova et al., 2019; Zarantoniello et al., 2020). The higher relative abundance of *Corynebacterium* spp. in the intestinal microbiota also was previously associated with the inclusion of full-fat BSFLM in the rainbow trout diet (Huyben et al., 2019). In that study, the authors hypothesized that the ability of this bacterium to produce lipase and the lipid composition of the diets promoted the proliferation of this genus, which may agree with the present findings, where the apparent higher abundance of *Corynebacterium* spp. in the intestinal microbiota of red drum fed BSFL-B diet could have happened by a higher inclusion of BSFL oil. The metabolic pathways detected in the red drum intestinal microbiota that were affected by the addition of BSFLMs were mainly related to the synthesis of nucleotides, vitamins (flavin and thiamin), and arginine, which are molecules with the potential to enhance intestinal health. An increase in the metabolism of arginine also was observed for the European seabass fed mealworms (*Tenebrio molitor*) (Panteli et al., 2021). While the microbial metabolic pathways described in our findings may have important roles in intestinal health, other methods are needed to verify the enrichment of these pathways in future studies, because those analyzed in the present study are predicted from the bacterial metagenome.

In conclusion, the present study demonstrated that the feeding substrate used to culture BSFL is a major variable that will ultimately determine the nutrient composition of the BSFLMs, and will also singularly affect the red drum production performance, intestinal microbiota, and whole-body nutrient composition. Overall, the inclusion of the BSFLMs in the experimental diets appeared to produce beneficial effects on the red drum intestinal microbiota, with a prospective nutraceutical property to reduce potentially pathogenic bacteria and increase in the relative abundance of bacteria that are usually considered beneficial for the intestinal health (*i.e.*, lactic acid bacteria and other bacilli). Further research is necessary to better understand what the nutritional differences between BSFLMs can be driving these changes, and pinpoint which aspects of these meals are negative influencing the production performance of red drum.

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CRediT authorship contribution statement

Fernando Y. Yamamoto: Study concept and design, conducted the experiment, assisted the sampling procedures, laboratorial analysis, Data curation, statistical analysis and interpretation of the data, drafting the original manuscript. **Delbert M. Gatlin III:** Funding acquisition, assisted the sampling procedures, draft the manuscript, critical revision. **Blaine A. Suehs:** Assisted the sampling procedures, laboratorial analysis, revised the manuscript. **Matthew Ellis:** Conducted the experiment, assisted the sampling procedures, revised the manuscript. **Paul R. Bowles:** Conducted the experiment, assisted the sampling procedures, revised the manuscript. **Caitlin E. Older:** Assisted with data curation and interpretation of the data, revised the manuscript. **Michael E. Hume:** Assisted with data curation and interpretation of the data, revised the manuscript. **Gabriel G. Bake:** Manufactured the raw ingredients, revised the manuscript. **Jonathan A. Cammack:** Manufactured the raw ingredients, revised the manuscript. **Jeffery K. Tomberlin:** Funding acquisition, critical revision.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2021.115179.

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Effect of rearing substrate on growth performance, waste reduction efficiency and chemical composition of black soldier fly (*Hermetia illucens*) larvae†

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Abstract

BACKGROUND: Wastes can be used as rearing substrate by black soldier fly (BSF) larvae, the latter being exploitable as a protein source in animal feed. This research aimed to assess the influence of four rearing substrates (Trial 1 (organic wastes): a mixture of vegetable and fruit (VEGFRU) *vs.* a mixture of fruits only (FRU); Trial 2 (agro-industrial by-products): brewery (BRE) *vs.* winery (WIN) by-products) on BSF larvae development, waste reduction efficiency and nutritional composition.

RESULTS: If respectively compared to FRU and WIN, VEGFRU and BRE larvae needed less time to reach the prepupal stage (22.0, 22.2, 20.2 and 8.0 days of trial, respectively) and had higher protein content (229.7, 257.3, 312.9 and 395.7 g kg^{-1} dry matter). The waste reduction index ranged from 2.4 (WIN) to 5.3 g d^{-1} (BRE). BRE larvae showed the lowest saturated and the highest polyunsaturated fatty acid proportions (612.4 and 260.1 g kg^{-1} total fatty acids, respectively).

CONCLUSION: Vegetable and fruit wastes and winery by-products can be used as rearing substrates for BSF larval mass production. Brewery by-products led to very promising larval performance and nutritional composition. However, given BRE limited availability, low BRE dietary inclusion levels could be used with the purpose of increasing larval performance.
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Keywords: organic waste; agro-industrial by-product; *Hermetia illucens*; animal feed; crude protein; fatty acid profile

INTRODUCTION

The world population is estimated to be around 7.3 billion, with a growth rate of about 83 million per year. This increase will generate an increment of food demand with a consequent rise in waste and by-product production.[1] Urgent and innovative solutions are needed for the management of the waste streams (WS) that currently are estimated to be around 1.3 billion and 100 million tons per year in the world and in the European Union, respectively.[1,2] Furthermore, EC Directive No. 2008/98 unequivocally establishes the order of priority in the choice of WS treatment, the first being their reuse and the last their landfill disposal.

Some WS could be valorized through the recovery of the residual bio-elements they contain, with a cost reduction both for the industry (disposal cost) and the environment (pollution).[3] The use of insects in the bioconversion of WS constitutes a new approach and an interesting example of sustainable circular economy. This bioconversion can generate new elements such as proteins and lipids for animal feeds,[4–7] biodiesel,[8] high-value products such as chitin[9] or antimicrobial peptides.[10]

Processed proteins from seven insect species have recently been approved for aquafeed by EC Regulation No. 2017/893, which also lists the licensed rearing substrates. Among authorized species, black soldier fly (BSF; Diptera: Stratiomydae) is one of the most promising, and research has recently aimed to increase knowledge on optimal rearing substrates for larvae and prepupae. In this respect, BSF has shown great flexibility as it can be used to reduce volume and add value to various wastes.[8,11,12] The available literature has highlighted that the BSF life cycle and nutritional composition are noticeably influenced by the rearing substrate,[13,14] with the crude protein (CP) content of the larvae ranging from about 317 to 630 g kg^{-1} dry matter (DM).[7,15,16]

In 2014, around 90 million tons of slaughter and vegetable WS were produced in Europe (EUROSTAT (http://

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appsso.eurostat.ec.europa.eu/nui/show.do?dataset=env_wasgen&lang=en)). Considering the Italian context, 54% of the total production of waste and agro-industrial by-products is generated by the manufacture of vegetable products.[17] About 1.5 million tons of winery by-products and 406 tons of brewery by-products are produced every year (EUROSTAT (http://ec.europa.eu/eurostat/tgm/table.do?tab=table&init=1&language=en&pcode=tag00034&plugin=1; EU Report (https://www.brewersofeurope.org/site/media-centre/index.php?doc_id=905&class_id=31&detail=true)).[18]

The aim of this research was to evaluate the effects of organic wastes (vegetables and fruits) and agro-industrial by-products (winery and brewery) generated by the Italian food sector as rearing substrates for BSF larvae on their development, waste reduction efficiency and chemical composition.

MATERIAL AND METHODS

Two trials were carried out at the Experimental Facility of the Department of Agricultural, Forest and Food Sciences (DISAFA; University of Torino, Torino, Italy).

Rearing substrates

In Trial 1, two organic wastes were compared:

- vegetable–fruit waste (VEGFRU) obtained from a street market (Turin, Italy) and containing a mixture of vegetables and fruits (celery 43.4%, oranges 28.9% and peppers 27.7%);
- fruit waste (FRU) obtained from a fruit market (Turin, Italy) and containing fruits only (apples 47.8%, oranges 15.5%, apple leftovers 13.8%, strawberries 7.1%, mandarins 4.8%, pears 4.1%, kiwis 3.4%, bananas 1.9% and lemons 1.6%).

In Trial 2, two agro-industrial by-products were used:

- winery by-product (WIN) obtained during the winemaking process, before alcohol extraction, from a private distillery (Distilleria Santa Teresa dei Fratelli Marolo Srl, Alba (CN), Italy) and containing grape seeds, pulp, skins, stems and leaves;
- brewery by-product (BRE) obtained during beer production (IFN 5-00-517 Barley brewers' grains wet) from a private brewery ('Birrificio dei Santi', Castelnuovo Don Bosco (AT), Italy).

Each substrate was ground with a 3 mm die meat mincer (FTS136; Fama Industrie Srl, Rimini, Italy) and carefully mixed.

A sample of each substrate was freeze dried and frozen at −80 °C for further chemical analysis, while the remainder was stored at −20 °C until it was fed to the larvae.

BSF eggs

BSF eggs laid on corrugated cardboard for less than 24 h were purchased from a private company (CIMI Srl, Cervasca (CN), Italy). The cardboard with the eggs was immediately transported to the DISAFA Experimental Facility. The cardboard was placed on plastic boxes (25 cm × 33 cm × 12 cm) which contained whole rye thoroughly mixed with water (60% moisture) as a rearing substrate for the newborn larvae. The plastic boxes were placed in climatic chambers under controlled environmental conditions (T: 27 ± 0.5 °C; RH: 70 ± 5%; 24:0, L:D photoperiod). The eggs hatched approximately 3 days after oviposition.

Experimental design and calculations

Larvae development and waste reduction efficiency

Six-day-old larvae were used in both trials. In each trial, for the evaluation of larval development (weight and length) and waste reduction efficiency, six replicates of 100 larvae were weighed (KERN PLE-N v. 2.2; KERN & Sohn GmbH, Balingen-Frommern, Germany; d: 0.001) and assigned to each rearing substrate. The method reported by Harnden and Tomberlin[19] was used to count the larvae. For each replicate, the larvae were placed in plastic containers (10 cm × 17.5 cm × 7 cm), directly on the rearing substrate (100 g per replicate). The containers were covered with a perforated cap with a black nylon grid and placed in a climatic chamber under controlled environmental conditions (T: 27 ± 0.5 °C; RH: 70 ± 5%; 24:0, L:D photoperiod).

Each replicate was monitored daily to control the quantity of available feed. If needed, as reported by Harnden and Tomberlin,[19] 50 g substrate per replicate was added in all replicates at the same time.

To avoid the effect of handling on the considered dependent variables,[13] weight and length data were collected every 4 days until the appearance of the first prepupae, and thereafter every day for the relative substrate. Thirty larvae were randomly sampled three consecutive times from each container to measure weight and length. As measurement was not destructive, the larvae were reintroduced into the containers between two consecutive samplings. The sampled larvae were individually cleaned, dried with a paper towel and weighed, and photographed orthogonally (Lumix G1; Panasonic Corp., Kadoma, Osaka, Japan) with a metric scale (mm). The images were analyzed with the ImageJ software package (v. 1.50b) to record larval length (i.e. from mouthpart to the bottom of the last abdominal segment).

For each container, weight and length data collection ended when 30% of the larvae reached the prepupal stage. The prepupae were removed from the containers. The remaining 70% of the larvae were hand counted, washed, dried with a paper towel and individually weighed and photographed. The total final biomass (larvae + prepupae) and the residual rearing substrate were also weighed. The following parameters were then calculated:

- larval mortality (LM)

$$\mathrm{LM} = [\text{initial number of larvae} - (\text{final number of larvae} + \text{number of prepupae})] / \text{initial number of larvae} \times 100;$$

- growth rate (GR),[20] readapted for this research substituting prepupal body weight (g) with larva body weight (g)

$$\mathrm{GR} = (\text{larva average final body weight (g)} - \text{larval initial body weight (g)}) / \text{days of trial (d)};$$

- substrate reduction (SR)[21]

$$\mathrm{SR} = [(\text{distributed substrate (g)} - \text{residual substrate (g)}) / \text{distributed substrate (g)}] \times 100;$$

- waste reduction index (WRI)[20]

$$\mathrm{WRI} = [(\mathrm{W} - \mathrm{R}) / \mathrm{W}] / \text{days of trial (d)} \times 100$$

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where W = total amount of rearing substrate distributed during the trial (g); R = residue substrate (g);

- efficiency of conversion of digested food (ECD)[20]

$$\text{ECD} = \text{total final biomass (g)} / (\text{total feed distributed (g)} - \text{residual substrate (g)})$$

where total final biomass = larvae + prepupae; residual substrate = undigested food + excretory products.

Parameters related to waste reduction efficiency (SR, WRI and ECD) were calculated on a fresh matter basis.

Larvae nutritional composition

For each trial, a second set of six replicates per rearing substrate was simultaneously prepared with the aim of rearing a sufficient amount of larvae to be analyzed for their proximate composition and fatty acid (FA) profile. Five hundred hand-counted 6-day-old larvae were placed in plastic containers of larger size (25 cm × 33 cm × 15 cm) than those used for the larval development and waste reduction efficiency test, following the same relationships between (i) number of larvae/container size surface, and (ii) amount of administered feed/larvae density. The larvae were not handled until the appearance of the first prepupa. Then, each container was checked daily and the identified prepupae were removed. The trial ended when 30% of the larvae reached the prepupal stage. The remaining larvae were then manually separated from the residual rearing substrate, washed, lightly dried with paper towel, weighed and frozen at −80 °C until being freeze dried.

Chemical analyses of rearing substrates and larvae

Samples of freeze-dried rearing substrates and larvae were ground using a cutting mill (MLI 204; Bühler AG, Uzwil, Switzerland). They were analyzed for DM, ash, CP and EE following AOAC International methods, as detailed in Gasco *et al.*[5] For the determination of the CP of whole BSF larvae, in addition to the conventional nitrogen-to-protein (N-factor) conversion factor of 6.25, the more accurate N-factor of 4.67 suggested by Janssen *et al.*[22] was used. Neutral detergent fiber (NDF) was analyzed according to Van Soest *et al.*[23] Acid detergent fiber and acid detergent lignin (ADF and ADL) were determined according to method No. 973·18 of AOAC International.[24] The residual nitrogen in ADF (ADFN) was determined according to method No. 984.13 of AOAC International.[24] Chitin (CHI, g kg^{-1} DM) was estimated as ash-free ADF (g kg^{-1}) − ADFN × N-factor (g kg^{-1}).[9] Gross energy (GE) was determined using an adiabatic calorimetric bomb (C7000; IKA, Staufen, Germany).

The FA composition of substrates and larvae was assessed.[25] The results were expressed as g kg^{-1} of total detected fatty acids (TFA) (Table 2).

Statistical analyses

Statistical analysis of data was performed using IBM SPSS Statistics v. 20.0 for Windows. The two trials were considered separately. Larval weights and lengths were subjected to a two-way mixed analysis of variance (ANOVA). The Shapiro–Wilk test was used to verify whether the dependent variables were normally distributed for each combination of the groups of within-subject (test day, considered as a repeated measure) and between-subject (rearing substrate) factors. Levene's test was used to verify the homogeneity of variances for each combination of the groups of within- and between-subject factors. Mauchly's test was used to verify the assumption of sphericity; if such an assumption was violated, the Greenhouse–Geisser or the Huynh–Feldt correction (in cases of estimates of sphericity lower or higher than 0.75, respectively) was applied to correct the degrees of freedom of the F-distribution. Final larval weights and lengths (average weight and length of the leftover 70% larvae after removing 30% of prepupae) were further subjected to independent-sample Student's t-test to assess differences between rearing substrates.

Differences in terms of larval growth performance, waste reduction efficiency, proximate composition and FA profile between substrates were also assessed using independent-sample Student's t-test.

The Kruskal–Wallis test was used to compare the time needed by the larvae to reach the prepupal stage. Significance was declared at $P < 0.05$.

RESULTS

Growth performances and waste reduction efficiency of the BSF larvae

The effect of rearing substrate on the development of BSF larvae over time is reported in Fig. 1 (Trial 1) and Fig. 2 (Trial 2). In both trials, results from the two-way mixed ANOVA showed that, for larval development (weight and length), rearing substrate, test day and their interaction were highly significant ($P < 0.001$), VEGFRU and BRE performing better than FRU and WIN, respectively.

In Trial 1, no differences were observed for larval weight (mean ± SD: 0.004 ± 0.0002 g) at the beginning of the trial (day 0; 6-day-old larvae) (Fig. 1a). Differences appeared after 4 days of trial, with a higher weight in the VEGFRU larvae (0.055 ± 0.0084 g) compared to the FRU larvae (0.037 ± 0.0058 g). Such a trend was maintained at each test day until day 16 (VEGFRU: 0.148 ± 0.0103 g; FRU: 0.120 ± 0.0094 g) when VEGFRU larvae started to enter the prepupal stage. The final weight of the larvae did not show any differences between the two rearing substrates. At the beginning of Trial 1, VEGFRU and FRU larvae showed length values of 5.7 ± 1.32 mm and 5.3 ± 1.17 mm, respectively ($P < 0.05$; Fig. 1b). VEGFRU larvae continued showing higher length values than FRU larvae until the last statistical assessment (day 16). VEGFRU and FRU larvae achieved a final length of 17.7 ± 0.46 mm and 17.8 ± 0.51 mm, respectively ($P > 0.05$).

At the beginning of Trial 2, no differences were observed between WIN and BRE for larval weight (0.007 ± 0.0011 g) (Fig. 2a). Remarkable differences were reported after 4 days of trial, with a higher weight in the BRE larvae compared to the WIN larvae (0.092 ± 0.0063 g and 0.017 ± 0.0018 g, respectively). The final weight of the larvae (reached after 8 and 26 days of trial for BRE and WIN, respectively) did not show differences between treatments. The mean length of 6-day-old larvae (day 0) was 6.5 ± 1.36 mm and 6.4 ± 1.24 mm for BRE and WIN, respectively ($P > 0.05$; Fig. 2b). After 4 days of trial, differences in larval length were highlighted, with recorded values of 15.1 ± 1.84 mm (BRE) and 8.7 ± 1.23 mm (WIN).

Dynamics of growth and waste reduction efficiency parameters are reported in Table 1. In Trial 1, VEGFRU larvae showed lower LM and time needed to reach the prepupal stage, as well as higher ECD than FRU larvae. In Trial 2, BRE larvae showed lower LM, time

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Figure 1. Trial 1: development (a: weight; b: length) of black soldier fly larvae reared on organic wastes (VEGFRU: 70% vegetable and 30% fruit waste; FRU: 100% fruit waste) generated by the Italian food sector. *P*-value: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Error bars represent the standard error of the mean.

Figure 2. Trial 2: development (a: weight; b: length) of black soldier fly larvae reared on agro-industrial by-products (WIN: winery by-product; BRE: brewery by-product) generated by the Italian food sector. *P*-value: $***P < 0.001$. Error bars represent the standard error of the mean.

needed to reach the prepupal stage and SR, and contemporarily higher total final biomass, GR, WRI and ECD than WIN larvae.

Proximate and fatty acid composition of the rearing substrates

The proximate and FA compositions of the rearing substrates are reported in Tables 2 and 3, respectively. In Trial 1, VEGFRU showed lower values of DM and NSC and higher contents of ash, CP, NDF and ADF than FRU, whereas comparable EE and ADL contents were found. In Trial 2, WIN showed higher DM, ash, NDF, ADF and ADL contents and lower CP and NSC contents than BRE. VEGFRU and FRU showed similar GE values, which were lower than those obtained in the second trial for WIN and BRE.

Total FA ranged from 10.04 (FRU) to 82.47 g kg^{-1} DM (BRE). VEGFRU showed higher total polyunsaturated fatty acids (PUFA) and lower total monounsaturated fatty acids (MUFA) than FRU. WIN had higher MUFA and lower SFA when compared to BRE. Linoleic acid (C18:2 n6) was the most abundant FA in all substrates.

Proximate and fatty acid compositions of the BSF larvae

The proximate and FA compositions of the BSF larvae are reported in Tables 4 and 5, respectively. Concerning Trial 1, ash, CP and ADF values in the VEGFRU larvae were higher than those in FRU larvae. Conversely, the FRU larvae showed higher DM, EE and NDF contents than the VEGFRU larvae. In Trial 2, the WIN larvae showed lower DM and CP contents when compared to the BRE larvae, while all the other parameters showed an opposite trend.

Considering the FA composition of the larvae, FRU larvae showed higher TFA than VEGFRU larvae. Conversely, in Trial 2 similar TFA contents were observed for BRE and WIN larvae. Significant differences between treatments were observed in both trials for almost all considered FA groups and individual FA. PUFA were higher in VEGFRU and BRE larvae when compared to FRU and WIN larvae, respectively, whereas an opposite trend was observed for SFA. The most represented individual FA in BSF larvae from all treatments was C12:0, which showed higher amounts in FRU and WIN when compared to VEGFRU and BRE, respectively. C18:1 c9 and C18:2 n6 were the most represented unsaturated FA in all treatments.

DISCUSSION AND CONCLUSIONS

Our study investigated, through two trials, the effects of different rearing substrates on development, waste reduction efficiency and nutritional composition of BSF larvae.

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Table 1. Dynamics of growth and waste reduction efficiency (on a fresh matter basis) of black soldier fly larvae reared on organic wastes (vegetables and fruits) and agro-industrial by-products (winery and brewery) generated by the Italian food sector (mean ± SD; $n = 6$)

	Trial 1 (organic wastes)			Trial 2 (agro-industrial by-products)		
	VEGFRU	FRU	*P*	WIN	BRE	*P*
Larval mortality (%)	11.2 ± 4.35	19.3 ± 5.24	0.015	24.8 ± 10.53	9.5 ± 5.68	0.011
Total final biomass (g)	10.42 ± 0.648	10.92 ± 2.057	0.584	9.90 ± 0.785	11.32 ± 0.864	0.014
Time needed to reach prepupal stage (days of trial)	20.2 ± 1.33	22.0 ± 0.89	0.031	22.2 ± 0.98	8.0 ± 0.01	0.003
Growth rate (g d^{-1})	0.006 ± 0.0018	0.007 ± 0.0007	0.451	0.006 ± 0.0009	0.014 ± 0.0009	0.000
Substrate reduction (%)	65.2 ± 5.54	70.8 ± 8.39	0.129	53.0 ± 5.28	42.5 ± 8.41	0.027
Waste reduction index (g d^{-1})	3.2 ± 0.26	3.2 ± 0.41	0.952	2.4 ± 0.32	5.3 ± 1.05	0.000
Efficiency of conversion of digested food	0.07 ± 0.009	0.05 ± 0.011	0.004	0.06 ± 0.002	0.14 ± 0.034	0.000

BRE, brewery by-product; FRU, 100% fruit waste; VEGFRU, 70% vegetable and 30% fruit waste; WIN, winery by-product.

