Universität Rostock Traditio et Innovatio

Aus der Professur für Ernährungsphysiologie und Tierernährung
der Agrar- und Umweltwissenschaftlichen Fakultät
und dem Forschungsinstitut für Nutztierbiologie (FBN)

Effect of supplementation of broilers with Black Soldier Fly Larvae (BSFL) reared on recycled phosphorus-rich substrates

Kumulative Dissertation

zur Erlangung des akademischen Grades

Doktor der Agrarwissenschaften (*doctor agriculturae*)

an der Agrar- und Umweltwissenschaftlichen Fakultät

der Universität Rostock

vorgelegt von

Seyed Mohammad M. Seyedalmoosavi

aus Mashhad, Iran

Dummerstorf, 2023

https://doi.org/10.18453/rosdok_id00004626

Gutachter:

Prof. Dr. Helen Louton, Universität Rostock, AUF, Tiergesundheit und Tierschutz

Prof. Dr. Cornelia Metges, Forschungsinstitut für Nutztierbiologie (FBN), Institut für Ernährungsphysiologie

Prof. Dr. Klaus Eder, Justus-Liebig-Universität Gießen, Institut für Tierernährung und Ernährungsphysiologie

Prof. Dr. Annette Zeyner, Martin-Luther-Universität Halle-Wittenberg, Professur Tierernährung

Jahr der Einreichung: 2023

Jahr der Verteidigung: 2024

Scientific Advisors:

Prof. Dr. rer. nat. habil. Cornelia C. Metges

Research Institute for Farm Animal Biology (FBN), Institute of Nutritional Physiology "Oskar Kellner"

Dr. sc. agr. Gürbüz Daş

Research Institute for Farm Animal Biology (FBN), Institute of Nutritional Physiology "Oskar Kellner"

Prof. Dr. med. vet. habil. Helen Louton

University of Rostock, Nutritional Physiology and Animal Nutrition, Faculty of Agricultural and Environmental Sciences, Rostock, Germany

Prof. Dr. Petra Wolf (until July 2022)

University of Rostock, Nutritional Physiology and Animal Nutrition, Faculty of Agricultural and Environmental Sciences, Rostock, Germany

Contents

List of figures

Abbreviations

∑n-3	Total n-3 PUFA
∑n-6	Total n-6 PUFA
AA	Amino acid
ADF	Acid detergent fiber
ADL	Acid detergent lignin
AI	Atherogenicity index
ALP	Alkaline phosphatase
AMP	Antimicrobial peptides
As	Arsenic
BAF	Bioaccumulation factor
BAI	Bioaccumulation index
BBM	Brush border membrane
BHT	t-butylhydroxytoluene
BSF	Black soldier fly
BSFL	Black soldier fly larvae
BW	Body weight
BWG	Body weight gain
Ca	Calcium
CC	Carcass characteristics
Cd	Cadmium
CF	Crude fibre
CLA	Conjugated linoleic acid
CP	Crude protein
Cu	Copper
CV	Coefficient of variation
DHA	Docosahexaenoic acid
DM	Dry matter

DMI	Dry matter intake
DPA	Docosapentaenoic acid
ECR	Energy conversion ratio
EPA	Eicosapentaenoic acid
FA	Fatty acids
FAO	Food and Agriculture Organization
FCD	Fold change difference
FCD_BW	FCD corrected for BW
FCR	Feed conversion ratio
FCR-1	Feed conversion ratio based on FMI
FCR-2	Feed conversion ratio based on DMI
FD	Gainesville fly diet
Fe	Iron
FER	Feed eating rate
FER_BW	FER was also adjusted for BW
FI	Feed intake
FMI	Fresh matter intake
FPD	Footpad dermatitis
GE	Gross energy
GECR	Gross energy conversion ratio
GGE	Greenhouse gas emissions
HB	Hock burn
HCA	Hierarchical two-way clustering analysis
Hg	Mercury
HH	Hypocholesterolemic / Hypercholesterolemic ratio
HPLC	High performance liquid chromatography
HR-GC	High-resolution gas chromatography
K	Potassium

LA	Lauric acid
LDL	Low-density lipoprotein
LER	Larvae eating rate
LER_BW	LER adjusted for the BW
LSMEANS	Least square means
LUFA	Long-chain unsaturated fatty acids
LW	Larval weight
MCFA	Medium chain fatty acids
ME	Metabolizable energy
Mg	Magnesium
Mn	Manganese
Mo	Molybdenum
MUFA	Mono-unsaturated fatty acids
Na	Sodium
NDF	Neutral detergent fiber
NEFA	Non-esterified FA
NVI	Nutritional Value Index
P	Phosphorus
Pb	Lead
PCA	Principal component analysis
PCR	Protein conversion ratio
PI	Peroxidability index
PROC GLM	linear model procedure of SAS
PROC MIXED	Linear mixed model
PUFA	Poly-unsaturated fatty acids
SE	Standard errors
Se	Selenium
SFA	Saturated fatty acids