Table 2. Proximate composition (g kg^{-1} dry matter, unless otherwise stated) of organic wastes (vegetables and fruits) and agro-industrial by-products (winery and brewery) generated by the Italian food sector and used as rearing substrates by black soldier fly larvae

	Trial 1 (organic wastes)		Trial 2 (agro-industrial by-products)	
	VEGFRU	FRU	WIN	BRE
Dry matter (g kg^{-1})	82.7	131.9	358.3	232.1
Ash	91.1	30.4	103.0	39.8
Crude protein	119.9	46.0	117.4	200.5
Ether extract	26.0	27.8	79.0	86.7
Neutral detergent fiber	178.0	139.3	566.4	447.1
Acid detergent fiber	110.5	91.1	462.4	225.3
Acid detergent lignin	12.9	13.1	323.5	62.1
Non-structural carbohydrates[a]	585.0	756.5	134.2	225.9
Gross energy (MJ kg^{-1} DM)	15.1	15.6	19.5	19.4

BRE, brewery by-product; FRU, 100% fruit waste; VEGFRU, 70% vegetable and 30% fruit waste; WIN, winery by-product.
[a] Calculated as: 1000 − (crude protein + ether extract + ash + neutral detergent fiber).

VEGFRU and BRE larvae showed higher weights after 4 days from the beginning of the trial, had lower mortality and needed less time to reach the prepupal stage than FRU and WIN larvae, respectively. Such results were obtained in spite of comparable GE values found in Trial 1 for VEGFRU and FRU and in Trial 2 for WIN and BRE substrates, and can be at least partly ascribed to the higher CP and moisture contents of VEGFRU and BRE, confirming the results obtained by other authors.[26,27]

The need for high dietary moisture content could be ascribed to the morphology of the mouthparts of BSF larvae, which resembles the characteristics of scavenger insects.[28,29] This kind of macerating mouth apparatus allows BSF larvae to scrape off the food from the feeding surface. By softening the feed solids, increased dietary moisture content makes it easier for the larvae to feed.[30]

The results obtained in our trials could also be reflective of possible differences between rearing substrates in terms of the content of nutrients other than CP (e.g. ether extract, structural and non-structural carbohydrates, amino acids) and/or in terms of nutrient digestibility. In both trials, the EE content of substrates (<90 g kg^{-1} DM) was far below the 200–260 g kg^{-1} found by Nguyen *et al.*[12,13] to have detrimental effects for the survival of BSF larvae and adults. BRE larvae showed very good performance despite the high structural carbohydrate content of the relative rearing substrate (NDF: 447 g kg^{-1} DM; ADF: 225 g kg^{-1} DM). Such a result clearly demonstrates that BSF larvae are also able to efficiently bioconvert wastes and by-products characterized by high fiber content, thanks to the presence, in the digestive tract of the insect, of intestinal bacteria able to degrade cellulose.[31] The amino acid composition of the rearing substrates was not analyzed in our trials, and little literature is available concerning the effects of dietary amino acids on development and nutritional composition of BSF larvae.[32,33] Studying the nutritional composition of BSF prepupae reared on different organic waste substrates, Spranghers *et al.*[32] showed that the amino acid content of the prepupae had narrow ranges, particularly when compared to the noticeable differences found in the amino acid composition of the rearing substrates. Concerning nutrient digestibility, to the best of our knowledge no studies are currently available. Further studies are necessary to deepen these aspects for the optimization of BSF feeding and nutrition.

In Trial 2 the differences in larval growth performance between treatments were very pronounced. We may speculate that the GE of the WIN substrate was not fully available for the larvae. The methodology used to grind the WIN substrate could have influenced the availability of the oil present inside the grape seeds. Indeed, a 3 mm grinder was used, and this size could not have completely milled the seeds. Moreover, the WIN substrate could have contained substances unsuitable for the BSF larvae development. Indeed, winery by-products usually contain high levels of polyphenols.[34] It is known that plants use polyphenolic compounds to protect themselves from herbivore insect attacks.[35] Some studies also showed how the grape seeds can accumulate high doses of pesticides and insecticides used in wine grapevine management.[35,36]

Hard *et al.*[37] reported that larval rearing density affects competition for food, low densities usually leading to highest larval weights. This was also reflected in our trial, as no (Trial 1) or slight (Trial 2) differences were observed for the total final biomass despite the differences found in LM between treatments. The observed LM for VEGFRU was lower than that reported by Nguyen *et al.*[13] using a vegetable and fruit rearing substrate.

In both trials, the differences highlighted in terms of LM and ECD were closely connected, and treatments leading to lower

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Table 3. Fatty acid composition (g kg^{-1} total fatty acids, unless otherwise stated) of organic wastes (vegetables and fruits) and agro-industrial by-products (winery and brewery) generated by the Italian food sector and used as rearing substrates by black soldier fly larvae

	Trial 1 (organic wastes)		Trial 2 (agro-industrial by-products)	
	VEGFRU	FRU	WIN	BRE
Total fatty acids (g kg^{-1} dry matter)	20.91	10.04	73.57	82.47
C12:0	0.73	3.52	0.93	0.80
C14:0	8.85	8.44	1.99	3.22
C16:0	184.90	192.71	100.49	252.48
C16:1 c9	5.89	5.96	4.43	1.75
C18:0	26.14	43.36	50.24	15.42
C18:1 c9	65.91	208.61	185.09	103.27
C18:1 c11	20.85	29.62	8.51	7.46
C18:2 n6	575.23	333.38	630.32	554.90
C18:3 n3	111.50	174.40	18.00	60.70
Saturated fatty acids	220.62	248.03	153.65	271.92
Monounsaturated fatty acids	92.65	244.19	198.03	112.48
Polyunsaturated fatty acids	686.73	507.78	648.32	615.60

BRE, brewery by-product; FRU, 100% fruit waste; VEGFRU, 70% vegetable and 30% fruit waste; WIN, winery by-product.

Table 4. Proximate composition (g kg^{-1} dry matter, unless otherwise stated) of black soldier fly larvae reared on organic wastes (vegetables and fruits) and agro-industrial by-products (winery and brewery) generated by the Italian food sector (mean ± SD; $n = 6$)

	Trial 1 (organic wastes)			Trial 2 (agro-industrial by-products)		
	VEGFRU	FRU	*P*	WIN	BRE	*P*
Dry matter (g kg^{-1})	219.6 ± 10.22	282.9 ± 6.57	0.000	265.4 ± 5.93	290.8 ± 6.96	0.000
Ash	129.8 ± 6.50	72.2 ± 2.22	0.000	145.7 ± 6.67	73.0 ± 1.89	0.000
Crude protein[a]	418.8 ± 13.24	307.5 ± 10.29	0.000	344.3 ± 7.63	529.6 ± 5.27	0.000
Crude protein[b]	312.9 ± 9.89	229.7 ± 7.69	0.000	257.3 ± 5.70	395.7 ± 3.94	0.000
Ether extract	262.8 ± 18.01	407.0 ± 18.83	0.000	322.2 ± 19.60	298.7 ± 6.49	0.031
Neutral detergent fiber	170.9 ± 16.49	197.9 ± 13.48	0.011	177.3 ± 13.08	87.0 ± 9.89	0.000
Acid detergent fiber	113.1 ± 20.09	93.4 ± 3.55	0.014	98.5 ± 10.16	64.8 ± 9.17	0.000
Acid detergent lignin	14.9 ± 7.75	8.9 ± 2.47	0.104	44.8 ± 17.80	8.3 ± 3.35	0.001
Chitin[c]	62.4 ± 19.63	56.0 ± 3.96	0.453	52.9 ± 9.25	14.2 ± 6.06	0.000
Chitin[d]	75.2 ± 19.7	65.5 ± 3.53	0.283	64.5 ± 9.48	27.0 ± 6.59	0.000

BRE, brewery by-product; FRU, 100% fruit waste; VEGFRU, 70% vegetable and 30% fruit waste; WIN, winery by-product.
[a] Calculated using the nitrogen-to-protein conversion factor of 6.25.
[b] Calculated using the nitrogen-to-protein conversion factor of 4.67.
[c] Calculated using the nitrogen-to-protein conversion factor of 6.25.
[d] Calculated using the nitrogen-to-protein conversion factor of 4.67.

mortality allowed us to obtain the best performance in terms of ECD. BRE larvae reduced a lesser quantity of substrate compared to WIN larvae; nevertheless, the WRI was higher in the BRE larvae as they took less time to reach the prepupal stage, which is also confirmed by the higher GR results. The SR was particularly high (above 65%) in Trial 1, showing the great potential of BSF larvae in the degradation of vegetable and fruit wastes.[12,13] Overall, the BRE larvae showed the best ECD combined with the absolute highest total final biomass production and the shortest developmental period.

The time needed by the larvae to reach the prepupal stage seemed to influence their chitin content. Such results agree with the findings of Diener *et al.*,[11] who reported how small larvae grown in 42 days showed a chitin level higher than heavy larvae grown in 16 days.

In both trials, substrates containing the highest CP and moisture contents (VEGFRU and BRE) allowed obtaining BSF larvae with the highest CP level, which is consistent with the results obtained by other authors.[26,27] Consistent with the findings of Janssen *et al.*,[22] the use of the conventional N-factor of 6.25 led to a CP overestimation of about 25%.

Despite comparable EE values of the rearing substrates, FRU larvae showed higher EE content than VEGFRU larvae, probably as a consequence of the higher NSC level of FRU.[38] Insects have the ability to convert carbohydrates into lipids.[32,39] Insects store lipids for two reasons: firstly, as energy reserve for the adult stage;[14] secondly, because, as the insect body presents an open blood system and a high surface compared to volume and the combination of these two factors could be a problem for the loss of water and the drying out process, lipids allow them to avoid transpiration and store non-imbibed water.[40] However, the influence of the NSC content of substrates on the EE content of BSF larvae should be further investigated as higher NSC in BRE substrate did not lead to higher EE content in BSF larvae in Trial 2.

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Table 5. Fatty acid composition (g kg^{-1} total fatty acids, unless otherwise stated) of black soldier fly larvae reared on organic wastes (vegetables and fruits) and agro-industrial by-products (winery and brewery) generated by the Italian food sector (mean ± SD; $n = 6$)

	Trial 1 (organic wastes)			Trial 2 (agro-industrial by-products)		
	VEGFRU	FRU	*P*	WIN	BRE	*P*
Total fatty acids (g kg^{-1} dry matter)	253.02 ± 18.512	398.40 ± 18.547	0.000	287.41 ± 16.973	282.93 ± 6.936	0.563
C12:0	520.61 ± 17.505	574.32 ± 11.060	0.000	346.91 ± 16.840	323.73 ± 9.277	0.014
C14:0	103.55 ± 3.303	96.39 ± 3.471	0.004	65.54 ± 4.283	66.49 ± 2.687	0.654
C16:0	138.95 ± 7.338	130.57 ± 3.846	0.040	189.36 ± 7.434	204.15 ± 5.772	0.003
C16:1 *c*9	33.57 ± 3.606	37.45 ± 0.956	0.046	60.63 ± 4.718	29.45 ± 2.639	0.000
C18:0	25.90 ± 1.693	17.51 ± 0.539	0.000	28.32 ± 2.139	18.07 ± 0.599	0.000
C18:1 *c*9	85.37 ± 4.075	93.19 ± 2.086	0.002	124.59 ± 4.280	92.23 ± 2.414	0.004
C18:1 *c*11	4.31 ± 0.381	2.79 ± 0.157	0.000	4.46 ± 0.261	5.75 ± 1.155	0.040
C18:2 n6	70.41 ± 7.408	40.70 ± 1.534	0.000	175.76 ± 14.935	235.47 ± 6.593	0.000
C18:3 n3	17.31 ± 1.370	7.06 ± 0.729	0.000	4.44 ± 0.392	24.65 ± 0.504	0.000
Saturated fatty acids	789.02 ± 10.854	818.81 ± 4.632	0.000	630.13 ± 16.745	612.45 ± 8.784	0.045
Monounsaturated fatty acids	123.26 ± 6.829	133.44 ± 2.773	0.013	189.68 ± 6.220	127.43 ± 5.354	0.000
Polyunsaturated fatty acids	87.72 ± 7.333	47.75 ± 2.083	0.000	180.19 ± 15.244	260.12 ± 6.843	0.000

BRE, brewery by-product; FRU, 100% fruit waste; VEGFRU, 70% vegetable and 30% fruit waste; WIN, winery by-product.

The FA composition of the rearing substrates did not directly affect the larval FA composition, which was also influenced by carbohydrates (starch and sugars), confirming other research studies.[26,32] Being of vegetable origin, all rearing substrates had PUFA as the most abundant FA group. Notwithstanding, as typically observed for Diptera, the BSF larval FA profile was dominated by SFA, mainly C12:0 (which showed the absolute highest values among individual detected FA), C14:0, C16:0 and C18:0.[32,41] The high presence of SFA in insects is connected with cold adaptation.[42] Indeed, larvae from some species showed a SFA decrease from summer to autumn, while PUFA increased, highlighting a correlation between the change in FA composition and the temperature due to seasonal change.[42–44] BSF is a subtropical species growing at high temperatures (27–32 °C), and the difficult adaptation to low temperatures was demonstrated by the lowest BSF survival rate at about 16 °C.[45] We can argue that the high SFA presence could be ascribed to BSF adaptation to the subtropical climate. In particular, the high content of lauric acid (melting point: 43.2 °C) could preserve BSF larvae from lipid oxidation and allow them to survive at temperatures above 40 °C.[41] Consistent with other findings,[7,15,32] C18:1 *c*9 was the main represented MUFA in the larvae, while C18:2 n6 and C18:3 n3 were the main represented PUFA n6 and PUFA n3, respectively. The low quantity of recovered PUFA n3 in the larvae could represent a problem if insect meals are intended to be used for animal feed. Indeed, research highlighted a decrease in nutritional product quality with the inclusion of insect meals in animal diets especially when full-fat meals are used.[5,6,46] Nevertheless, BSF larvae can be enriched in PUFA n3 through the substrate.[33,47] Authors[10,48] reported that C12:0 is a good inhibitor of bacterial strains and could be of great interest in the reduction of the use of antibiotics in animal feeding.[10,49,50] In this context, BSF larvae reared on organic wastes showed very interesting results, with up to 574 g kg^{-1} TFA of lauric acid.

Especially in southern Europe, the wide availability of vegetable and fruit wastes (mainly from markets and supermarkets) may allow the development of BSF larval mass production, enabling also economic and environmental benefits from the sustainable management of organic wastes. Regarding the considered agro-industrial by-products, the use of winery by-products as a rearing substrate for BSF larvae could be conditioned, both from a technological and economical point of view, by the need for preliminary processing to remove or reduce polyphenol, pesticide and insecticide contents, which could exert a negative influence on the growth performance of the larvae. Remarkable positive results were obtained in terms of overall development time, growth performance and nutritional composition for the larvae reared on brewery by-products, which should therefore be considered promising rearing substrates. However, as brewery by-products are characterized by a more limited availability than vegetables, fruits or winery by-products, it could be advisable to use BRE at low dietary inclusion levels with the purpose of increasing BSF larvae performance.

Overall, our results show that the performance and chemical composition of BSF larvae are largely affected by the chemical composition of the provided substrate. This clearly demonstrates that insects, like farm animals, have nutritional requirements which have to be met for optimal performance.

The performance obtained in our benchtop trials may vary when transferred to an industrial scale. For instance, the large volumes of waste used as well as the high larval concentration could result in environmental oxygen depletion and heat production.[51] Attention should then be given to the airflow to guarantee appropriate rearing conditions. In addition, to optimize land use, insect breeding should exploit the verticality of the breeding structure. However, this can lead to the stratification of temperature, and particular attention must be given to an adequate air circulation to guarantee a homogeneous temperature in all parts of the building. At an industrial level, the production system would also require a constant supply of substrates, possibly with a fairly constant chemical composition, in order to obtain BSF larvae with a relatively constant nutrient profile.

Future studies should be designed to assess the nutritional requirements of BSF larvae and to evaluate other agro-industrial by-products, as well as the effect of mixing different organic wastes and agro-industrial by-products, to obtain optimal BSF larval performance in terms of development, waste reduction efficiency and nutritional composition.

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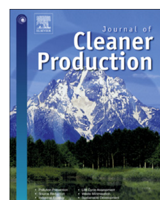

Effects of feedstock on larval development and process efficiency in waste treatment with black soldier fly (*Hermetia illucens*)

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A B S T R A C T

Global population growth has led to an urgent need for more efficient food production systems. Moreover, as income levels increase, dietary preferences are shifting to more animal-based products. However, current feed protein sources deplete wild fish populations and contribute to rainforest deforestation. Capturing the resources in organic waste could help alleviate environmental impacts of food production. The larvae of the black soldier fly (*Hermetia illucens*) are ferocious feeders on decomposing organic material and could be used as protein source in animal feed. This study evaluated development of black soldier fly larvae on eight urban organic waste fractions and two control substrates. Principal component analysis was conducted to identify substrate properties that contributed to treatment efficiency and larval development. The main treatment factors found to be affected by substrate were waste-to-biomass conversion ratio, larval development time and final prepupal weight. The substrate properties with the greatest impact on biomass conversion ratio and larval development time were content of total volatile solids and protein content, while only total volatile solids content affected final prepupal weight. It was concluded that black soldier fly larvae are versatile in their feedstock preferences and can be used to treat a variety of organic waste streams, provided that the total volatile solids and nitrogen content are sufficiently high to support larval development. Abattoir waste, food waste, human faeces and a mixture of abattoir waste – fruits & vegetables are waste streams that are highly suitable for fly larvae treatment.

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1. Introduction

The world's population will have exceeded 9 billion by 2050, with more than half of this global population growth occurring in Africa (United Nations, 2015). According to FAO estimates, food production will have to increase by 70% in order to feed the global population and this growth will be accompanied by increased income levels, and thus a shift in food consumption with a higher demand for meat, especially fish and poultry (van Huis et al., 2013). However, instead of consuming fish directly, around one-third of global fish catches are processed into fish oil and fishmeal for use in livestock and aquaculture feeds (Tveterås and Tveterås, 2010). For example, with a Fish-in-Fishout ratio of 1.9–2.9 for carnivorous fish aquaculture, as conservatively calculated by IFFO (International Fishmeal and Fish Oil Organisation) (Seafish, 2011), the pressure on wild fish is increasing with the growing aquaculture industry. From 1990 to 2014, worldwide aquaculture production grew by 7.8% annually (FAO, 2014). As a consequence, the price of fishmeal, today a major ingredient in feed, tripled from 2000 to 2015 and it will become increasingly unaffordable for fish and meat production. Already today, 60–70% of production costs in animal husbandry derive from feed purchasing (animal meal, fishmeal, soybean meal). Meeting these needs will require the development of new animal feed production systems (FAO, 2009), so alternative sources of protein of animal origin are therefore being explored.

Insect farming can be a viable new source of animal protein. Insects can be farmed in high densities with small space requirements and they have a high bioconversion ratio (Oonincx and de Boer, 2012). Furthermore, many insects can be reared on waste streams, which keeps the environmental footprint low and assists in recycling of refuse (Smetana et al., 2016). Consequently, the use of fly larvae in waste management has started to gain attention (Čičková et al., 2015; Pastor et al., 2015). One species that has gained more attention than others is the black soldier fly (BSF), *Hermetia*

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illucens L. (Diptera: Stratiomyidae), whose polyphagous larvae (BSFL) are known to feed and develop on a wide range of feed sources, such as kitchen waste (Diener et al., 2011; Nguyen et al., 2015), dairy manure (Myers et al., 2008b), chicken manure (Zhou et al., 2013) and human faeces (Banks et al., 2014; Lalander et al., 2013). One reason why this species is of particular interest is because the fly does not feed and thus is not a vector in disease transmission (Sheppard et al., 2002). Another reason is that, when the larvae stop feeding in the final larval stage, they are higher in fat than other fly larvae (Čičková et al., 2015). This makes BSFL of particular interest for use as a protein and fat source in animal feed (Wang and Shelomi, 2017) and for the production of biodiesel (Surendra et al., 2016).

However, the economic feasibility of a BSF system depends, among other factors, on the larval biomass produced from a certain amount of waste, in other words, the waste-to-biomass conversion ratio. This varies with the nature of input materials and may range from as little as 3% for biogas digestate to 23% on a wet-weight basis when working with fresh human excreta (Banks et al., 2014; Lalander et al., 2015; Newton et al., 2005; Spranghers et al., 2016).

There are many factors that affect the growth of insects. Assuming unlimited access to a selected material, development of insect larvae depends in the first instance on the presence of essential nutrients (Table 1).

Shortages, or even lack, of essential nutrients result in reduced growth and lowered survival rates (Cohen et al., 2004). However, even when the nutrients are present, they have to be bioavailable to the animal. Bioavailability is greatly dependent on the species of the animal in question and the type of food in which the nutrient is present. Unfortunately, almost nothing is known of the bioavailability of nutrients in insects. The majority of fly larvae, just like larvae of many species from other insect groups (*e.g.* beetles, true bugs, hymenoptera), feed on materials that are originally solids and converted into a liquid slurry before ingestion (Cohen et al., 2004). Such solid-to-liquid feeding or extraoral digestion is necessary as they lack biting and chewing mouthparts.

The main objectives of this study were to investigate the effect of different substrates on fly larvae composting in terms of larval growth (biomass conversion ratio, final larval weight), larval development time, waste reduction and amino acid profile of the larvae, and to identify the substrate parameters that influence the fly larvae composting process.

2. Material and methods

2.1. Animals

Eggs of the black soldier fly, *H. illucens* L. (Diptera: Stratiomyidae), were obtained from a laboratory colony located at the Research Institute of Organic Agriculture (FiBL, Switzerland). Newly hatched larvae used for the experiments were reared on chicken feed (Granngården Hönsfoder Start, metabolisable energy content = 11.2 MJ kg^{-1}, 80% moisture) for around 10 days. They were then transferred to one of the 11 substrates listed below.

2.2. Substrates

Poultry feed: Dry poultry feed (Granngården Hönsfoder Bas, ME = 10.9 MJ kg^{-1}) was dissolved in water to 40% dry matter (DM).

Dog food: Dry dog food (Purina Pro Plan puppy, ME = 16.0 MG kg^{-1}) was dissolved in water to 40% DM.

Food waste: Food waste was collected from the local restaurant at Ultuna campus (Swedish University of Agricultural Sciences), Uppsala, and minced in a grinder (Palmia) with grinder plates Ø 5 mm.

Fruit & vegetables: Lettuce (50%), apples (30%) and potatoes (20%) were minced in a food processor (Moulinex, Masterchef 3000).

Abattoir waste: Abattoir waste was collected from a sheep farm in mid-west Sweden and was chopped with a knife and mixed manually in order to represent the content of abattoir waste in low and middle-income countries. It comprised 48% stomach contents, 16% blood (cattle blood bought in a retail outlet), 12% manure, 16% meat and 8% organs (lungs, heart).

Abattoir-fruit & veg. waste: Abattoir waste was mixed with fruit and vegetables in a 1:1 ratio based on wet weight.

Poultry manure: Fresh poultry manure (laying hens) was collected from the Swedish University of Agricultural Sciences experimental farm at Funbo-Lövsta, Uppsala.

Human faeces: Human faeces were collected fresh in plastic bags and stored at −20 °C.

Primary sludge: Dewatered primary wastewater sludge was sent under cooling from Hammarby sjöstadsverken in Stockholm.

Undigested sludge: Sewage sludge was retrieved before the digestion step (activated sludge) at Uppsala municipal wastewater treatment plant (Kungsängens reningsverk, Uppsala). The DM content was very low at the time of collection and the sludge was dewatered through a cloth, achieving a DM content of around 8%.

Digested sludge: Dewatered, anaerobically digested wastewater sludge was collected at Uppsala municipal sewage treatment plant (Kungsängens reningsverk, Uppsala).

All substrates were divided into feeding portions, bagged in freezer bags and stored at −20 °C until use.

2.3. Experimental set-up

The experiments were conducted in triplicate in plastic containers (Smartstore classic 2, 21 cm × 17 cm x 11 cm) with netted lids, kept at 28 °C. In each box, 200 larvae (>0.2 cm in size, 10 ± 2 d old) were placed, giving a larval density of 0.6 larvae cm^{-2}. The substrates were applied every second or third day, with a feeding rate of 40 mg DM $larva^{-1}$ d^{-1}. The substrate portions were thawed and brought to room temperature before feeding. When 50% of the larvae had transformed into prepupae, feeding was stopped but the experiment continued until all larvae had either turned into prepupae or had died. The survival rate was determined at the end of the experiment by enumerating all emerging prepupae and dividing this number by the total number of larvae added at the start of the experiment.

2.4. Sampling and analysis

All substrates were weighed when applied, as was the total remaining material at the end of the experiment. On all feeding occasions, the combined weight of 10 larvae was recorded. These

Table 1
Essential nutrients in insect diets (Cohen et al., 2004).

Amino acids	Lipids	Vitamins	Minerals
Arginine	Cholesterol	Ascorbic acid (C)	Calcium
Histidine	B-Sitosterol	Thiamine (B_1)	Chlorine
Isoleucine	Stigmasterol	Riboflavin (B_2)	Copper
Leucine	Campesterol	Pyridoxine (B_6)	Iron
Lysine	24-Methylcholesterol	Nicotinic acid (B_3)	Magnesium
Methionine	Linoleic acid	Pantothenic (B_5)	Manganese
Phenylalanine	Linoenic acid	Biotin (B_7)	Phosphorus
Threonine		Folic acid (B_9)	Potassium
Tryptophan		Choline	Sodium
Valine		Cyanocobalamin (B_{12})	Sulfur
		Inositol	Zinc

larvae were collected, washed in water, dried on a piece of paper and weighed, after which they were placed back on the substrate. All emerged prepupae were counted and weighed within one day of emerging. When no prepupae emerged on two subsequent analysis occasions, the remaining prepupae and larvae found in the material were picked out, counted and weighed. All dead larvae were counted, but were not taken into account when calculating the substrate-to-biomass conversion ratio (BCR).

Samples of all substrates and of the compost residue at the end of the experiments were taken for analysis of dry matter (DM) and total volatile solids (VS). Samples were dried at 80 °C for 48 h. After drying, the material was combusted in a muffle oven at 550 °C for 4 h for determination of VS. The pH was analysed five days after the start of the experiment, once a week during the duration of the experiment and at the end of the experiment. For these pH measurements, a radiometer electrode at room temperature was used: 10 g of sample were diluted with 50 mL deionised water and left to settle for 1 h at room temperature prior to analysis. At the end of the experiment, the prepupae were dried at 50 °C for 48 h and sent to Eurofins Food & Agro Testing Sweden AB (Swedac accredited lab) for amino acid profiling. The Swedish standard method (ISO 13903:2005) was followed for amino acid profiling.

For analysis of total nitrogen, the samples were boiled in concentrated sulphuric acid according to the method described in Lalander et al. (2015). Following acid boiling, the samples were neutralised to pH > 3 using 10 M NaOH, diluted 50-fold in deionised water and then digested using Spectroquant® Crack-Set 20 (1.14963.0001). The nitrate concentration in the digested diluted sample was determined at 340 nm using Spectroquant® nitrate test with concentration range 0.4–25 mg L^{-1} (1.09713.0002).

2.5. Calculations

The percentage material reduction on a dry matter basis (Mat. $red._{DM}$) was calculated as:

$$\text{Mat. red.}_{DM} = \left(1 - \frac{\text{sub.in}_{DM}}{\text{mat.out}_{DM}}\right) \times 100, \quad (1)$$

where sub.in_{DM} and mat.out_{DM} was the dry matter of the substrate fed and of the residue after the experiment, respectively.

The percentage waste-to-biomass conversion ratio on a dry matter basis (BCR_{DM}) was calculated as:

$$BCR_{DM} = \frac{pp_{DM}}{\text{sub.in}_{DM}} \times 100, \quad (2)$$

where pp_{DM} and sub.in_{DM} was the total dry matter in the prepupae (pp) and the substrate (sub.in), respectively.

The percentage protein conversion ratio on a dry matter basis ($PrCR_{DM}$) was calculated as:

$$PrCR_{DM} = \frac{pp_{DM} \times \%Pr_{pp}}{\text{sub.in}_{DM} \times \%Pr_{sub.in}} \times 100, \quad (3)$$

where pp_{DM} and sub.in_{DM} was the total dry matter and $\%Pr_{pp}$ and $\%Pr_{sub.in}$ was the percentage crude protein (% of DM) in the prepupae (pp) and the substrate (sub.in), respectively.

Values for carbohydrate content of similar substrates were taken from the literature. The total protein content (% of DM) was used to verify the similarity between the substrates tested in this study and those described in the literature. No reliable comparable literature value was found for the digested sludge. Based on the literature values of protein and carbohydrate concentration, the carbohydrate to protein ratio (CHO/Pr) was calculated.

The carbon to nitrogen ratio (C/N) was calculated by dividing the percentage of organic carbon by the percentage of total nitrogen, on a dry matter basis. The percentage of organic carbon was estimated by dividing the percentage of VS by 1.8 (Haug, 1980).

2.6. Statistical analysis

The Brown-Forsythe-Levene test was performed to verify equal variance between the data on the different substrates. Analysis of variance (ANOVA) with 95% confidence interval was performed to identify statistically significant differences between substrates. When a significant difference was found, Tukey post-hoc test with 95% confidence interval was performed. Principal component analysis (PCA) was performed to find the variables that mostly contributed to the data variance, while generalised linear regression was used to evaluate the variables selected in the PCA. All statistical analyses and graphical illustrations were carried out using RStudio (RStudio Team, 2016).

3. Results

The dry matter content of the different substrates varied between 41% for poultry feed and 8% for undigested sludge, while the VS content on a dry matter basis varied between 93% for dog food and 63% for undigested sludge (Table 2). Dog food had the highest protein concentration (40% of DM) and fruit & vegetables the lowest (13% of DM). The pH after five days of BSF composting ranged between 4.3 (dog food, fruit & vegetables) and 8.9 (poultry manure). Fruit & vegetables had the highest CHO/P ratio and slaughterhouse waste the lowest. Poultry feed had the highest C/N ratio, while abattoir waste had the lowest.

The highest waste-to-biomass conversion ratio (BCR) was achieved with the abattoir waste (15% DM) and the highest material reduction occurred for poultry manure, of which 85% was reduced on a DM basis (Table 3). Undigested sludge had the lowest conversion ratio (2% DM) and material reduction (13% DM). Poultry feed had the highest protein conversion ratio (PrCR), while digested sludge had the lowest. The larvae developed rapidly on abattoir waste, abattoir waste-fruit & veg., dog food, poultry feed, food waste, human faeces and poultry manure (Fig. 1). By day 14 of the experiment, the first prepupae had emerged from these substrates and by day 19, 50% or more of the prepupae had emerged (Table 3). The prepupae in these substrates were quite large in all cases (>210 mg $larva^{-1}$) except those reared on poultry manure (165 mg $larva^{-1}$). The largest prepupae were those reared on abattoir waste-fruit & vegetables, which weighed just over 250 mg $larva^{-1}$ on average. Larval development was slowest on the digested sludge, where it took 30 days for the first prepupa to emerge and around 50 days for 50% of the prepupae to emerge. The prepupae that emerged from the different sludges were smaller than those on the other substrates; around 140 mg $larva^{-1}$ for those reared on undigested and primary sludge and 70 mg $larva^{-1}$ for those on digested sludge. Larval development was also slow on the fruit & vegetables substrate, 28 days for the first prepupa to emerge and around 28 days for 50% of the prepupae to emerge. However, in that case the average prepupal weight was quite high (220 mg $larva^{-1}$).

Principal component analysis was conducted in order to distinguish the most important parameters affecting BSF composting (Fig. S1). The BSF composting variables found to be affected by substrate properties were waste-to-biomass conversion ratio (% DM), larval development (time for first prepupa and for 50% of prepupae to emerge) and prepupal final weight (mg $prepupa^{-1}$). The survival rate did not vary greatly between the different substrates. The variables found to contribute most to BCR and larval development were VS and protein feeding rates. Volatile solids

Table 2
Physico-chemical and nutritional properties of substrates tested for their suitability as feed for BSFL. Experimentally determined values, presented as mean ± standard deviation (n = 3). (Literature (Lit.) values presented for comparison).

	Dry matter (%)	Total volatile solids (% of DM)	N-tot (mg g^{-1})	Crude protein (Pr) (% of DM)**	Lit. crude protein (% of DM)	Lit. carbohydrate (CHO) (% of DM)	CHO/Pr ratio	C/N ratio***	pH‡
Poultry feed	41.2 ± 0.0	90.0 ± 0.0	9.6 ± 0.9	17.3	17.8[a]	60[a]	3.4[a]	18.1	4.9 ± 0.0
Dog food	37.7 ± 0.0	93.3 ± 0.3	20.4 ± 0.4	33.9	32.6[b]	36.4[b]	1.1[b]	9.6	4.3 ± 0.0
Food waste	24.3 ± 1.9	89.8 ± 4.0	8.6 ± 0.7	22.2	22.3[c]	55[c]	2.5[c]	14.0	6.5 ± 0.4
Fruits & veg.	11.1 ± 0.5	93.0 ± 2.0	2.3 ± 0.3	13.2	12.3[d]	72.6[d]	5.9[d]	24.4	4.3 ± 0.2
Abattoir waste	18.0 ± 3.0	89.3 ± 4.0	16.2 ± 0.5	56.3	53.4[e]	2[e]	0.1[e]	5.5	8.5 ± 0.1
Abattoir -fruits & veg.*	14.5	91.2	9.3	34.8	27.9	37.4	1.3	9.0	6.8 ± 0.7
Poultry manure	31.2 ± 0.3	83.6 ± 0.3	11.4 ± 0.9	22.8	24.2[f]	38.0[f]	1.5[f]	12.7	8.9 ± 0.1
Human faeces	21.9 ± 0.2	87.1 ± 0.1	12.4 ± 3.5	35.5	38.8[g]	31.0[g]	0.8[g]	8.5	7.3 ± 0.2
Primary sludge	17.5 ± 0.6	77.8 ± 0.2	4.7 ± 0.5	16.9	21.8[h]	38.9[h]	1.8[h]	16.0	8.6 ± 0.0
Undigested sludge	8.3 ± 0.1	77.1 ± 0.5	4.2 ± 0.1	31.5	28[†,i]	18[‡, i]	0.6[i]	8.5	6.8 ± 0.1
Digested sludge	28.7 ± 0.1	63.2 ± 0.2	6.8 ± 0.2	14.7	na	na	na	14.9	8.8 ± 0.0

* Average of abattoir waste and Fruits and veg.; ** N-tot x 6.25; † % of COD; ***Org-C/N-tot, org-C estimated as VS (% of DM)/1.8 (Haug, 1980), ‡ Determined after five days in treatment.
[a] Granngården Hönsfoder Bas.
[b] Purina Pro Plan puppy.
[c] Kjos et al. (2000).
[d] Livsmedelsinfo.nu.
[e] Skrede and Nes (1988).
[f] Feedipedia.
[g] Rose et al. (2015).
[h] Nielfa et al. (2015).
[i] Raunkjær et al. (1994).

Table 3
Process efficiency and larval development properties to the final larval stage prepupae (pp) in BSFL composting with different substrates. Values within columns marked with same letters are not significantly different ($p < 0.05$). Values presented are mean ± standard deviation (n = 3).

	Process efficiency			Larval development properties			
	Biomass conversion ratio (% DM)	Protein conversion ratio (%)	Material reduction (% DM)	Time to first pp (d start of exp.)	Time to 50% pp (d start of exp.)	Prepupal weight (mg)	Survival rate (%)
Poultry feed	12.8 ± 0.7[a,d]	80.4 ± 1.2	84.8 ± 3.6[d]	14	16	251±6[a]	93.0 ± 2.9[a,b]
Dog food	13.4 ± 0.9[a,b]	46.3 ± 2.8[a,b]	60.5 ± 1.5[b,e]	15	18	252±6[a]	89.3 ± 6.6[a,b,c]
Food waste	13.9 ± 0.3[a,b]	58.7 ± 1.3	55.3 ± 4.1[a,b,e]	14	19	212±4[b]	87.2 ± 0.5[a,b,c]
Fruits & veg.	4.1 ± 0.2[e,f]	34.3 ± 1.1[c]	46.7 ± 3.1[a,b,e]	28	42–47	218±4[b]	90.7 ± 5.6[a,b,c]
Abattoir waste	15.2 ± 1.6[a]	30.8 ± 2.8[c]	46.3 ± 2.9[a]	12	17	248±3[a]	101.5 ± 2.8[a]
Abattoir waste –fruits & veg.	14.2 ± 1.9[a,b]	47.7 ± 6.6[a]	61.1 ± 10.7[a,b,e]	12	17	252 ± 13[a]	96.3 ± 5.2[a,b]
Poultry manure	7.1 ± 0.6[e]	37.8 ± 3.4[b,c]	60.0 ± 2.3[a,b,e]	14	19	164 ± 14	92.7 ± 3.3[a,b]
Human faeces	11.3 ± 0.3[b,d]	31.6 ± 0.6[c]	47.7 ± 1.1[a,b]	12	19	245±5[a]	91.8 ± 4.5[a,b,c]
Primary sludge	2.3 ± 0.1[c,f]	15.0 ± 0.5[d]	63.3 ± 1.9[b,e]	16–21	28–32	137±5[c]	81.0 ± 1.5[b,c]
Undigested sludge	2.2 ± 0.2[c,f]	7.8 ± 0.6[d,e]	49.2 ± 3.7[a,b,e]	30	46–51	145±5[c]	76.2 ± 7.1[c]
Digested sludge	0.2 ± 0.0[c]	1.9 ± 0.3[e]	13.2 ± 0.8[c]	39–42	NA	70 ± 5	39.0 ± 4.4

feeding rate (VS_r) was found to be the most important parameter, contributing to 60% of the variance in BCR (Table 3). When the protein feeding rate (Pr_r) was included in the linear model, close to 80% of the variance in BCR was explained (Fig. 2). The model including both VS and protein feeding rate took the form $y_{vs+Pr} = a \times VS_r + b \times Pr_r$, where a and b are model-derived constants representing the estimated slope in the correlations of VS_r and Pr_r with response variables. This model was not as strong in explaining the development time, but the degree of explanation increased greatly from the simpler model (y_{vs}), from 30% to 60% (Fig. 2c and d). For the prepupal final weight, VS feeding rate was by far the strongest factor; including protein feeding in the model only increased the model strength from 0.87 to 0.9 (Table 4; Fig. S2). The only substrate property investigated that was found to explain the variation in PrCR was VS feeding rate, while inclusion of protein feeding rate did not improve the model.

The average crude protein content of the prepupae in this study was 41.2% (Table 5) and it did not vary greatly between the prepupae reared on the different substrates. The highest protein content was found for the prepupae reared on abattoir waste (44%) and the lowest for those reared on human faeces (39%). The combined amino acid component of the dry matter comprised 36 ± 0.6% (Table S1). The differences in amino acid profiles in the prepupae reared on different substrates were not large, although some were significant (Table 5).

4. Discussion

This study investigated how different substrates affect BSFL composting in terms of larval growth (biomass conversion ratio, final larval weight), larval development time and waste reduction. It has already been established in other studies that larval density, feeding rate and feeding frequency have a great impact on the efficiency of the process (Banks et al., 2014; Parra Paz et al., 2015), so those parameters were not analysed in this study. The same larval density (0.6 larvae cm^{-2}), feeding rate (40 mg DM day^{-1} $larva^{-1}$)

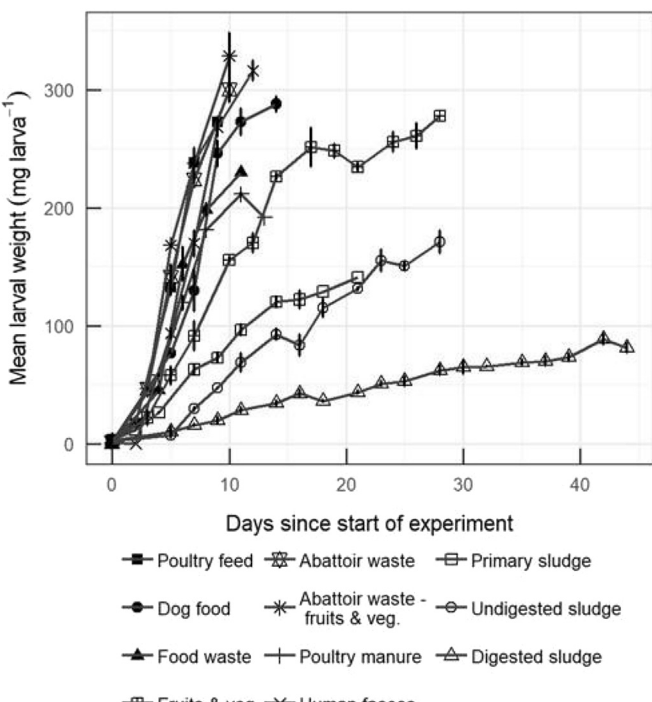

Fig. 1. Larval mass gain (mg) over time (days) in the different substrates: points represent the mean (n = 3) and error bars the standard error of the mean.

and feeding frequency (every 2nd or 3rd day) were employed for all 11 substrates tested, in order to investigate the impact of the substrate itself. These variables are by no mean the most optimised, but were those used in this study. Dog food and poultry feed were included in the study, since these substrates have been used as model substrates in previous studies.

4.1. Substrate properties affecting the process

The two main factors found to contribute to the BSFL composting process were VS content of the substrate and nitrogen feeding rate (mg larva^{-1}) (Table 4). In contrast, the C/N ratio was not found to directly correlate to the response variables (Fig. S1). Rehman et al. (2017b) stressed the importance of a good balance between VS and nitrogen in BSFL substrates. They found that BCR in BSFL composting was higher on soybean curd residue than on dairy manure, while mixtures of the two substrates yielded even higher BCR, which those authors attributed to better nutrient balance (C/N ratio) in the composite substrate. In this study, human faeces and undigested sludge had the same C/N ratio (8.5), but the BCR for human faeces was around 11%, while that of undigested sludge was just over 2% (Table 3). This is probably because the VS content of the undigested sludge was too low, even though the ratio of nitrogen to carbon was within a range similar to other substrates with higher BCR (dog food and abattoir - fruits & veg.). As the feeding rate was regulated by dry matter content in this study, the VS and protein feeding rates varied for the different substrates. To capture the variations in the amount of VS and protein received by the larvae reared on the different substrates, daily VS and protein feeding rates were calculated. The model including VS and protein feeding rates (y_{VS+Pr}) was able to capture the variations in BCR for the different substrates (Fig. 2b). However, that model did not describe the variations in larval development time as well, *e.g.* larvae reared on the fruit & vegetables substrate, which had a high VS and low protein content, had a longer development time than estimated by the model for that VS and protein feeding rate. The BCR did not correlate with the protein conversion ratio (PrCR): although there was no significant difference in the PrCR of fruit & vegetables, abattoir waste, poultry manure and human faeces, the BCR of these waste fractions varied between 4% (fruit & veg.) and 15% (abattoir waste). Interestingly, the PrCR for the mixture of abattoir waste and fruit & veg. (48%) was higher than that of the pure abattoir waste fraction (31%), although the BCR of the two substrates was almost the same (14–15%). The nutrient balance was better in the mixture of abattoir waste-fruit & veg., as the carbon added by the fruit & vegetable fraction balanced the high nitrogen content of the abattoir waste, enabling the larvae to utilise the available nutrients to a higher degree.

4.2. Factor affecting larval size and development

The larvae grew largest on abattoir waste (including that mixed with 50% fruit & vegetables) and were smallest on undigested sludge (Table 3). On all substrates, the larval weight gain appeared to be linear. The correlation between VS feeding rate and the final weight of the prepupae was very strong (Table 4; Fig. S2), and is likely the strongest factor controlling the final prepupal weight.

The development of BSFL on cattle manure has been shown to be slower and the larvae considerably smaller (Myers et al., 2008a) than found for poultry feed (Diener et al., 2009). As observed in the present study, Nguyen et al. (2013) also found that the development of BSFL on fruit & vegetable waste was quite slow. However, the larvae fed fruit & vegetables in this study were considerably larger than in their study. The energy and protein content of the fruit & vegetables substrate in their study was lower than that of manure, yet the larvae fed fruit & vegetables grew larger and developed more quickly. This could be because, although the energy content was higher in manure, it consisted to a large extent of lignin, which the larvae could not degrade. Rehman et al. (2017a) found considerably lower BCR for dairy manure than for chicken manure even though the dairy manure had higher total organic carbon, because the proportion of lignin, cellulose and hemicellulose was higher. Another reason why the larvae fed the fruit & vegetables substrate were smaller for Nguyen et al. (2013) could be because they were handled to a greater extent (daily), which according to those authors can cause stress and thereby hamper growth.

The larvae reared on fruit & vegetables waste were quite large, but their development was slow compared with values reported in other studies on similar substrates. For example, in Spranghers et al. (2016) the required development time for the first prepupae to emerge was 19 days from the first feeding and in Meneguz et al. (2018) it was 20 days, compared with 28 days in this study. Fruit and vegetables are high in VS but low in protein, so the slow development could be due to a larger amount of substrate being required in order to attain a sufficient amount of protein for development. Interestingly, Meneguz et al. (2018) found a larger difference in development time between winery by-product waste and brewery waste (22 d and 8 d, respectively), where the winery by-product waste had similar properties to the fruit & vegetable waste in this study (8.3% DM, 90.9% VS and 12% protein on a DM basis), while the brewery waste had a considerably higher VS and protein content (23.2% DM, 96% VS and 20.1% protein on a DM basis).

It was also observed in the present study that, when a substrate was high in protein, the development was faster even when the VS content was not high. However, the larvae did not grow as large, as in the case of poultry manure. It appears that the larvae accumulated enough protein to continue their development, while consuming less energy, but this resulted in smaller larvae.

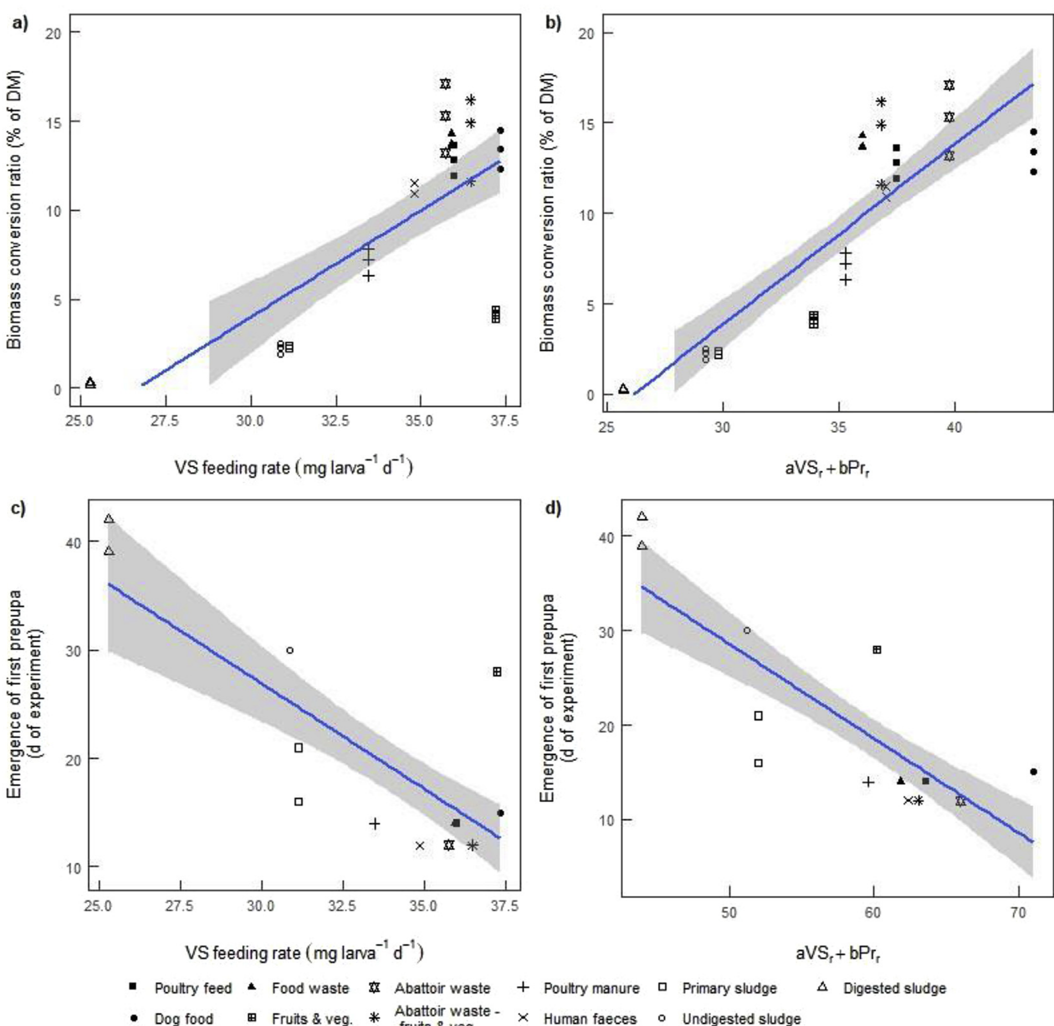

Fig. 2. Graphical representations of the correlations between biomass conversion ratio (% of dry matter) and a) volatile solids (VS) feeding rate (VS_r, mg larva^{-1} d^{-1}) and b) VS_r and the protein feeding rate. (Pr_r) and the correlation between the emergency of first prepupa (days of experiment) and c) VS_r, d) VS_r and Pr_r.

Table 4
Model strength (R^2) and significance level of models describing the correlation between volatile solids (VS_r) and protein (Pr_r) feeding rate, alone and in combination, on biomass and protein conversion ratio (BCR and PrCR, respectively), prepupal emergence rate and prepupal weight.

	Model strengths (R^2) for response variables (y)			
	BCR_{DM}	$PrCR_{DM}$	Prepupal weight	Prepupal emergence
VS feeding rate (VS_r) $y_{VS} = a \times \mathbf{VS_r}$	0.58^{***}	0.55^{***}	0.87^{***}	0.54^{***}
Protein feeding rate (Pr_r) $y_{Pr} = b \times \mathbf{Pr_r}$	0.51^{***}	0.15^{*}	0.31^{***}	0.33^{***}
VS and protein feeding rate $y_{VS+Pr} = a \times \mathbf{VS_r} + b \times \mathbf{Pr_r}$	0.77^{***}	0.56^{***}	0.90^{***}	0.63^{***}

Significance level of model coefficients: $p < 0.001^{***}$, $p < 0.5^{*}$

Spranghers et al. (2016) found that rearing BSFL on digested vegetable waste resulted in almost 40% lower larval biomass yield compared with rearing them on undigested vegetable waste, while the development time was not affected by consuming digested waste. In the present study, the larvae reared on digested sludge were the smallest (70 mg larva^{-1}) and had the longest development time, with BCR reduced by >90% compared with undigested sludge (Table 3). During anaerobic digestion, easily available carbon is reduced to methane, while nitrogen largely remains in the digestate (Zhang et al., 2014). The digested sewage sludge in this study had a low VS as well as protein feeding rate (Fig. 2), while the digested vegetables in Spranghers et al. (2016) had a lower VS but higher protein content, as compared to the vegetable waste. The larvae reared in digestate were thus smaller but the development time unaffected. This supports our suggestion that the VS content influences the size of the larvae, while the VS and protein content together affect the development time of the larvae.

4.3. Other factors that could have an impact on larval growth

Volatile solids and protein content seemed to explain the variations in BCR and larval development to a great extent. However, other factors are also likely to contribute. The fat content was not analysed in this study, but is likely to have an impact, since BSF

Table 5
The crude protein content (% of DM) and amino acid profile of the larvae (g kg^{-1} crude protein) reared in the different substrates. Where a significant difference between the larvae reared in the different substrates exist, values emphasised in bold font represent highest value for a given amino acid and the superscripted *i* represent significant difference ($p > 0.05$) to the highest value. Values are presented as mean (n = 3) ± standard deviation. The essential amino acids for the larvae are in italic.

	Abattoir waste	Abattoir -fruits & veg.	Dog food	Digested sludge	Food waste	Fruits & veg.	Human faeces	Poultry feed	Poultry manure	Primary sludge	Undigested sludge
Crude protein (% of DM)***	**44.2 ± 0.2**	44.1 ± 0.6	42.8 ± 0.2	42.6 ± 0.3	39.2 ± 2.5^{i}	41.3 ± 1.0^{i}	39.1 ± 0.3^{i}	39.6 ± 0.2^{i}	41.6 ± 1.5^{i}	39.6 ± 0.3^{i}	40.0 ± 1.5^{i}
Alanine***	57.2 ± 1.1^{i}	52.2 ± 0.9^{i}	59.4 ± 1.1	48.2 ± 2.3^{i}	59.3 ± 0.9	54.8 ± 2.5^{i}	**65.3 ± 0.9**	58.8 ± 0.9	56.5 ± 3.7^{i}	55.7 ± 5.4^{i}	56.6 ± 1.6^{i}
Arginine	49.6 ± 0.7	46.5 ± 1.2	49.5 ± 0.6	44.9 ± 4.3	48.9 ± 0.7	45.4 ± 3.4	51.0 ± 1.8	48.3 ± 4.7	49.2 ± 3.2	49.4 ± 3.7	49.4 ± 0.8
Aspartic acid	92.8 ± 1.2	88.0 ± 2.4	91.1 ± 1.3	82.6 ± 7.5	90.8 ± 0.8	80.6 ± 7.2	90.0 ± 1.8	83.8 ± 7.2	92.6 ± 5.9	88.5 ± 7.8	89.0 ± 2.3
Cysteine***	6.0 ± 0.1	5.7 ± 0.1	6.5 ± 0.0	6.5 ± 0.5	5.3 ± 0.1^{i}	5.0 ± 0.6^{i}	6.7 ± 0.5	5.9 ± 0.5^{i}	5.5 ± 0.0^{i}	6.6 ± 0.8	**6.7 ± 0.1**
Glutamic acid**	100.0 ± 0.2	92.2 ± 1.2^{i}	104.2 ± 1.5	95.7 ± 5.6	98.4 ± 0.3	95.4 ± 4.9	**105.9 ± 1.1**	94.6 ± 2.8	96.6 ± 5.2	100.4 ± 9.5	103.8 ± 2.8
Glycine***	5 8.0 ± 0.7	54.1 ± 0.9^{i}	56.2 ± 0.6	48.3 ± 2.5	52.7 ± 1.1	52.4 ± 3.1	**59.2 ± 0.4**	51.1 ± 1.2	54.1 ± 3.5	56.0 ± 5.3	55.5 ± 1.6
Histidine	35.3 ± 0.6	31.6 ± 0.7	25.3 ± 8.1	19.8 ± 8.4	29.1 ± 11.3	26.3 ± 1.9	33.0 ± 1.2	29.2 ± 0.7	30.9 ± 11.3	29.7 ± 11.3	31.3 ± 1.0
Hydroxyproline	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Isoleucine**	46.7 ± 1.3	45.3 ± 3.5	**47.6 ± 0.9**	39.0 ± 4.3^{i}	41.3 ± 0.3	43.0 ± 3.8	46.7 ± 0.7	45.3 ± 3.0	41.0 ± 2.9	45.0 ± 4.3	44.7 ± 1.9
Leucine**	68.4 ± 0.6^{i}	67.1 ± 0.6^{i}	**80.1 ± 1.8**	62.5 ± 2.2^{i}	67.9 ± 0.4^{i}	66.7 ± 5.8^{i}	70.0 ± 1.7	73.4 ± 8.9	66.1 ± 4.4^{i}	65.5 ± 5.5^{i}	75.6 ± 1.6
Lysine ***	64.0 ± 0.5^{i}	57.6 ± 1.3^{i}	61.7 ± 0.9^{i}	61.7 ± 4.0^{i}	**82.5 ± 5.0**	51.4 ± 3.2^{i}	61.7 ± 1.1^{i}	59.5 ± 1.7^{i}	70.7 ± 10.0	59.4 ± 5.7^{i}	64.2 ± 2.1^{i}
Methionine**	17.7 ± 0.2	17.3 ± 0.7^{i}	18.1 ± 0.3	18.1 ± 2.0	18.4 ± 0.4	15.3 ± 1.3^{i}	18.5 ± 0.9	17.1 ± 1.2^{i}	**20.5 ± 0.4**	18.1 ± 1.7	18.3 ± 0.5
Ornithine	0.3 ± 0.1	0.4 ± 0.1	0.7 ± 0.0	<0.25	<1.3	<0.25	0.6 ± 0.1	0.6 ± 0.1	<1.3	<0.25	<0.25
Phenylalanine**	35.9 ± 1.6^{i}	38.6 ± 1.6	41.4 ± 0.9	37.3 ± 4.3	40.3 ± 0.3	34.5 ± 3.1^{i}	**44.2 ± 3.7**	38.6 ± 2.6	42.0 ± 3.0	37.6 ± 2.9	39.9 ± 1.1
Proline	50.6 ± 0.8	45.1 ± 1.5	58.7 ± 8.9	46.5 ± 1.9	51.2 ± 1.2	52.8 ± 2.8	57.9 ± 2.9	48.5 ± 3.3	52.0 ± 6.2	55.9 ± 3.1	53.5 ± 2.2
Serine	40.5 ± 1.4	38.7 ± 0.1	41.4 ± 0.8	36.6 ± 1.3	41.3 ± 0.9	38.6 ± 3.2	43.9 ± 4.9	39.9 ± 3.2	38.4 ± 2.6	40.6 ± 2.9	42.5 ± 0.4
Threonine	37.0 ± 0.4	35.9 ± 0.4	38.0 ± 0.7	33.1 ± 2.4	38.6 ± 0.2	34.6 ± 3.3	37.6 ± 1.5	36.8 ± 2.3	38.3 ± 2.3	36.0 ± 2.7	37.0 ± 0.7
Tryptophan***	16.7 ± 0.3	16.6 ± 0.5	13.6 ± 0.2^{i}	**17.9 ± 0.3**	13.8 ± 0.4^{i}	14.0 ± 0.3^{i}	16.9 ± 0.1	15.2 ± 0.9^{i}	16.2 ± 0.3^{i}	16.5 ± 0.1^{i}	17.1 ± 0.6
Tyrosine***	86.2 ± 2.6^{i}	57.0 ± 1.0^{i}	61.1 ± 0.3^{i}	62.6 ± 3.5^{i}	60.2 ± 0.3^{i}	55.0 ± 3.3^{i}	**108.8 ± 0.6**	58.4 ± 1.1^{i}	62.1 ± 4.4^{i}	60.2 ± 3.9^{i}	65.6 ± 2.6^{i}
Valine*	63.2 ± 1.8	61.7 ± 1.1	62.9 ± 1.0	52.8 ± 5.5	58.4 ± 1.9	59.5 ± 6.0	**65.9 ± 1.1**	61.3 ± 3.8	59.7 ± 3.5	61.6 ± 4.9	60.8 ± 2.7

larvae need to accumulate energy in the form of fat for the adult phase (Sheppard et al., 1994). A substrate too rich in fat could also be detrimental, *e.g.* Memon (2010) found that blowfly larvae reared on a high fat diet lived for a shorter time and did not survive to adulthood. In the present study, no factors affecting the survival rate were identified and quite a high survival rate was found for all substrates (Table 3). On the other hand, emergence to flies was not monitored. Moreover, it is difficult to compare BSF with other insects, even other flies, as they do not feed as adults.

4.4. Material reduction

In this study, no particular substrate property was found to contribute to the material reduction. One factor that was not taken into account in this study, but which could contribute, was the amount of easily available carbon relative to the amount of slowly digestible carbon compounds, such as long-chain fibre compounds *e.g.* cellulose, lignocellulose and lignin (Pérez et al., 2002). Agricultural wastes, such as cattle manure, contain high proportions of cellulose and lignin and generally a lower proportion of easily available carbon. Li et al. (2011) found that 50% of cellulose and nearly 30% of hemicellulose was reduced after 21 days of BSF composting, while the relative lignin contain increased because lignin was not degraded. In a later study, Li et al. (2015) found a small reduction in lignin content after BSFL composting of anerobically digested corncobs, but pointed out that bacteria could be responsible for the lignin degradation, rather than the larvae themselves. The residence time in their fly larvae composting system was not reported. Lignin degradation is quite complex and is performed by either fungi or a consortium of bacteria (Brown and Chang, 2014). A material with high carbon content that comprises a great proportion of lignin could be expected to be broken down less well in BSFL composting, which is generally a quite fast process.

4.5. Protein content and amino acid profile of the prepupae

In the concept of waste management with black soldier flies, the larvae or prepupae could be used either as animal feed (Wang and Shelomi, 2017) or for production of biodiesel (Leong et al., 2016). For use as feed, the protein content and the amino acid profile of the larvae/prepupae are important. In this study, the importance of waste substrate on the protein content and amino acid profile of the larvae was investigated. The results showed that protein content did not vary greatly on a DM basis, ranging between 44% (abattoir waste) and 39% (food waste and human faeces) (Table 5), while the combined amino acid component of the DM comprised 36 ± 0.6% (Table S1). That is well in line with data presented by Liland et al. (2017), who showed that the deviation in gross protein and actual protein is related to the nitrogen content in the chitin compounds in the larvae, giving a misleading gross protein value. No factor was found to correlate with the protein content of the larvae (Fig. S1). Despite the protein content of the larvae not varying by much, the size of the larvae/prepupae varied considerably (70–250 mg $larva^{-1}$), as discussed above (Table 3).

There were some differences in the amino acid profile of the larvae/prepupae reared in different substrates (Table 5). The content of the non-essential amino acids tyrosine and lysine varied by 40–50% between the different substrates, while there was no significant difference in the essential amino acids arginine, histidine and threonine. The variation in the other amino acids was smaller (±20%). On comparing the results of this study to average values found in other studies using swine and dairy manure as feedstocks (Kroeckel et al., 2012; St-Hilaire et al., 2007; Stamer, 2015; Zhang et al., 2007), some differences were found. In general, the literature values were higher than the average obtained for the substrates evaluated in this study (Table S1). The methionine content found here was 1.8% of the crude protein content, while the average of the above studies was 2.1% of the crude protein content. The prepupae grown on the poultry manure substrate had a methionine content of around 2% of the crude protein content, which is comparable to that found in other studies. However, the methionine content of the larvae reared on poultry manure only differed significantly from that of the larvae reared on abattoir waste-fruit & veg., poultry feed and fruit & vegetables. So although substrate type appears to have some effect on the amino acid profile of BSF larvae, this effect does not appear to be large, confirming findings in other

studies (Spranghers et al., 2016).

4.6. Fly larvae treatment for different waste fractions

Based on the findings of this study, the most suitable substrates in terms of biomass yield for BSFL composting are those that contain a large proportion of easily available carbon and a sufficiently high protein content to support larval development. Abattoir waste, abattoir - fruits & vegetables, food waste and human faeces are examples of substrates that provide good conditions for larval growth, and for which BSFL composting would be a good option. Substrates that contain a high proportion of easily available carbon, but a low content of nitrogen, do not support larval development and thus the efficiency of the process is reduced. Thus in this study, the BCR of the fruit & vegetables substrate was 4%, compared with 15% for abattoir waste. The total biomass, and hence the potential revenue from treating fruit & vegetables waste in a BSFL composting system, would thus be much smaller. As the development time for the larvae would be considerably longer, the efficiency would be even lower, resulting in quite expensive treatment for small revenue.

The BSFL composting process was not optimised in this study, and thus the efficiencies can be expected to increase for some of the more promising waste fractions, *e.g.* abattoir waste, food waste and human faeces (Table 3). One way of increasing the possible revenue in waste management operations is by mixing waste fractions that have a high protein content with fractions that are high in easily available carbon, so that the larvae to a higher degree can make use of available nutrients (as in the abattoir waste-fruit & veg. mixture in this study). The conversion into larval biomass of protein-poor fractions could be increased, and the treatment process time decreased, by addition of a protein-rich substrate, while higher utilisation of the available nutrients could be expected on combining a substrate rich in easily available carbon with a protein-rich substrate.

For any waste substrate, the total revenue from potential products would have to be evaluated based on the current demand and market value at the production location (Lohri et al., 2017). For example, it might be more profitable to anaerobically digest the waste prior to BSF treatment in locations where vehicle gas has a higher value than animal feed protein, whereas the opposite may be true in a different location (Lalander et al., 2018).

5. Conclusions

The main substrate properties affecting BSFL composting were found to be VS and protein content of the substrate. The BSFL were effectively reared on many waste streams, including food waste, human faeces and abattoir waste. However, the larvae did not grow particularly well on different sewage sludges, as their VS content was too low. Larval growth on fruit & vegetable waste was slower and biomass conversion ratio quite low compared with other substrates, but the prepupae grew large. The amino acid profile of the prepupae did not vary greatly, with only smaller variations in the amino acid profiles of the prepupae reared on different substrates. The protein content of larval biomass varied only slightly (39–44% of DM), while great variations were found in final larval weight. The larvae of BSF are robust and can feed on a variety of substrates, provided that the VS and protein contents are sufficiently high to support larval development. Abattoir waste, food waste, human faeces and a mixture of abattoir waste – fruits & vegetables are highly suitable substrates for BSFL composting, while pure fruit & vegetable waste and different sewage sludges are less suitable.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2018.10.017.

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Update

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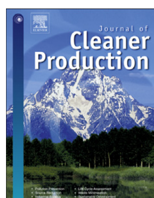
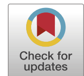


Corrigendum to “Effects of feedstock on larval development and process efficiency in waste treatment with black soldier fly (*Hermetia illucens*)” [J. Clean. Prod. 208 (2019) 211–219]



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The authors regret that there was a mistake in Equation (1) in the original article. The correct from of Equation (1) for the calculation of the percentage material reduction was:

$$\text{Mat. red.}_{DM} = \left(1 - \frac{\text{mat.out}_{DM}}{\text{sub.in}_{DM}}\right) \times 100, \qquad (1)$$

where $mat.out_{DM}$ and $sub.in_{DM}$ were the dry matter of the substrate fed and of the residue after the experiment, respectively.

The authors would like to apologise for any inconvenience caused.

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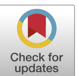

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ABSTRACT

Black soldier fly larvae (*Hermetia illucens;* BSFL) can convert organic wastes into a nutrient-rich biomass suitable in animal feed, which could be a way to achieve more sustainable production of food. However, little is known about how the diet fed to BSFL affects their nutritional value, especially their fatty acid composition. In this study, BSFL were fed 11 diets based on four different organic waste sources (mussels, bread, fish and food waste). Fatty acid and proximate composition (dry matter, crude fat, crude protein and ash) were analysed in the diets, in two-week-old larvae and substrate residues. Larval weight, survival and feed conversion were also recorded. The diet was found to affect all parameters investigated. Irrespective of diet, the larval fat consisted mainly of lauric acid and other saturated fatty acids and these were found to be synthesised by the larvae. However, both the fatty acid composition of the substrate, and the larval weight were found to affect the fatty acid profile of the larvae. In general, larvae with a higher weight contained a higher percentage of saturated fatty acids and a lower percentage of unsaturated fatty acids, such as eicosapentaenoic (EPA) and docosahexaenoic acid (DHA). It was concluded that the possibilities to tailor the fatty acid composition of the BSFL through the diet are limited; thus, the BSFL fat may not be suitable to replace fish oil, but has potential of inclusion in other food, feed and fuel products.
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1. Introduction

In the coming 30 years, the global population is estimated to reach 10 billion (UN, 2017). By then, global food production will need to produce sufficient food and nutrients for the increased population and also address the environmental impacts of food production, such as greenhouse gas emissions, loss of biodiversity and unsustainable use of water (Foley et al., 2011). One approach for achieving this is to use insects as feed and food (van Huis et al., 2013). Insects are associated with high feed conversion efficiency and lower emissions of greenhouse gases than conventional livestock, as well as a nutritional composition that makes them potentially suitable as food and animal feed (van Huis and Tomberlin, 2017). The larvae of the black soldier fly (BSF; *Hermetia illucens* (L.), Diptera: Stratiomyidae) have received great attention for their ability to convert organic waste into high-value biomass (van Huis et al., 2013).

Black soldier fly larvae (BSFL) are able to feed on a variety of organic materials (Tomberlin and Cammack, 2017). Examples include cow manure, fish offal, brewery by-products, restaurant waste and sewage sludge (Lalander et al., 2019; Meneguz et al., 2018; Spranghers et al., 2017; St-Hilaire et al., 2007a). Their nutritional composition can vary depending on the rearing substrate, but in general BSFL contain around 40% protein and 30% fat on a dry matter basis (Barragan-Fonseca et al., 2017). Because of their nutritional composition, BSFL have been used as an ingredient in feed for various animals such as poultry and fish (Schiavone et al., 2018; St-Hilaire et al., 2007b). However, under current legislation in Europe and the US, use of BSFL in animal feed is permitted mainly within aquaculture (Meneguz et al., 2018; Tomberlin and Cammack, 2017). Since the growing aquaculture industry has a currently unsustainable dependency on fish meal and fish oil (Tocher, 2015), BSFL could be a more sustainable alternative source of both protein and fat in feeds used in aquaculture.

Earlier studies have shown that BSFL meal can replace 50% of fish meal in the diet of Atlantic salmon (Lock et al., 2016) and 25% of fish meal in the diet of rainbow trout (St-Hilaire et al., 2007b), without negatively affecting growth of the fish. One prob-

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lem is that inclusion of BSFL in the feed decreases the content of α-linolenic acid (ALA; C18:3), eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6) in fish fillets (St-Hilaire et al., 2007b). Omega-3 (n-3) fatty acids, especially EPA and DHA, are essential for optimal growth and reproduction in both fish and shrimps (Tocher, 2015), and are also associated with health benefits for humans upon consumption (WHO, 2003). Therefore, lower concentrations of n-3 fatty acids in fish fed BSFL could be a problem for both producers and consumers.

In comparison with other insects, BSFL have been found to contain a high amount of fat, especially in the form of saturated fatty acids (SFA) (Ramos-Bueno et al., 2016). However, it may be possible to modify the nutritional composition of BSFL through the diet (Barragan-Fonseca et al., 2017; Meneguz et al., 2018; Spranghers et al., 2017). Furthermore, BSFL fed fish offal and algae have been found to incorporate significant amounts of EPA and DHA (Liland et al., 2017; St-Hilaire et al., 2007a). However, while findings in these earlier studies suggest that it is possible to modify the fatty acid composition of BSFL, it is not known which fatty acids the larvae produce by themselves, or to what extent the fatty acid composition of the diet influences the final fatty acid composition of the BSFL. Knowledge of these characteristics is of great importance if BSFL are to be grown on different waste substrates and used as an ingredient in animal feed. The aim of this study was thus to investigate how the properties of the diet affect the fatty acid and proximate composition of BSFL biomass, larval growth and feed utilisation.

2. Materials and methods

2.1. Materials

2.1.1. Black soldier fly larvae

Black soldier fly larvae from a colony continuously maintained since 2015 at the Department of Energy and Technology, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden, were used in the study.

2.1.2. Experimental diets

In Trial 1, four substrates (bread, food waste, fish and mussels) were used to create six diets. These diets contained: **bread**, coarsely mixed reclaimed bread obtained from the bread company Fazer (Uppsala, Sweden); **food waste**, homogenised household waste obtained from Eskilstuna waste treatment facility (Strängnäs Energi och Miljö AB; Eskilstuna, Sweden); **fish**, rainbow trout (*Onchorhynchus mykiss*) from the Department of Animal Nutrition and Management at SLU (Uppsala, Sweden) mixed to a 5:1 ratio (by wet weight) with wheat bran from Lantmännen Foder (Uppsala, Sweden); **fresh mussels**, crushed blue mussels (*Mytilus edulis*) from St. Anna mussel farm (Vattenbrukscentrum Öst; Inre Kärrö, Sweden) used upon arrival at the BSFL facility; **ensiled mussels**, crushed blue mussels ensiled with 3% formic acid and stored at room temperature (30 °C) for 2 weeks; and **rotten mussels**, crushed blue mussels stored at room temperature (30 °C) for 1 week.

In Trial 2, five diets were prepared from **bread and mussels** by mixing crushed blue mussels (*Mytilus edulis*) from Ecopelag (Värmdö, Sweden) with reclaimed bread received from the bread company Fazer (Uppsala, Sweden) in ratios where the mussels contributed 10, 20, 30, 40 and 50% of the total volatile solids content in the mixture.

2.1.3. Chemicals

For fatty acid analysis, the following chemicals were used: chloroform (VWR, CAS No. 67-66-3), methanol (Merck Millipore, CAS No. 67-56-1), sodium chloride (Merck Millipore, CAS No. 7647-14-5), sodium hydroxide (Merck Millipore, CAS No. 1310732), anhydrous methanol (Merck Millipore, CAS No. 67-56-1), 20% boron trifluoride-methanol complex in methanol (VWR, CAS No. 373-57-9) and hexane (Fisher Chemicals, CAS No. 110-54-3). Methyl 15-mehylheptanedecanoate (Larodan, Sweden) was used as internal standard in gas chromatography. Methyl laureate (Larodan, Sweden) and the fatty acid methyl ester mix GLC68D (Nu-Chek Prep Inc., Minnesota) were used to identify fatty acids through retention time in chromatograms. All fatty acid methyl esters used were of *cis*-configuration.

2.2. Experimental set-up

In total, 11 diets were evaluated in this study (Fig. 1). In Trial 1, the impact of bread, food waste, fish and fresh, ensiled and rotten mussels was investigated. In Trial 2, five diets with bread and mussels were investigated, in order to: (1) gain a better understanding of how different concentrations of EPA and DHA in the diet affect incorporation into larval fat; and (2) identify the ratio at which BSFL production on mussels is feasible in terms of technical process characteristics (separation of larvae from the substrate residues). Fatty acid and proximate analysis (dry matter, ash, crude fat and crude protein) was carried out on the diets and on the surviving BSFL and, in Trial 2, also on substrate residues (remains of the diet). The larvae were weighed and counted after the experiments.

In both trials, newly hatched larvae were raised on chicken feed for 5 d prior to being introduced to the diets. Before the start of the experiments, samples were collected of the 5-day-old larvae (triplicate) and the diets (singlets) and stored at −80 °C until further analysis. All experiments were carried out in triplicate at 28 °C for 14 d, a time period earlier found sufficient for development of BSF prepupae (on substrates suitable for the BSFL process) by Lalander et al. (2019). In Trial 1, the larvae fed bread, food waste and fish received 200 mg volatile solids per larva, with a larval density of 3–6 larvae cm^{-2} (Table S1). Due to differences in substrate properties, the larvae fed fresh, rotten and ensiled mussels received 1-2 g volatile solids per larva, and the larval density was 0.3 larva cm^{-2}. In Trial 2, all larvae received 300 mg volatile solids per larva, with a larval density of 2 larvae cm^{-2}. At the end of the experiments, the larvae were separated from the substrate residues by sieving, rinsed with water and dried on paper towels. The total weight was recorded for all surviving larvae and the substrate residues. Approximately 50 larvae were counted and their total weight was recorded in order to estimate the average larval weight. One sample of larvae and one sample of substrate residues (only in Trial 2) were collected in each of the three replicate experiments. These samples were stored at −80 °C until further analysis.

2.3. Chemical analysis

2.3.1. Lipid extraction and fatty acid analysis

Lipids were extracted using a modified version of the method described by Folch et al. (1957) followed by methylation according to Appelqvist (1968) and analysis by gas chromatography. A sample of 2 g was homogenised for 3 × 30 s in 50 mL chloroform:methanol (2:1, v/v) using an Ultra-Turrax T25 homogeniser (Janke and Kunkel, Germany). The sample was filtered, 12.5 mL 0.9% NaCl solution were added (giving a chloroform:methanol:water ratio of 8:4:3) and the mixture was transferred to a separating funnel. The lower phase (containing the lipids) was transferred to a round bottle flask. The chloroform and methanol were evaporated using a rotary evaporator (Büchi Labortechnik, Switzerland). From the resulting lipid extract, 5 mg were weighed into a centrifugation tube and 1 mg internal standard (methyl 15-methylheptadecanoate) and 2 mL

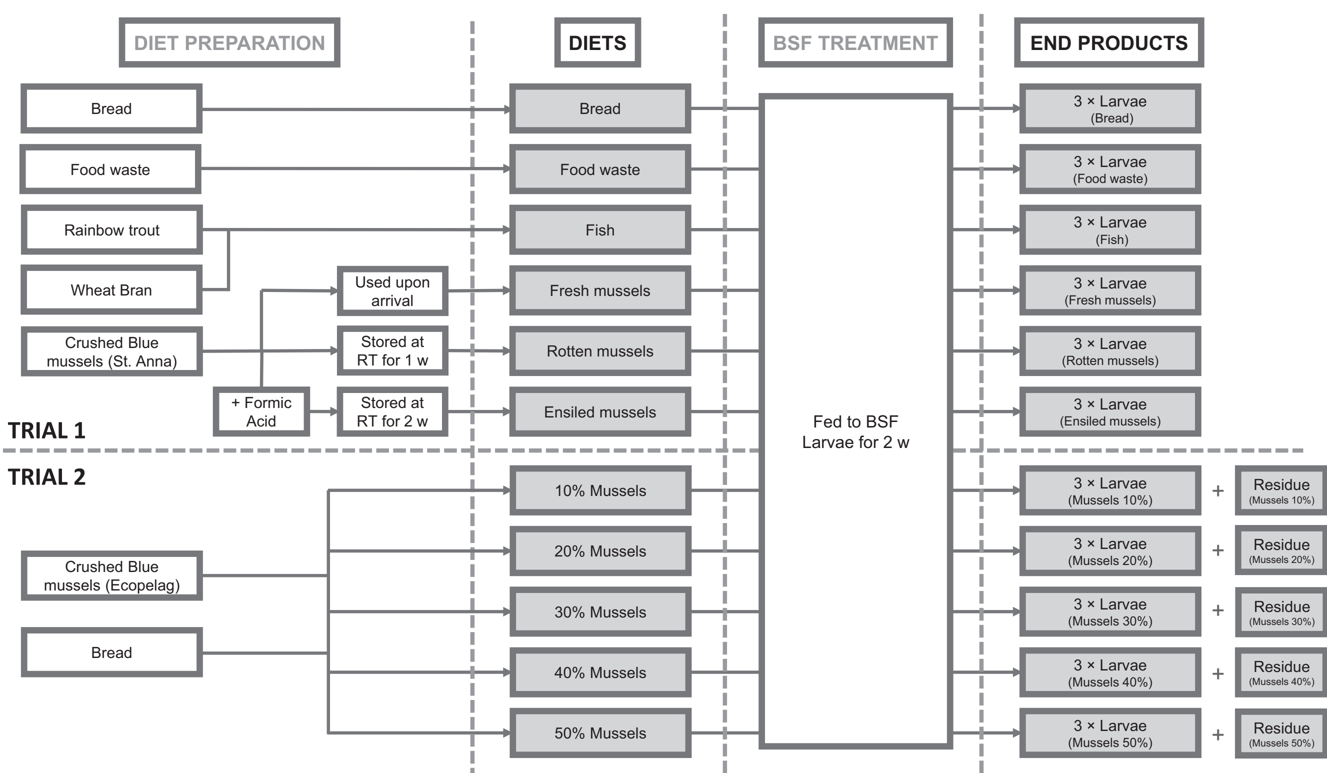

Fig. 1. Schematic illustration of the experimental set-up used for Trial 1 and Trial 2.

0.01 M NaOH in water-free methanol were added. Methylation was catalysed by 3 mL 20% BF3-methanol complex in methanol. To extract the fatty acid methyl esters, 2 mL 20% NaCl and 2 mL hexane were added, followed by centrifugation for 5 min at 500 xg in a Z383 K centrifuge (Hermle Labortechnik, Germany). The upper hexane phase (containing the fatty acid methyl esters) was removed and 1 µL was injected (split ratio 1:10) with an Agilent 7683 auto sampler (Agilent, California) onto a Agilent 6890 system with a flame ionisation detector (FID) attached (Agilent, California). Hydrogen was used as the carrier gas at a constant flow of 1 mL min^{-1} and separation was conducted on a SGE BPX70 capillary column (50 m × 0.22 mm × 0.25 µm; SGE/Trajan, Australia). The oven was maintained at 158 °C for 5 min, ramped up to 220 °C at 2 °C min^{-1} and held for 8 min. The temperature of the FID was 250 °C, with flow rates of hydrogen, oxygen and N_2 (make-up gas) of 40, 400 and 50 mL min^{-1}. Fatty acids were identified from retention times of standards (see Section 2.1.3). The weight of each fatty acid was estimated using the internal standard, and the fatty acid composition was expressed as percentage of identified fatty acids.

2.3.2. Proximate analysis

Proximate analysis was carried out at the Department of Animal Nutrition and Management, SLU (Uppsala, Sweden). All samples were freeze-dried for 72 h and milled before further analysis. Pre-dried samples were dried at 103 °C for 16 h (Trial 1) or 80 °C for 48 h (Trial 2) to determine the dry matter content, followed by incineration at 550 °C for 3 h to determine the ash content. Total nitrogen was measured using the Kjeldahl method in accordance with NMKL (1976). To estimate the protein content, a conversion factor of 4.76 was used, as suggested by Janssen et al. (2017) for more accurate estimation of the protein content in BSFL. Crude fat content was determined by hydrolysis in hydrochloric acid followed by extraction in light petroleum, as described by European Commission (1998).

2.3.3. Larval survival and process efficiency

The larval survival ratio (SR) was calculated as:

$$SR = \left(\frac{Lv_{Out}}{Lv_{In}}\right) \times 100 \quad (1)$$

where Lv is the total number of larvae at the beginning (*In*) and end of the experiment (*Out*).

The waste-to-biomass conversion ratio (BCR) was calculated on a wet weight (*WW*) and a volatile solids (*VS*) basis. The nitrogen conversion ratio (NCR) was calculated similarly, but on a total nitrogen (*N*) basis. The conversion ratio was calculated as:

$$BCR_{WW/VS} \quad \text{or} \quad NCR = \left(\frac{Lv_{WW/VS/N}}{Sub_{WW/VS/N}}\right) \times 100 \quad (2)$$

where *WW/VS/N* is the total wet weight (*WW*), weight of volatile solids (*VS*) and nitrogen (*N*) content of the larval biomass (Lv) at the end of experiments and in the initial substrate (Sub).

2.3.4. Fatty acid production value and retention

The fatty acid production value (FAPV) was calculated as described by Liland et al. (2017) for each fatty acid in the larvae within Trial 2 as:

$$FAPV = \frac{FA_{Lv}}{FA_{Sub} - FA_{Res}} \quad (3)$$

where FA_{Lv}, FA_{Sub} and FA_{Res} are the total mass of a specific fatty acid in the total larval biomass (*Lv*), the initial substrate (*Sub*) and the substrate residues (*Res*), respectively.

To estimate how much of a specific fatty acid was transferred from the substrate to the larvae and how much was left in the residues, retention values in larvae (Ret_{Lv}) and substrate residues (Ret_{Res}) were calculated as described by Liland et al. (2017) :

$$Ret_{Lv/Res} = \frac{FA_{Lv/Res}}{FA_{Sub}} \times 100 \quad (4)$$

where FA is the total mass of a specific fatty acid in the total larval biomass (*Lv*), the substrate residues and the initial substrate (*Sub*).

2.4. Statistical analysis

Minitab (Minitab Inc., Pennsylvania) was used to create linear regression models between larval weight and the fatty acid composition of substrates and larvae. It was also used for one-way analysis of variance (ANOVA) to identify statistically significant differences between the larvae. The level of significance was set at $p < 0.05$. A Tukey *post-hoc* test was performed in cases with statistically significant differences. None of the R^2-values presented are adjusted. Microsoft Excel 2013 (Microsoft, Washington) was used for creating graphical representations of the data.

3. Results

3.1. Larval growth and feed conversion

In Trial 1, the highest larval weight (230 mg) and highest survival rate (89%) were observed for the BSFL fed fresh mussels (Table 1). The highest biomass conversion ratio (volatile solids basis; 35%) and nitrogen conversion ratio (66%) were achieved for the BSFL fed food waste. The lowest larval weight (25 mg), survival (11%) and conversion ratio (<0.1%) were observed for the BSFL fed ensiled mussels. Low survival rates were also found for the larvae fed fish (18%) and rotten mussels (55%). Differences were also observed in the colour of the larvae: the larvae fed with bread and fish had a lighter colour, while the larvae fed with food waste and mussels were darker, indicating that they had started developing into prepupae. In Trial 2, the highest biomass conversion ratios (11–13%) and nitrogen conversion ratios (26–32%) were found for the BSFL fed a diet with $\leq$20% mussels. No significant differences were found in larval weight or survival rate, but numerical differences were observed: BSFL fed the diet with 50% mussels were found to have the lowest larval weight (130 mg) and survival rate (54%), in addition to the lowest biomass and nitrogen conversion ratio. The larvae in Trial 2 had lower weight and survival rate, but higher conversion ratio than those fed only fresh mussels. Compared with BSFL fed only bread, the larval weights and survival were higher or similar in Trial 2, while the conversion ratio was generally lower.

3.2. Proximate composition

In Trial 1, variations were observed especially in the crude fat (11–58%) and ash content of BSFL (4–33%) fed the different diets (Table 2). The extremes were represented by the larvae fed bread and ensiled mussels. In this trial, dry matter content ranged between 27 and 36% and crude protein content between 28 and

Table 1
Final larval weight, survival rate, bio-conversion ratio and nitrogen conversion ratio of the larvae in Trial 1 and Trial 2. Values shown are mean ± standard deviation (SD; n = 3). Different letters represent significant differences column-wise within the trials (Trial 1: lower-case, Trial 2: upper-case), with the level of significance set to p < 0.05.

	Larval weight (mg)	Survival rate (%)	BCR_{WW} (%)	BCR_{VS} (%)	NCR (%)
Trial 1					
Bread	$137 \pm 7^{b,c}$	69.8 ± 9.8^{b}	24.1 ± 3.3^{a}	13.4 ± 1.8^{b}	39.5 ± 7.2^{b}
Fish	$89 \pm 18^{c,d}$	18.4 ± 2.6^{c}	3.2 ± 0.1^{c}	2.4 ± 0.2^{c}	2.9 ± 0.1^{c}
Food waste	$191 \pm 19^{a,b}$	89.1 ± 6.0^{a}	18.9 ± 2.4^{b}	34.8 ± 3.8^{a}	66.4 ± 6.5^{a}
Fresh mussels	235 ± 15^{a}	89.3 ± 6.8^{a}	0.8 ± 0.1^{c}	3.5 ± 0.3^{c}	2.9 ± 0.2^{c}
Ensiled mussels[1]	25^{d}	11.0 ± 4.5^{c}	$<0.1^{c}$	$<0.1^{c}$	–
Rotten mussels	106 ± 29^{c}	55.1 ± 11.2^{b}	0.2 ± 0.0^{c}	1.0 ± 0.0^{c}	0.6 ± 0.0^{c}
Trial 2					
Bread and mussels (10%)	133 ± 39	86.2 ± 2.1	11.5 ± 3.6^{A}	13.0 ± 3.4	31.7 ± 8.4^{A}
Bread and mussels (20%)	181 ± 10	82.1 ± 13.2	10.5 ± 1.4^{A}	13.0 ± 1.9	$25.5 \pm 3.8^{A,B}$
Bread and mussels (30%)	168 ± 2	68.0 ± 13.2	$7.4 \pm 1.1^{A,B}$	10.4 ± 1.7	$16.5 \pm 3.1^{B,C}$
Bread and mussels (40%)	138 ± 21	77.1 ± 25.2	$5.7 \pm 2.1^{A,B}$	8.8 ± 3.4	$12.9 \pm 5.3^{B,C}$
Bread and mussels (50%)	131 ± 20	54.3 ± 34.4	3.6 ± 2.6^{B}	6.1 ± 4.0	8.2 ± 6.1^{C}

BCR_{WW} = bio-conversion ratio on wet weight basis; BCR_{VS} = bio-conversion ratio on volatile solids basis; NCR = nitrogen conversion ratio.
[1] Due to poor growth, the amount of material was not sufficient for analysis of nitrogen. Larval weight was estimated on a small sample (<10 larvae).

Table 2
Proximate composition of larvae in Trial 1 and Trial 2. All values shown are mean ± standard deviation (SD; n = 3) for percentage on a wet weight ($\%_{WW}$) or dry matter basis ($\%_{DM}$). Different letters represent significant differences column-wise within the trials (Trial 1: lower-case, Trial 2: upper-case), with the level of significance set to $p < 0.05$.

	Dry matter ($\%_{WW}$)	Crude fat ($\%_{DM}$)	Crude protein (factor 4.76 ($\%_{DM}$)	Crude protein (factor 6.25) ($\%_{DM}$)	Ash ($\%_{DM}$)
5-day larvae	32.7 ± 5.0	9.7 ± 3.8	34.1 ± 2.6	44.7 ± 3.4	15.9 ± 3.1
Trial 1					
Bread	35.5 ± 1.1^{a}	57.8 ± 1.5^{a}	$29.8 \pm 2.0^{b,c}$	$39.2 \pm 2.6^{b,c}$	3.9 ± 0.3^{d}
Fish	27.0 ± 2.1^{b}	46.7 ± 1.5^{b}	40.1 ± 1.7^{a}	52.6 ± 2.2^{a}	5.7 ± 0.3^{d}
Food waste	$33.0 \pm 1.3^{a,b}$	40.7 ± 2.3^{c}	27.9 ± 0.2^{c}	36.6 ± 0.3^{c}	16.3 ± 1.8^{c}
Fresh mussels	$31.3 \pm 0.8^{a,b}$	33.1 ± 1.2^{d}	34.0 ± 1.1^{b}	44.6 ± 1.4^{b}	$18.7 \pm 1.4^{b,c}$
Ensiled mussels[1]	$27.3^{a,b}$	11.2^{e}	–	–	33.0^{a}
Rotten mussels	27.5 ± 0.4^{b}	29.7 ± 0.3^{d}	32.2 ± 0.3^{b}	42.3 ± 0.4^{b}	22.6 ± 1.2^{b}
Trial 2					
Bread and mussels (10%)	40.5 ± 1.1^{A}	20.4 ± 0.7^{A}	25.0 ± 0.8^{C}	32.8 ± 1.0^{C}	30.9 ± 3.2
Bread and mussels (20%)	35.7 ± 0.8^{B}	19.6 ± 1.1^{A}	$26.1 \pm 0.2^{B,C}$	$34.2 \pm 0.3^{B,C}$	31.1 ± 0.5
Bread and mussels (30%)	34.2 ± 1.4^{B}	$17.9 \pm 0.3^{A,B}$	$25.8 \pm 0.5^{B,C}$	$33.8 \pm 0.7^{B,C}$	30.7 ± 1.1
Bread and mussels (40%)	33.2 ± 1.6^{B}	$17.9 \pm 0.8^{A,B}$	$27.5 \pm 0.4^{A,B}$	$36.1 \pm 0.5^{A,B}$	30.9 ± 1.4
Bread and mussels (50%)	32.6 ± 1.1^{B}	16.1 ± 2.3^{B}	28.8 ± 1.8^{A}	37.9 ± 2.3^{A}	29.6 ± 2.7

[1] Due to poor growth, the amount of material was only sufficient for analysis of dry matter, crude fat and ash, using a single sample.

40% (conversion factor 4.76), with the larvae fed fish having the highest protein content (40%). In Trial 2, significant differences ($p < 0.05$) were found for all parameters except ash content, albeit smaller than those found in Trial 1. Compared with larvae fed only bread and fresh mussels in Trial 1, the crude fat (16–20%) and crude protein content (25–29%; conversion factor 4.76) were in general lower in Trial 2, while the dry matter (33–41%) and ash content (30–31%) were higher. The larvae fed the diet with 10% mussels contained the highest percentage of dry matter and crude fat and the lowest percentage of crude protein in Trial 2.

3.3. Fatty acid composition

Most of the larvae contained a high percentage of SFA (up to 76%), followed by monounsaturated fatty acids (MUFA; up to 32%) and polyunsaturated fatty acids (PUFA; up to 23%) (Table 3). Of the SFAs, lauric acid (C12:0) was a major constituent (up to 52%). In addition, palmitic acid (C16:0) and oleic acid (C18:1 n-9) made up a high percentage of the fatty acids identified in the larvae (12–22% and 10–25%, respectively). Fatty acids with a carbon chain length of 12–18 carbons (C12:0 to C18:3) were found in all larvae, irrespective of diet. Arachidonic acid (C20:4) and EPA (C20:5) were found in all larvae except those fed bread. DHA (C22:6) was not found in the larvae fed bread or those fed food waste.

3.3.1. Factors impacting larval fatty acid composition

The results from Trial 1 and 2 were combined to investigate possible predictors of larval fatty acid composition. Larval weight was found to be positively correlated to the percentage of lauric acid (C12:0; $R^2 = 0.8$) and total SFA ($R^2 = 0.7$) in the larvae, and negatively correlated to total MUFA ($R^2 = 0.5$) and PUFA ($R^2 = 0.7$) (Fig. 2).

The larval weight and the concentration of fatty acids in the diets were combined in the following model:

$$FA\%_{Lv} = a + b \times LW + c \times FA\%_{Sub} \quad (5)$$

where FA% is the percentage of a specific fatty acid in the larvae (*Lv*) and the corresponding substrate (*Sub*), and LW is the weight of the larvae. This model had a better fit to the measured data for content of EPA (C20:5; $R^2 = 0.9$), DHA (C22:6; $R^2 = 0.8$) and total n-3 PUFA ($R^2 = 0.8$) in the larvae (Fig. 3). The percentage of these three fatty acids in the larvae was negatively correlated to larval weight and positively correlated to their percentage in the diet (coefficient $c > 0$).

3.3.2. Fatty acid production and retention values

Fatty acid production values (FAPV) and retention values were calculated for larvae and residues in Trial 2. A FAPV above 1.0 indicates net production of a particular fatty acid, which was found for four SFA and three MUFA, but no PUFA (Table S4). In the case of lauric acid (C12:0), the FAPV was above 100, and in some cases above 1000. However, for vaccenic acid (C18:1 n-7) the FAPV was above 1.0 only for the larvae fed the diet with 20–30% inclusion of mussels.

According to the larval retention values, up to 40% of the initial EPA (C20:5) and 10% of the DHA (C22:6) in the substrate were transferred to the larval fat (Table S5). The highest retention was found for larvae fed the diet with 10% mussels. However, when calculated on a weight basis, the larvae fed the diet with 30–50% mussels contained the greatest amount of EPA and DHA. The residue retention values indicated that 20–60% of the EPA and DHA remained in the substrate residues. A higher percentage of EPA and DHA remained in the substrates with higher inclusion of mussels.

Table 3
Content of selected fatty acids[1] in the larvae in Trial 1 and Trial 2. Each fatty acid (C12:0-C22:6) and the fatty acid totals (SFA, MUFA, PUFA, n-3 PUFA) are presented as percentage of identified fatty acids. Identified fatty acids are presented on a dry matter basis (%DM). All values shown are mean ± standard deviation (SD; n = 3). Different letters represent significant differences row-wise within the trials (Trial 1: lower-case, Trial 2: upper-case), with the level of significance set to $p < 0.05$.

	Trial 1 – Different substrates							Trial 2 - Bread and mussels				
	5 d larvae	Bread	Fish	Food waste	Fresh mussels	Ensiled mussels	Rotten mussels	10%	20%	30%	40%	50%
Identified fatty acids ($\%_{DM}$)	6.7 ± 2.9^{d}	54.8 ± 5.6^{a}	39.3 ± 1.5^{b}	34.5 ± 1.2^{b}	26.5 ± 0.9^{c}	7.0 ± 0.7^{d}	21.5 ± 2.6^{c}	15.5 ± 1.0	15.5 ± 0.6	14.1 ± 0.9	14.1 ± 0.3	13.8 ± 1.3
C12:0	7.5 ± 1.9^{c}	51.8 ± 3.3^{a}	28.6 ± 2.1^{b}	39.9 ± 5.1^{b}	52.1 ± 1.8^{a}	13.4 ± 3.9^{c}	32.3 ± 7.6^{b}	47.4 ± 0.8^{A}	47.6 ± 1.1^{A}	43.6 ± 2.6^{A}	42.0 ± 4.3^{A}	35.3 ± 1.6^{B}
C14:0	2.3 ± 0.4^{d}	9.5 ± 0.9^{a}	6.1 ± 0.4^{c}	6.7 ± 0.3^{b,c}	8.0 ± 0.5^{b}	5.8 ± 0.4^{c}	10.1 ± 0.1^{a}	8.7 ± 0.2^{A}	8.5 ± 0.1^{A,B}	8.0 ± 0.2^{B,C}	7.8 ± 0.4^{C}	6.8 ± 0.3^{D}
C14:1 (n-5)	0.0 ± 0.0^{d}	0.2 ± 0.0^{c}	0.2 ± 0.0^{c}	0.2 ± 0.0^{c}	0.3 ± 0.0^{b}	0.4 ± 0.1^{c}	0.6 ± 0.0^{a}	0.2 ± 0.0^{B}	0.2 ± 0.0^{B}	0.2 ± 0.0^{A,B}	0.3 ± 0.0^{A}	0.2 ± 0.0^{A,B}
C16:0	19.2 ± 1.9^{a,b}	12.7 ± 0.8^{c,d}	12.6 ± 0.4^{d}	16.3 ± 1.0^{b,c}	11.9 ± 1.3^{d}	21.9 ± 0.6^{a}	19.8 ± 2.1^{a,b}	13.9 ± 0.4^{B}	14.3 ± 0.4^{B}	14.7 ± 0.7^{A,B}	14.6 ± 0.9^{A,B}	15.8 ± 0.3^{A}
C16:1 (n-7)	0.8 ± 0.1^{f}	2.8 ± 0.1^{e}	4.8 ± 0.5^{d}	2.6 ± 0.3^{e}	6.9 ± 0.8^{c}	14.1 ± 0.4^{a}	9.8 ± 1.2^{b}	3.1 ± 0.2^{D}	3.8 ± 0.1^{C,D}	4.6 ± 0.3^{B,C}	5.2 ± 0.4^{A,B}	5.9 ± 0.7^{A}
C18:0	6.9 ± 0.6^{a}	1.5 ± 0.3^{d}	2.2 ± 0.2^{c,d}	2.1 ± 0.6^{c,d}	1.6 ± 0.3^{d}	4.0 ± 0.3^{b}	3.3 ± 0.6^{b,c}	2.7 ± 0.2	2.9 ± 0.2	2.7 ± 0.2	2.8 ± 0.4	3.1 ± 0.3
C18:1 (n-9)	26.6 ± 3.4^{a}	12.0 ± 1.2^{c}	25.1 ± 0.7^{a}	19.1 ± 2.1^{b}	10.3 ± 2.3^{c}	14.0 ± 0.4^{b,c}	12.9 ± 2.1^{c}	13.3 ± 0.4^{A,B}	11.7 ± 0.3^{B}	12.3 ± 0.6^{A,B}	13.2 ± 1.2^{A,B}	14.0 ± 1.0^{A}
C18:1 (n-7)	1.1 ± 0.1^{b}	0.1 ± 0.1^{c}	1.1 ± 0.1^{b}	0.4 ± 0.1^{c}	1.3 ± 0.3^{b}	2.2 ± 0.2^{a}	1.5 ± 0.2^{b}	0.8 ± 0.0^{B}	1.2 ± 0.1^{A}	1.2 ± 0.1^{A}	1.2 ± 0.2^{A}	1.5 ± 0.2^{A}
C18:2 (n-6)	31.4 ± 1.4^{a}	7.7 ± 0.3^{d}	12.5 ± 0.3^{b}	9.9 ± 0.8^{c}	2.6 ± 0.3^{e}	4.5 ± 0.4^{e}	4.2 ± 0.7^{e}	5.6 ± 0.4^{A,B}	4.5 ± 0.0^{C}	4.8 ± 0.1^{B,C}	4.5 ± 0.5^{C}	5.9 ± 0.2^{A}
C18:3 (n-3)	3.6 ± 0.3^{a}	1.6 ± 0.1^{b}	3.4 ± 0.1^{a}	1.8 ± 0.2^{b}	1.3 ± 0.2^{b}	3.6 ± 0.6^{a}	1.1 ± 0.1^{b}	2.6 ± 0.1^{B,C}	2.2 ± 0.1^{C}	2.7 ± 0.1^{B}	2.6 ± 0.2^{B}	3.4 ± 0.1^{A}
C20:4 (n-6)	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.1 ± 0.0^{b,c}	0.2 ± 0.0^{b}	0.2 ± 0.0^{b,c}	1.3 ± 0.2^{a}	0.1 ± 0.0^{b,c}	0.1 ± 0.0^{D}	0.1 ± 0.0^{C,D}	0.2 ± 0.0^{B,C}	0.3 ± 0.1^{B}	0.4 ± 0.0^{A}
C20:5 (n-3)	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	1.7 ± 0.1^{b}	0.5 ± 0.0^{c}	2.0 ± 0.2^{b}	8.2 ± 1.0^{a}	1.9 ± 0.2^{b}	0.9 ± 0.0^{C}	1.7 ± 0.2^{C}	3.0 ± 0.4^{B}	3.5 ± 0.7^{B}	4.8 ± 0.5^{A}
C22:6 (n-3)	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.7 ± 0.0^{b}	0.0 ± 0.0^{b}	0.5 ± 0.0^{b}	4.5 ± 0.9^{a}	0.3 ± 0.0^{b}	0.3 ± 0.0^{D}	0.5 ± 0.1^{C,D}	0.9 ± 0.1^{B,C}	1.1 ± 0.3^{B}	1.7 ± 0.2^{A}
ΣSFA	36.2 ± 4.6^{d}	75.6 ± 1.4^{a}	49.6 ± 1.6^{c}	65.2 ± 3.7^{b}	73.7 ± 1.6^{a,b}	45.3 ± 3.5^{c,d}	65.6 ± 4.7^{b}	72.8 ± 0.8^{A}	73.5 ± 0.6^{A}	69.2 ± 1.8^{A,B}	67.4 ± 3.5^{B}	61.3 ± 1.2^{C}
ΣMUFA	28.7 ± 3.4^{a,b}	15.1 ± 1.4^{d}	31.8 ± 1.2^{a}	22.4 ± 2.6^{b,c}	19.5 ± 1.4^{c,d}	32.1 ± 0.4^{a}	26.2 ± 3.7^{a,b}	17.6 ± 0.3^{C}	17.2 ± 0.4^{C}	18.8 ± 1.0^{B,C}	20.3 ± 1.8^{A,B}	22.2 ± 0.5^{A}
ΣPUFA (all)	35.0 ± 1.6^{a}	9.3 ± 0.2^{c,d}	18.7 ± 0.4^{b}	12.4 ± 1.1^{c}	6.8 ± 0.3^{d}	22.6 ± 3.1^{b}	8.2 ± 1.0^{d}	9.6 ± 0.5$^{B;C}$	9.3 ± 0.2^{C}	12.0 ± 0.8^{B}	12.2 ± 1.8^{B}	16.6 ± 1.0^{A}
ΣPUFA (n-3)	3.6 ± 0.3^{b,c}	1.6 ± 0.1^{c}	5.9 ± 0.2^{b}	2.3 ± 0.3^{c}	3.8 ± 0.3^{b,c}	16.5 ± 2.5^{a}	3.4 ± 0.3^{b,c}	3.8 ± 0.1^{C}	4.4 ± 0.2^{C}	6.7 ± 0.6^{B}	7.2 ± 1.1^{B}	9.9 ± 0.8^{A}

ΣSFA = sum of all saturated fatty acids; ΣMUFA = sum of all monounsaturated fatty acids; ΣPUFA (all) = sum of all polyunsaturated fatty acids; ΣPUFA (n-3) = sum of all omega-3 polyunsaturated fatty acids.
[1] The content of C20:0, C20:1, C20:2, C20:3, C22:0, C22:1, C24:0 and C24:1 is not presented due to low (<0.5%) concentrations.

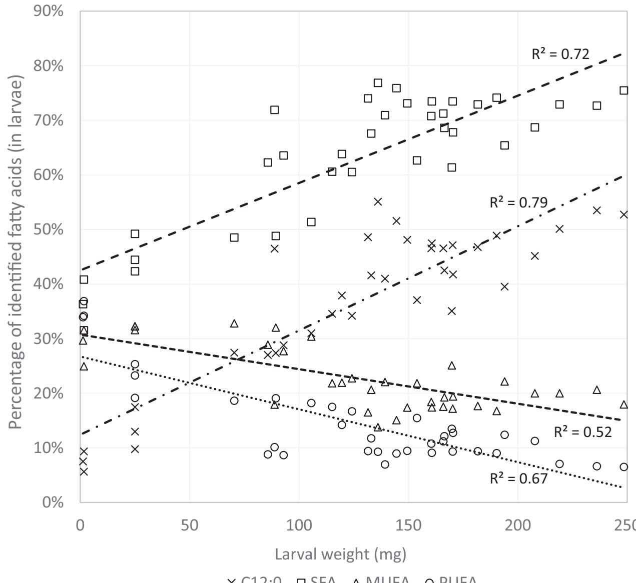

Fig. 2. Graphical representation of the percentage of lauric acid (C12:0) and total SFA, MUFA and PUFA in the larvae, in relation to larval weight. The model for each displayed component displayed along with the model fit (R^2).

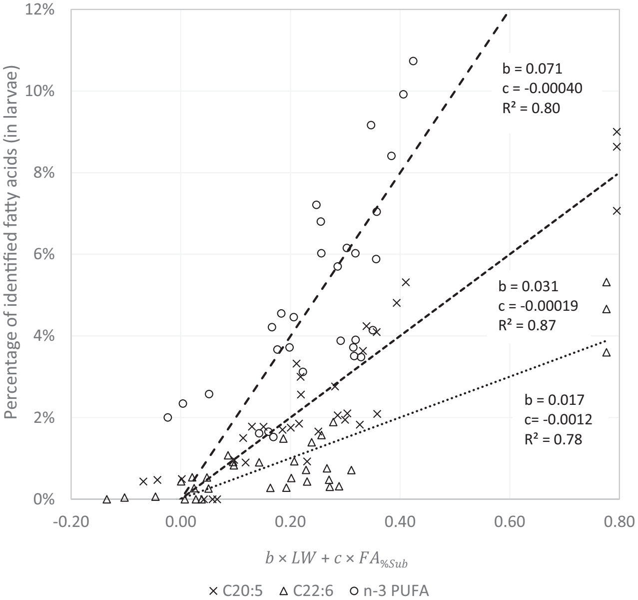

Fig. 3. Graphical representation of percentage of EPA (C20:5), DHA (C22:6) and total n-3 PUFA in the larvae, in relation to predicted percentages (of the same fatty acids in the larvae) from the model described in Eq. (5) based on larval weight (LW) and percentage of the specific fatty acid in the substrate ($FA\%_{Sub}$). Model parameters (constants b and c; and model fit through R^2) for each displayed fat component presented.

4. Discussion

4.1. Main fatty acid components in the larvae

Irrespective of diet, SFA was found to be the main group of fatty acids in all larvae (Table 3) the single most common fatty acid was lauric acid (C12:0), followed by palmitic (C16:0) and oleic acid (C18:1 n-9). These results are in line with findings in several earlier studies (Barroso et al., 2017; Liland et al., 2017; Meneguz et al., 2018; Spranghers et al., 2017; St-Hilaire et al., 2007a). In the studies by Barroso et al. (2017) and Liland et al. (2017), linoleic acid (C18:2) was also found in high percentages (12–24%) in larvae fed diets of fish meal and chicken feed, or wheat and brown algae. This is slightly higher than the levels found in this study (3–13%). There were great similarities between the fatty acid composition of the larvae and the substrate: palmitic, oleic and linoleic acid were some of the most common fatty acids found in the substrates (range 8–32%, 6–42% and 4–36%, respectively; Table S2). These are some of the most commonly found fatty acids in plant and animal tissues (Tvrzicka et al., 2011). Therefore, the chances of excluding these fatty acids from the larval diet are low, and thus it seems as though the main fatty acid components in BSFL will be very similar, irrespective of the diet composition.

Lauric acid (C12:0) was present at high levels (13–52%) in all larvae, but in very limited amounts in the diets (0–1%; Table S2). This strongly indicates that it is synthesised by the larvae, as suggested by Spranghers et al. (2017). Those authors also suggest that the larvae synthesise lauric acid from the carbohydrates in the substrate. This would explain why BSFL grown on bread (79% carbohydrates; Table S2) contained the highest levels of lauric acid (28% on dry matter basis). A positive correlation was found between larval weight and percentage of lauric acid (C12:0) and total SFA in the larvae (Fig. 2), suggesting that these fatty acids accumulate most as the larvae gain weight. Similar trends were observed by Liu et al. (2017), who found that BSFL in later stages of development contained higher percentages of SFA and lauric acid. A high content of SFA has been demonstrated to differentiate BSFL from other insect larvae (Ramos-Bueno et al., 2016). Since adult BSF do not feed (Tomberlin and Sheppard, 2002), the fatty acids in the larvae are most likely a way to store energy for this later life stage. The reason for storing energy in the form of SFA in particular could be because these fatty acids are less prone to oxidation than unsaturated fatty acids, as suggested by Ushakova et al. (2016).

Besides lauric acid (C12:0), the FAPVs obtained in this study also indicated that six other fatty acids (C14:0, C14:1, C16:0, C16:1, C18:0 and C18:1 n-7) are synthesised by BSFL (Table S4). While Liland et al. (2017) did not delve into specific fatty acids, the FAPVs they present indicate that SFAs, and to some extent MUFAs, are synthesised by BSFL. Most insect species have been found to synthesise myristic (C14:0), palmitic (C16:0) and stearic acid (C18:0) *de novo*, and possess the enzymes required for production of myristoleic (C14:1), palmitoleic (C16:1) and oleic acid (C18:1 n-9) (Stanley-Samuelson et al., 1988). It is therefore surprising that the FAPVs obtained indicated that vaccenic acid (C18:1 n-7), and not oleic acid (C18:1 n-9), is produced by BSFL. The enzyme Δ11 desaturase, which enables production of vaccenic acid (C18:1 n-7), has only been found in a few insect species (Stanley-Samuelson et al., 1988), which makes it less likely that BSFL are able to produce this fatty acid. It is possible that the levels of vaccenic acid found in the larvae in Trial 2 (0.8–1.5%) were too low to allow accurate estimates of whether this fatty acid is produced by BSFL. However, this hypothesis needs further testing.

4.2. Polyunsaturated fatty acids

The FAPVs do not suggest that the BSFL are able to synthesise PUFA (Table S4), a conclusion also drawn by Liland et al. (2017). Therefore, linoleic acid (C18:2) and ALA (C18:3), as well as EPA (C20:5) and DHA (C22:6), found in larvae most likely originated from the substrate. To date, the enzymes required for production of linoleic acid and ALA (the precursor for EPA and DHA) have been found mainly in plants and marine algae (Tvrzicka et al., 2011). Therefore it seems unlikely that BSFL can produce these fatty acids. Some insect species, e.g. crickets and cockroaches, have been found capable of producing linoleic acid *de novo* (Stanley-Samuelson et al., 1988). However, these species are not very closely related

to BSF (Diptera), since they are of different taxonomic orders (Blattodea and Orthoptera).

One difference in the larval fatty acid composition in this study was the high percentages of EPA (C20:5) and DHA (C22:6) (Table 3). The larvae fed ensiled mussels contained 8% EPA and 5% DHA, which is considerably higher than the 2% EPA and 2% DHA reported previously (Barroso et al., 2017; Liland et al., 2017; St-Hilaire et al., 2007a). According to the linear regression model LW/FA (Eq. 5; Fig. 3), the percentage of EPA and DHA increased in the larvae when they represented a higher percentage of the total fatty acids in the diet, while it decreased as the larvae grew. Similar trends have been found by others, e.g. Liland et al. (2017) found a positive correlation between the percentage of EPA in total fatty acids in the larvae and in the diet, while Liu et al. (2017) found a decrease in unsaturated fatty acids over time as BSFL grew older. The decrease in EPA and DHA is most likely because lauric acid and SFA increase at a higher rate as BSFL grow.

The larvae that incorporated the highest percentage of EPA (40%) and DHA (10%) were those fed the diet with 10% mussels (Table S5). When a higher percentage of mussels was mixed into the diet, the larval retention decreased and most of these fatty acids remained in the residues. This suggests that production of BSFL with high levels of EPA and DHA would result in less efficient utilisation and higher waste of these fatty acids. There seems to be a difference in retention between EPA and DHA. It is possible that there are mechanisms in BSFL that favour incorporation of EPA over DHA, as suggested by Barroso et al. (2017).

4.3. Other nutritional components of the larvae

Considerable differences in crude fat, crude protein and ash were observed between the BSFL in this study. This is in line with earlier findings by Meneguz et al. (2018), but contradicts findings by Lalander et al. (2019) and Spranghers et al. (2017) of only minor differences in the protein content of BSFL fed different substrates. However, one important difference between these studies and this study is the larval stage of the larvae analysed. Their larvae were collected in the prepupal stage, whereas all larvae in this study were analysed after two weeks. Since larval development time is affected by parameters such as the substrate (Lalander et al., 2019) and feeding rate (Diener et al., 2009), the larvae in this study were most likely at different larval stages. Differences were observed in the colour – from light to dark – of the larvae after two weeks, especially in Trial 1. This indicates that the larvae fed with some substrates had reached the prepupal stage, while others had not. Significant differences have previously been found in crude fat, crude protein and ash content of BSFL at different larval stages (Liu et al., 2017).

The crude fat content seems to be closely linked to the percentage of "identified fatty acids" in the larvae. This suggests that the fraction termed "crude fat" in the larvae mainly consists of fatty acids. Besides stage of larval development, it also seems that the carbohydrate content of the diet impacts the crude fat content of the larvae. Spranghers et al. (2017) found a strong correlation between the crude fat of prepupae and non-fibrous carbohydrate content in the substrate. This could explain why the larvae fed bread were so high in fat. Moreover, Li et al. (2015) found increasing amount of lipids in BSFL on adding increasing levels of glucose and xylose to the diet.

The larvae in this study were found to contain up to 33% of ash on a dry matter basis (Table 2), which is much higher than the level reported previously (Liland et al., 2017; Meneguz et al., 2018; Spranghers et al., 2017). Spranghers et al. (2017) found a strong correlation between the ash content of the substrate and BSF prepupae. Therefore it is likely that the high levels of ash found in many larvae are explained by the high levels of ash in the diets with mussels (26–84%; Table S2). No mussel shells were observed in the intestine of the larvae, but it is still possible that small fractions of shells were included in the analysis of ash content.

4.4. Implications for future use of larval fat

In this study two different factors were found to affect the fatty acid composition of the BSFL: the fatty acid composition of the substrate and the larval weight. Both of these two factors can be linked to the substrate, since the larval growth is affected by substrate properties (Lalander et al. 2019). This opens up for the possibility to engineer the fatty acid profile of the BSFL, by substrate selection and BSFL harvesting time.

Depending on the substrate, most larvae in this study contained EPA (C20:5) and DHA (C22:6) at different levels, fatty acids that are of great interest for aquaculture. In theory it seems possible to design the fat profile of BSFL to contain a higher percentage of these n-3 fatty acids by altering the fatty acid profile of the diet. However, the percentages of EPA and DHA were low in larvae of higher weight, and the retention decreased when higher amounts were included in the diet. Based on these results, it seems that the possibilities for modification of BSFL fatty acid composition through the diet are limited, as earlier suggested by Spranghers et al. (2017) and Oonincx et al. (2015).

It could be possible, to alter the percentage of MUFA and PUFA in the larvae by selecting the larval stage by which they are harvested, since these fatty acids were negatively correlated to the larval weight. However, there are some issues with this method. First of all, a lower larval weight would result in a lower yield of larvae, which makes the process less efficient from a waste management perspective. Also, a higher percentage of a certain fatty acid does not necessarily mean that the total weight of the fatty acid is higher, it all depends on the fat percentage and the total biomass. For these two reasons, the harvesting of larvae at the earlier larval stages may not be an efficient way for altering the fatty acid composition of the BSFL.

The possibilities for modification of the fatty acid profile appear to be somewhat limited, thus it may be advisable to focus on the major fatty acid constituent, *i.e.* the SFA, in particular lauric acid, rather than the minor constituents (such as EPA and DHA) when evaluating possibilities for future applications of the BSFL fat. Replacement of fish oil with BSFL fat, as suggested by St-Hilaire et al. (2007a), may not be a viable option, whereas the replacement of vegetable oils could be a fruitful route to take. Li et al. (2016) found that it was possible to replace 100% of the soybean oil in the diet of Jian carp (*Cyprinus carpio* var. Jian) with BSFL fat, without negative impacts on growth performance. Schiavone et al. (2018) report similar results, but for broilers. It has also been found that the fatty acid content of BSFL is similar to that of palm oil and coconut fat, and therefore it may be possible to use BSFL fat within the food industry and other relevant industries where these fats are used (Matthäus et al., 2019). Furthermore, Surendra et al. (2016) found that the fatty acids in BSFL are suitable for the production of high-quality biodiesel. Thus, there seems to be good potential for using BSFL fat in the production of feed, food and fuel.

5. Conclusions

In this study, BSFL were fed 11 different diets composed of mussels, bread, fish and food waste. The larvae, diets and residues were analysed for proximate and fatty acid composition, and larval growth and feed conversion were measured. The fatty acid composition of the BSFL was found to be affected by the diet and the larval weight, but the possibilities for modification were hypothesised to be somewhat limited. The larvae contained mainly

SFA (especially lauric acid) and MUFAs, fatty acids which the larvae seem able to produce by themselves. It is possible to incorporate n-3 fatty acids such as EPA (C20:5) and DHA (C22:6) from the diet into the larval fat, but when the larvae gain weight, the percentage of these fatty acids decreases. Thus the potential for using BSFL fat as a substitute for fish oil seems small. Instead, based on its major fatty acid components, it is recommended that BSFL fat be used e.g. as a substitute for vegetable oils in food and feed, and for the production of biofuel.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2019.10.014.

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Fungal Fermented Palm Kernel Expeller as Feed for Black Soldier Fly Larvae in Producing Protein and Biodiesel

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Abstract: Being the second-largest country in the production of palm oil, Malaysia has a massive amount of palm kernel expeller (PKE) leftover. For that purpose, black soldier fly larvae (BSFL) are thus employed in this study to valorize the PKE waste. More specifically, this work elucidated the effects of the pre-fermentation of PKE via different amounts of *Rhizopus oligosporus* to enhance PKE palatability for the feeding of BSFL. The results showed that fermentation successfully enriched the raw PKE and thus contributed to the better growth of BSFL. BSFL grew to be 34% heavier at the optimum inoculum volume of 0.5 mL/10 g dry weight of PKE as compared to the control. Meanwhile, excessive fungal inoculum induced competition between BSFL and *R. oligosporus*, resulting in a reduction in BSFL weight. Under optimum feeding conditions, BSFL also registered the highest lipid yield (24.7%) and protein yield (44.5%). The biodiesel derived from BSFL lipid had also shown good compliance with the European biodiesel standard EN 14214. The high saturated fatty acid methyl esters (FAMEs) content (C12:0, C14:0, C16:0) in derived biodiesel made it highly oxidatively stable. Lastly, the superior degradation rate of PKE executed by BSFL further underpinned the sustainable conversion process in attaining valuable larval bioproducts.

Keywords: black soldier fly larvae; palm kernel expeller; *Rhizopus oligosporus*; fermentation; protein; biodiesel

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1. Introduction

In recent years, the reported studies associated with the biodiesel derived from black soldier fly larvae (BSFL) have intensified, offering a new and sustainable feedstock to the renewable energy industries. In this regard, various organics, usually in the form of wastes such as sewage sludges, agricultural by-products, and food wastes, have been administered to BSFL in challenging the capability of larvae to valorize and later assimilate wastes into its body biomass [1,2]. Furthermore, the exploitation of organic wastes as larval feed could mitigate the untoward environmental impacts should the wastes be disposed of indiscriminately. In such a situation, BSFL serve as an agent to reduce organic wastes, managing the waste volume in an environmentally friendly manner, whilst producing a useful larval biomass feedstock for biofuel industries. Shortcomings arise when BSFL confront its valorization capability limits, i.e., when the larvae are fed with recalcitrant organics that contain a high composition of lignocellulosic materials or organic wastes impoverished of essential nutrients. In both cases, the growth of BSFL will be retarded, and there is a high possibility the larvae will require a longer duration to reach its higher instars, should the larvae survive until the pupation stage. In enriching the organic wastes of this class, the fermentation process has been introduced to fortify the nutritional contents of the larval feed. Indeed, various fermented larval feeds have been explored to grow BSFL positively, and these include maize straw, coconut waste, pulp and paper bio-sludge, dried distiller grains, rice straw, duck manure, etc. [3–7].

In general, fermentation is a process carried out under aerobic or anaerobic conditions by a single or group of microorganisms that catalyze the breakdown of biopolymers in the organics, while producing secondary metabolites as well as fulfilling other physiological activities of the fermenters [8]. The fermentation process has been widely employed, mostly in food industries to produce fermented foods, including bread, kimchi, soy sauce, tempeh, cheese, etc. Accordingly, different inoculations of microorganisms, e.g., bacteria and fungi, are introduced into the mediums during fermentation [9]. The prime intention of fermentation is to help unleash the nutrients that are naturally found in the starting materials to become more accessible and digestible for the later consumers upon the completion of the fermentation process [10]. On the other hand, besides food industries, the fermentation process has also been applied in bioprocesses such as the bioremediation and biodegradation of hazardous organic materials, the bioconversion of biomasses, the biotransformation of agricultural residues, and the biopulping and synthesis of valuable metabolites, e.g., antibodies and enzymes [11].

Rhizopus oligosporus is a zygomycete that has been broadly used to ferment soybean, turning it into tempeh [12]. Through fermentation, the protein-nitrogen content will decrease and amino-nitrogen and ammoniacal nitrogen concentrations will conversely increase in soybean product after having been altered by the proteolytic activities of *R. oligosporus* [13]. In fact, *R. oligosporus* is capable of exuding enzymes that can hydrolyze the protein, lipid, and starch, as well as change the physical and functional properties of the fermented soybean [14]. The presence of *R. oligosporus* also inhibits the growth of pathogenic bacteria such as *Helicobacter pylori* that could otherwise cause stomach inflammation, chronic gastritis, and even worse, gastric cancer. [15]. Moreover, researchers found that the compositions of protein and protein solubility, in vitro protein digestibility, and essential amino acids in tempeh increase after the fermentation process [16], signifying nutritional enrichment. The solid-stage fermentation by *R. oligosporus* was also found to fortify the nutritional indicators, including the protein efficiency ratio, protein digestibility, and corrected amino acid score in chicken pea-fermented flour [17]. In this regard, ideally, *R. oligosporus* is a good fermenter, and therefore was chosen as an inoculant in this study.

Palm kernel expeller (PKE) is a by-product generated by palm oil mills and is currently serving as the feed for ruminants and broilers in Malaysia [18]. It has been reported that it was possible to feed absolute PKE to the ruminants without any negative impacts on growth, while also providing ample amounts of calcium and vitamins [19]. On the other hand, the incorporation of PKE into broilers' feed has shown prebiotic features and lowered

the *Escherichia coli* population in the digesta [20]. PKE is well-known as an acceptable feed replacement for ruminants' and non-ruminants' diets since it serves as a good protein source. Furthermore, it is cost effective and abundantly available in Malaysia throughout the year [18,19]. Thus, the main objective of this study was to enhance the nutritional characteristics of PKE not only to suit farm animals, but also the palatability of BSFL, regarding the excessive quantities produced from palm oil mills, since Malaysia is the second-largest producer of palm oil in the world [21]. This was achieved through prior fermentation of PKE with different inoculations of *R. oligosporus* spore suspensions before BSFL feeding. Accordingly, BSFL performances, including the valorization of fermented PKE as well as larval biochemical productions when feeding with different fermented PKE, were unveiled in this study. The enriched PKE feed was expected to promote the growth of BSFL, and subsequently, translate into high BSFL-based biodiesel production.

2. Materials and Methods

2.1. Preparation of Rhizopus oligosporus Spore Suspension

To produce the *R. oligosporus* spore suspension, Potato Dextrose Broth (Sigma-Aldrich) and Potato Dextrose Agar (Merck) were prepared beforehand. The sterile Potato Dextrose Broth was prepared by adding 6 g of the dehydrated medium into 250 mL of distilled water and autoclaved at 121 °C for 15 min. The sterile Potato Dextrose Agar was prepared by adding 39 g of the dehydrated medium into 1 L of distilled water followed by autoclaving at 121 °C for 15 min. *R. oligosporus* activation was carried out by inoculating 20 g of the dried culture of *R. oligosporus* (Raprima Brand) into the 250 mL sterile Potato Dextrose Broth and leaving it for incubation in an incubator shaker operating at 180 rpm and 30 °C for 48 h. Then, 1 mL of the activated *R. oligosporus* culture was transferred into the sterile Potato Dextrose Agar, spread, and incubated at 30 °C for approximately 7 days until the presence of black spores could be observed. For *R. oligopsorus* spore harvesting, a desired amount of sterile distilled water was slowly added to the agar plate, and the spores were dislodged using a sterile inoculating loop. The final concentration of the spore suspension was adjusted to approximately 1.0×10^6 spores per mL as determined from the cell counting plate [22,23]. All these steps were carried out under aseptic conditions to prevent contamination.

2.2. Rearing of Black Soldier Fly Larvae

Freshly laid BSF eggs were procured from MLF Ingredient Sdn Bhd located in Johor, Malaysia. The eggs were immediately transported and transferred to a sterile Petri dish with the moisture controlled by wet filter paper. The dish was left for incubation at 27 °C until the neonate had closed after about 4 days. The newly hatched larvae were reared with raw palm kernel expeller waste up to 6 days old prior to their use in the experiments.

The initial moisture of PKE was adjusted to 70% by adding adequate sterile distilled water. The fermentation of PKE was carried out by adding different volumes (0.1, 0.5, 1, 2, 3, 4, 5 mL) of the *R. oligosporus* spore suspension into each 10 g moisture-adjusted PKE sample. The samples were then homogenized by shaking and left for incubation at 30 °C for 72 h until the presence of white mycelium could be observed.

Upon completion of fermentation, 20 6-day-old BSFL were allowed to inoculate in 10 g of fermented PKE containing various *R. oligosporus* inoculum volumes. A 10 g control of PKE that was free from *R. oligosporus* spores was also set up and inoculated with 20 6-day-old BSFL. Throughout the larval rearing period, PKE moisture was maintained at 60–70% for all setups. The rearing of BSFL was terminated when half of the larvae had reached the late 5th instar stage as identified from its body colors and head sizes [24]. The separation of BSFL from the PKE medium was performed, and the harvested BSFLs were washed with distilled water, deactivated at −20 °C, and then dried at 60 °C to a constant weight. The residues of PKE were also separately dried at 105 °C until reaching constant weights.

2.3. Growth Performances of Black Soldier Fly Larvae

The growth of BSFL was measured in terms of biomass gained after the rearing period. The efficiency of the conversion of digested feed (ECD) was also recorded to signify the efficacy of ingested PKE being assimilated into larval biomass [4]. Finally, the treatment of PKE in terms of a reduced quantity was measured by the degradation rate.

$$\text{Biomass gained (g)} = \text{Final BSFL dry weight (g)} - \text{Initial BSFL dry weight (g)} \quad (1)$$

$$\text{ECD (\%)} = \text{Biomass gained (g)}/\text{Total feed consumed (g)} \times 100\% \quad (2)$$

$$\text{Degradation rate (\%)} = (\text{Initial PKE dry weight (g)} - \text{Final PKE dry weight (g)})/(\text{Initial PKE dry weight (g)}) \times 100\% \quad (3)$$

2.4. Biochemical Analyses

2.4.1. Lipid

Using petroleum ether as a solvent, the lipid from BSFL biomass was extracted via the immersing technique. Initially, 100 mg of ground larval biomass was added with 20 mL of petroleum ether and agitated for 24 h. To separate the larval lipid from petroleum ether, the mixture was filtered through filter paper, and the filtrate was dried in a rotary evaporator. Finally, the weight of the extracted lipids was recorded and used to compute the BSFL lipid yield and lipid productivity.

$$\text{Lipid yield (\%)} = (\text{Dry weight of extracted lipid (g)})/(\text{Dry weight of BSFL biomass (g)}) \times 100\% \quad (4)$$

2.4.2. Protein

The protein content of BSFL biomass was calculated by multiplying the nitrogen content of the larvae by a factor of 6.25 [25]. Using the Perkin Elmer CHNS/O Elemental Analyzer 2400 Series II, the larval nitrogen content and PKE nitrogen content were determined via the Dumas combustion technique. Initially, 1 mg of ground BSFL biomass or PKE was encapsulated in tin foil. The sample was then oxidized at 965 °C in the combustion chamber before being reduced to 640 °C in the reduction chamber. The BSFL protein yield was subsequently calculated as below.

$$\text{Protein yield (\%)} = \text{Nitrogen content in BSFL biomass (\%)} \times 6.25 \quad (5)$$

2.4.3. Fatty Acid Methyl Ester

A two-step reaction with methanol yielded a variety of fatty acid methyl esters (FAMEs) from the extracted BSFL lipid, specifically acid-catalyzed esterification, followed by base-catalyzed transesterification. The procedures were carried out according to Wong et al. [4]. The Shimadzu GC-2010 plus equipped with a flame ionization detector and a polythene glycol capillary column BPX-BD20 (30 m × 0.32 mm × 0.25 m) was used to analyze the larval FAMEs mixture. Finally, the larval FAME profile was calculated as reported by Lim et al. [26].

$$\text{FAME in biodiesel (\%)} = (A_{FAME}/A_{ISTD}) \times (C_{ISTD} \times V_{ISTD})/m \times 100\% \quad (6)$$

where A_{FAME} represents the peak area of a specific FAME species, A_{ISTD} represents the peak area of the internal standard, C_{ISTD} represents the concentration of the internal standard (1.00 mg/mL), V_{ISTD} represents the volume of the internal standard (1 mL), and m represents the dry weight of biodiesel mixed with the internal standard (mg).

3. Results and Discussion

3.1. Growth of BSFL Fed with Various Fermented PKE

The experimental results indicated that PKE could be used as a substrate for feeding BSFL. For the control set in which no *R. oligosporus* was inoculated, the weight gained by BSFL was 1.08 g for a total of 20 mature larvae. In addition, as shown in Figure 1, the inoculations with *R. oligosporus* could clearly improve the growth of BSFL ($p < 0.05$), across all tested volumes. The optimum volume as illustrated from the figure was 0.5 mL. When the *R. oligosporus* population was increased gradually from 0 to 0.5 mL, BSFL could attain a maximum weight gain of 1.45 g. Exceeding the optimum volume, the weight gained by BSFL abruptly dropped by about 12% to 1.27 g. The subsequent addition of a higher fungal volume for fermentation did not significantly affect the growth of BSFL, with their weight gain hovering around 1.25 to 1.30 g. However, when excessive *R. oligosporus* was added at 5 mL, the weight gained by BSFL again dropped drastically to 1.13 g, which was close to the control set.

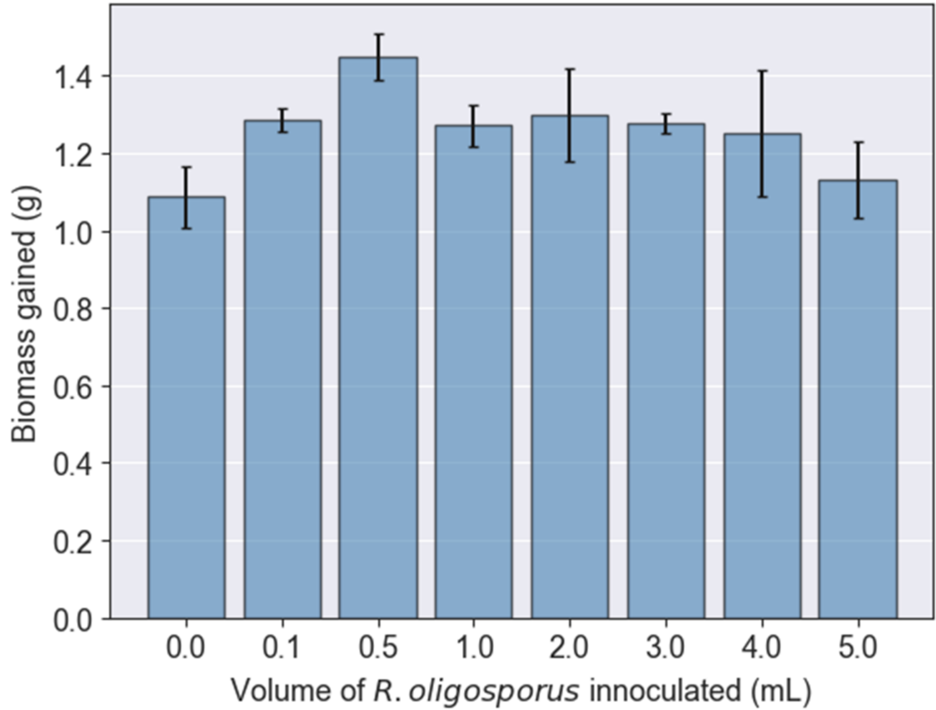

Figure 1. Total weight of biomass gained by black soldier fly larvae fed palm kernel expeller containing various inoculation volumes of *R. oligosporus*.

When the volume of fungal inoculation was increased from 0 to 0.5 mL, the ECD also increased from 17.61% to 24.37% (Figure 2). However, it was notable that the feed consumed by BSFL remained almost constant at this stage. In other words, BSFL were consuming a similar amount of feed but managed to grow heavier when the volume of fungal inoculation increased from 0 to 0.5 mL. This indicated that the fermentation process was effective in improving the palatability of PKE, in which it enriched the nutritional content available per unit of feed for growing BSFL. Lateef et al. [27] had proven that fermentation using *R. stolonifer* could also reduce the crude fiber in PKE by 44.5%, while increasing the crude protein content by 33.2%. This also incontrovertibly justified the better growth of BSFL while being administered fermented PKE with increasing fungal volume from 0 to 0.5 mL (Figure 1).

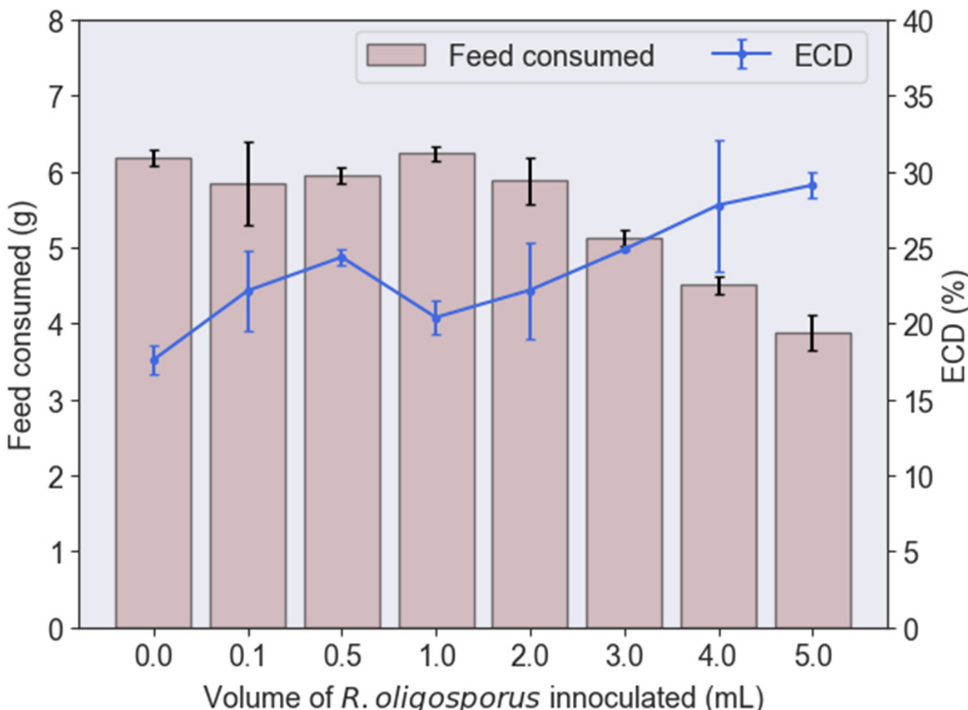

Figure 2. Total feed consumed and the efficiency of conversion of digested feed (ECD) by black soldier fly larvae fed with palm kernel expeller containing various inoculation volumes of *R. oligosporus*.

Beyond the optimum volume of 0.5 mL, the ECD dipped to 20.40%. The dip occurred at 1 mL of fungi with the same amount of feed consumed, but a lower BSFL biomass gained as opposed to 0.5 mL. This could signify that the *R. oligosporus* added was overwhelming. The excessive population of *R. oligosporus* would compete for nutrients with the BSFL. Furthermore, this could accelerate the exhaustion of nutritional contents within the feed, resulting in an impoverished substrate that was no longer enriching for BSFL consumption. This was also exemplified by early work [27] in which the lipid content in fermented PKE was reduced by 15.4% after the fermentation process carried out by *R. stolonifer*. Another plausible rationale could be the imbalance of nutrients in the PKE substrate. Raw PKE consisted of 16% protein, 9.3% fat, and the remaining 73% was neutral detergent fiber (namely, lignin, cellulose, and hemicellulose) [28]. However, with the aid of excessive *R. oligosporus*, a similar phenomenon concerning the enhancement of crude protein content could occur [27], which would further elevate the total composition of crude protein in PKE. In contrast, besides having a balanced amount of protein, the recommended BSFL substrate should consist of plenty of non-fiber carbohydrates as they can be easily digested and converted into larval body lipids [29].

A substrate that was only high in protein and low in carbohydrates had been proven to be underperforming in growing BSFL effectively. Lim et al. [26], for instance, had shown that beyond the optimum protein content required, BSFL growth would present a reverse trend as the larvae performed the proteinogenic nitrogen detoxification process in order to survive the high protein content in the feed. This detoxification process was energy-intensive, and hence, would stunt or even reverse the growth rate of BSFL. The high intake of protein by BSFL with 1 to 5 mL inoculum volumes can be seen from Figure 3, where the nitrogen contents of PKE residue dropped by nearly 28%, i.e., from 3.63 to 2.55 wt%. The reduction in nitrogen contents from the PKE residue was directly translated into the intake of nitrogen in the form of protein by BSFL.

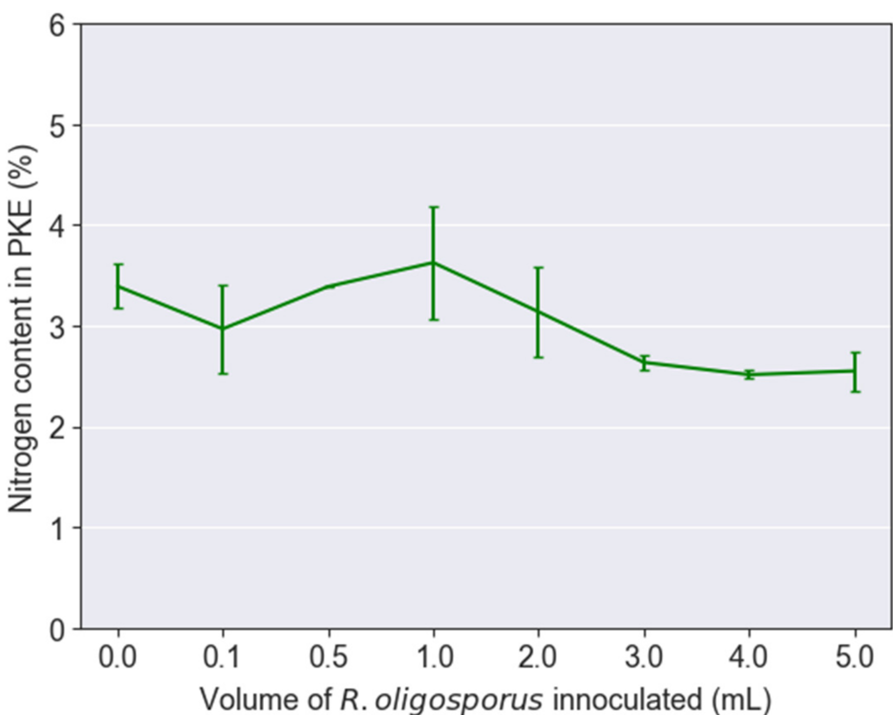

Figure 3. Nitrogen contents in palm kernel expeller residues after feeding to black soldier fly larvae.

Interestingly, the ECD climbed from a low of 20.40% to 29.11% when the volume of *R. oligosporus* inoculated was increased from 1mL to 5mL. This occurred primarily because of the reduction in feed consumed, while the weight of biomass gained remained almost constant. When the volume of *R. oligosporus* inoculated was increased from 1mL to 5mL, the feed consumed by BSFL dropped gradually from around 6.24 g to 3.88 g ($p < 0.05$). Similar to other microorganisms, *R. oligosporus* perform respiration to generate energy to sustain their cellular activities. At the same time, respiration also produces carbon dioxide and water as by-products. Hence, the presence of an excessively high *R. oligosporus* volume in PKE feed could result in intense aerobic respiration, which resulted in increasing the water content in the substrate. Hence, when volumes of *R. oligosporus* higher than 1 mL were inoculated, BSFL could have been ingesting the same amount of substrate as before, but with higher water content. This could have been the cause of the declining amount of feed consumed when *R. oligosporus* was increased from 1 to 5 mL, since the feed consumed was measured in terms of dry weight.

3.2. Protein and Lipid to Biodiesel from BSFL Fed with Various Fermented PKE

The ANOVA analysis proved that the presence of *R. oligosporus* enhanced the protein yield ($p < 0.05$) and lipid yield ($p < 0.05$) as compared with non-fermented PKE. As illustrated in Figure 4, both protein and lipid yields presented a similar trend when the volume of *R. oligosporus* was increased from 0 to 0.5 mL. Protein yield increased from 38.2% to 44.5%, which was the highest protein yield recorded across the entire set of samples. Subsequently, the protein yield of BSFL registered a slight decline with more fungi introduced but did not drop further to below 41%. On the other hand, the lipid yield increased from 18.6% to a maximum of 24.7% at a 0.5 mL fungal volume. Again, the lipid yield declined slightly with more fungal inoculation, but still registered a higher lipid yield in comparison with the unfermented PKE. The protein and lipid yields in Figure 4 were in conformity with the findings from the previous section in which a 0.5 mL fungal inoculation was singled out as the optimum inoculum size that could achieve the highest larval biomass gained. Accordingly, not only was the maximum BSFL weight gained, but it also had the highest protein and lipid yields as opposed to the other inoculum sizes. At the optimum inoculation

volume of 0.5 mL, the protein yield had increased by 16.5%, the lipid yield had increased by 32.8%, and the biomass gained had increased by 34.2%.

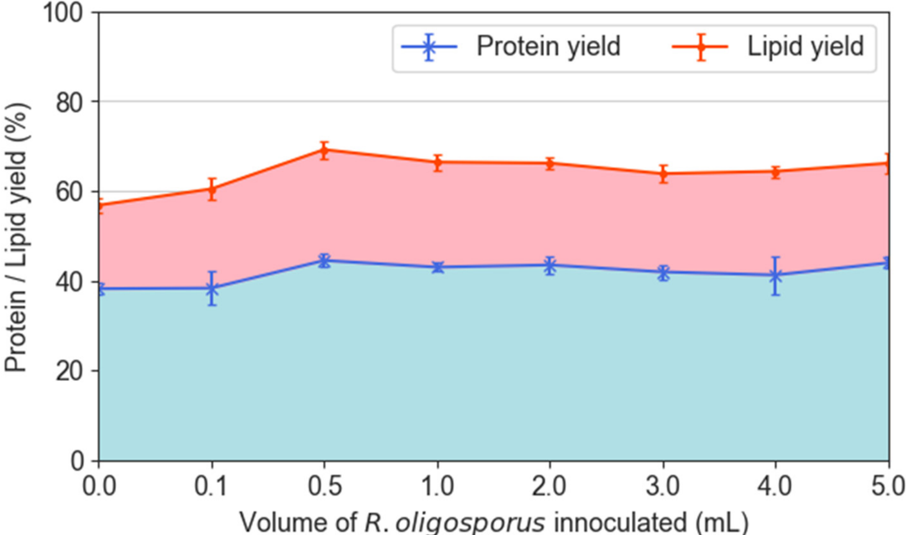

Figure 4. Protein and lipid yields from black soldier fly larvae fed palm kernel expeller containing various inoculation volumes of *R. oligosporus*.

In comparison with the previous work on BSFL rearing administered with fermented coconut endosperm waste, the lipid yield of BSFL always ranged around 40%, which was consistently higher than the protein yield [23,30]. However, among all the inoculum sizes in this work, the opposite was observed. The protein yields had been consistently approximately two times higher than the respective lipid yields from BSFL upon feeding with PKE. This could primarily stem from the different substrates being fed to BSFL, proffering a different spectrum of nutrients. The mature coconut endosperm had been reported to consist of 28.7 wt% carbohydrates, 63.2 wt% fat, and only 6.28 wt% protein [31]. This means the coconut endosperm is a highly energy-dense substrate due to the high amounts of fat and carbohydrate. Meanwhile, as stated early, PKE had only 16 wt% protein and 9.3 wt% fat that were easily digestible, while the remaining 73 wt% existed as neutral detergent fiber (namely, lignin, cellulose, and hemicellulose) [28,32]. Although the high protein composition in PKE had boosted the BSFL protein yield, the lack of easily digestible carbohydrates could be one of the reasons for low BSFL lipid yield in this work.

The FAME profiles derived from BSFL fed the controlled PKE (0 mL), the optimum inoculum size PKE (0.5 mL), and the random inoculum size PKE (1.0 mL) were investigated. From Figure 5, it ca be deduced that the fermentation process executed by *R. oligosporus* had no significant effect on the composition of FAME upon feeding BSFL with PKE. The C12:0 was consistently the highest FAME at approximately 47–53 wt% of the total FAME composition regardless of either the absence or presence of *R. oligosporus*. This was then followed by C14:0, C16:0, and C18:1. These four types of FAMEs made up 87–90 wt% of the entire FAME composition. The sum of saturated FAMEs ranged from 79–83 wt%, thereby making BSFL-derived biodiesel highly oxidatively stable. In comparison, other typical biodiesels derived from soybean, rapeseed, and oil palm were inferior in the sense that they had a low saturated FAME composition of 7.4–49 wt% [33]. Their FAME compositions and respective saturated FAME contents are summarized in Table 1. Furthermore, the larval biodiesel attained in this work also met the European specification (EN-14214) where the poly-unsaturated FAME ($\geq$4 π bonds) was lower than 1 wt% [33]. The linolenic acid methyl ester content (2.17–4.02 wt%) was also well below the standard of 12 wt%. Even though the larval biodiesel produced possessed desirable traits, the *R. oligosporus*-fermented PKE seemed to be a more suitable substrate for producing high-protein-yield larvae instead of high-lipid-yield larvae to produce biodiesel.

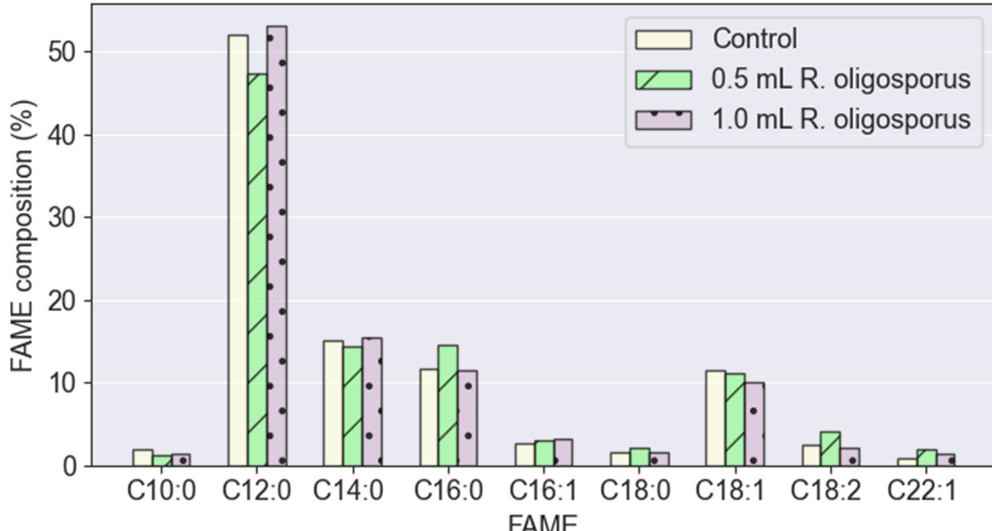

Figure 5. FAME profiles derived from black soldier fly larvae fed with palm kernel expeller containing various inoculation volumes of *R. oligosporus*.

Table 1. FAME compositions in biodiesel derived from well-established feedstock.

FAME	BSFL (This Work at 0.5 mL) (%)	Soybean (%) [33]	Rapeseed (%) [33]	Oil Palm (%) [33]
C10:0	1.2	0.0	0.6	0.5
C12:0	47.5	0.1	0.1	0.3
C14:0	14.4	0.1	0.0	1.1
C16:0	14.6	11.6	4.2	42.5
C16:1	3.1	0.2	0.1	0.2
C18:0	2.1	3.9	1.6	4.2
C18:1	11.1	23.7	59.5	41.3
C18:2	4.0	53.8	21.5	9.5
C22:1	2.0	0.1	0.5	0.0
SFA	79.8	16.5	7.4	49.0
MUFA	16.2	24.7	62.8	41.6
PUFA (<4 π bonds)	4.0	59.7	30.0	9.8
Total	100.0	100.9	100.2	100.4

SFA: Saturated FAME; MUFA: Mono-unsaturated FAME; PUFA: Polyunsaturated FAME.

3.3. Degradation Rate of PKE by BSFL

The PKE degradation rate was influenced by the inoculation volume of *R. oligosporus* ($p < 0.05$). Nonetheless, the post-Tukey test results as demonstrated in Figure 6 evidenced that there was little difference among the first few samples in which the inoculation volumes were less than 2 mL since they belonged to the same category. Thereafter, the higher inoculation volumes had manifested a significant difference in degradation rates. The degradation rates were found to decline when the fungal inoculation was more than 2 mL. Initially, the degradation rates of PKE fluctuated around 58.4–62.4%. However, when the inoculation volumes increased to 3, 4, and 5 mL, the degradation rates dropped drastically to 51.3%, 45.1%, and, finally, 38.8%, respectively. The decline in degradation rates could be attributed to the wetter substrate conditions at higher inoculum volumes. The presence of denser populations of *R. oligosporus* at higher inoculation volumes could have resulted in higher respiration rates, generating more moisture within the PKE substrate. This resulted in a moister and more diluted substrate, engendering BSFL to feed on less PKE measured as dry matter. In comparison with other substrates that had been fed to BSFL, the degradation rates, ranging from 58.4–62.4%, that were achieved by the control and samples with lower inoculum volumes (<2 mL) were deemed attractive, having been positioned at relatively

high values. The degradation rates of other known substrates for BSFL treatments are shown in Table 2 in which the PKE was found comparable to coconut endosperm waste and soybean curd residue, whilst being higher than wheat bran and food waste.

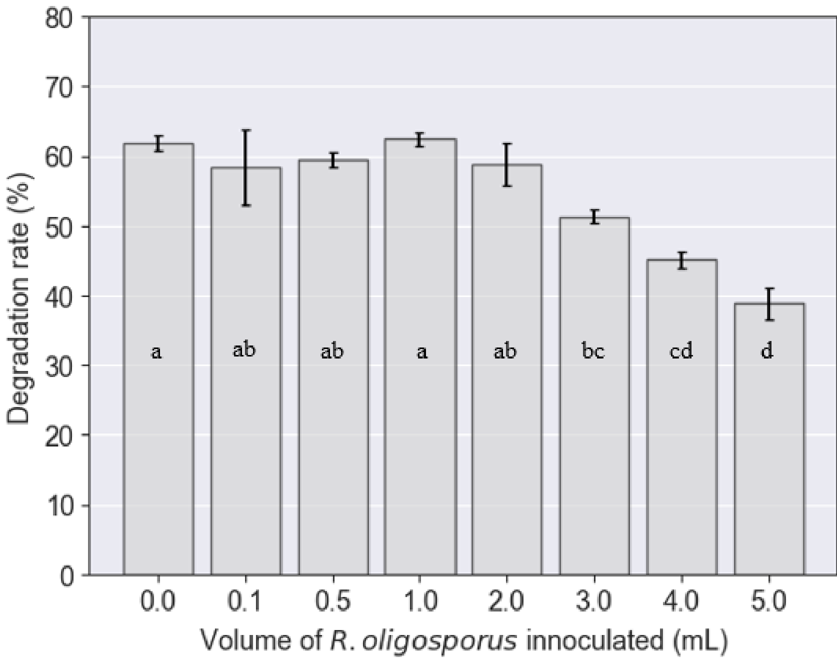

Figure 6. Degradation rates of palm kernel expeller executed by black soldier fly larvae upon inoculation with various volumes of *R. oligosporus*.

Table 2. Degradation rates of different organic substrates after black soldier fly larvae treatments.

Substrate	Degradation Rate (%)	Reference
Palm kernel expeller	38.8–62.4	This work
Coconut endosperm waste	52.0–75.0	[23,26]
Soybean curd residue	64.0–72.4	[26,34]
Cow manure	25.8	[34]
Corn stover	39.9	[35]
Fermented maize straw	48.4	[3]
Wheat bran	55.0	[3]
Fruits and vegetable waste	46.7–49.5	[2,36]
Food waste	50.3–55.3	[2,36]

4. Conclusions

The fermentation of PKE through the inoculation of *R. oligosporus* evidently enhanced the growth of BSFL. The optimum inoculum volume was found to be 0.5 mL/10 g dry weight of PKE. At this inoculum condition, the biomass gained by BSFL improved remarkably by 34%. The highest lipid and protein yields from BSFL were recorded at 44.5% and 24.7%, respectively, which transpired under the optimum inoculum volume as well. The biodiesel quality derived from BSFL, on the other hand, was not significantly affected by the presence or absence of the fermentation process. Accordingly, biodiesels were all of good quality, oxidatively stable, and fulfilled the FAME requirements as decreed by European biodiesel standard EN 14214. Despite positive enhancements from PKE fermentation for BSFL growth and later larval biodiesel characteristics, the resulting BSFL biomass had an almost two-fold higher protein yield than lipid yield. While more future work can be conducted to enrich the carbohydrate and lipid contents in PKE substrate to boost the lipid yield from BSFL, thus far, fermented PKE seems to be more ideal for the production of high protein content rather than larval lipid for biodiesel. The high protein accumulation via

rapid BSFL growth was also underpinned by the high PKE degradation rate via assimilation into the larval body weight, heralding PKE palatability for BSFL feeding as opposed to other substrates. The high content of protein in larval biomass could then be applied as an alternative nutritional food for the substitution of conventional animal feed. Meanwhile, biodiesel-derived larvae could be further improved in terms of lipid yield and utilized as a new generation of biodiesel feedstock in energy industries due to their good quality of FAME compositions.

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