SGR	Specific growth rate
SS	Sewage sludge
SSP	Single Superphosphate
SSR	Sewage sludge recyclates
TI	Thrombogenicity Index
TSL	Time spent by birds eating larvae
Zn	Zinc

1. Literature overview

1.1. Introduction

The United Nations Population Prospects suggest that the global human population will increase to 9.1 billion by 2050, which is 34% more than today (United Nations, 2015). The Food and Agriculture Organization (FAO) commented on this issue and suggested that to provide food for this growing global human population, production of food must be increased by 70 percent. Therefore, there would be a high demand for consumption of meat, milk, and eggs rising at the expense of staple foods, which will put significant pressure on the current land and will impact all the environmental aspects, including water, soil, and air resources (Van Huis et al., 2013). Besides this, to meet the increasing demand for animal products with the current feed sources, 280 million hectares of additional land will be needed by 2030 (Pahlow et al., 2015). The current allocation of crops to animal feed causes different environmental impacts as it mainly expands in tropical areas. According to estimations, around 80% of tropical forest fragments are surrounded by cropland. This expansion is problematic as the expansion of agriculture in tropical areas is together with reducing biodiversity and increasing greenhouse gas (GHGE) emissions. Tropical deforestation has been considered as a significant source of GHG which is a critical component of climate change mitigation and depleting critical ecosystem services (Foley et al., 2011). Increasing food production without agriculture expanding implies that we must increase production on our existing land. Therefore, studies for low-cost and sustainable feeding alternatives have gained increasing attention (Ferrer Llagostera et al., 2019).

1.2. Phosphorus in living organisms and environment

In all animal species, phosphorus (P) has a critical role in cellular metabolism, as a component of the energy reservoir of the cell, in cellular regulatory mechanisms, and in bone development

and mineralization (Bolan et al., 2010). In living organisms, P is available as phosphoric acid ($H_2PO_4^-$ and HPO_4^{2-}) in the form of sodium (Na) and potassium (K) salts or its less soluble calcium (Ca) salt such as hydroxyapatite and is one of the essential minerals for all forms of life (Chande and Bergwitz, 2018). Poultry consume around 50% of global animal feed phosphate (Devereux et al., 1994). Generally, feed phosphates account for most of the P content in broiler diets; meeting more than 60% of the P requirements of poultry (Li et al., 2016). However, P utilization efficiency by poultry is low and its suggested that only one-third of feed P is utilized by poultry, and the rest of feed P is excreted trough manure and applied to the soil for cultivation (Patterson et al., 2005).

Phosphorus is taken up into vesicles of the intestinal brush border membrane (BBM) via active and passive transport pathways (Sabbagh et al., 2011, Proszkowiec-Weglarz and Angel, 2013). Active transport accrues in the proximal small intestine through Na-dependent phosphate co-transporter, whereas passive transport occurs in the jejunum and ileum through a dependent diffusion process in which P moves from the lumen which has a higher P concentration, to blood which has a lower P concentration (Sabbagh et al., 2011). However, it has been reported that the epithelial membrane of the intestine is not effectively permeable to P (Cross et al., 1990).

Transepithelial active transport of P is associated with Na^+ concentration in the intestinal lumen, renal tubules, or intestinal tissue, whereas passive transport is correlated with P concentration (Proszkowiec-Weglarz and Angel, 2013). Therefore, low dietary P intake is expected to result in dominant active transport of P through the apical BBM by the Na-dependent co-transporter and translocation across the cell, whereas passive transport being the dominant mechanism at high dietary P intakes (Danisi and Straub, 1980). Transportation of P across enterocytes involves three different major steps: (i) P entry across the luminal BBM into the

enterocyte; (ii) transfer of P to the serosal compartment, and (iii) P transport from the epithelial cell into the extracellular space (Yan et al., 2007).

Homeostasis of P is determined through gastrointestinal P absorption, reabsorption and excretion of P in kidney, and the exchange of P between extracellular and bone storage pools (Penido and Alon, 2012). Phosphorus homeostasis is regulated within a constrained physiological range by a complex mechanism involving vitamin D3 and the action of numerous ions, hormones, and their respective receptors on the intestine, kidneys, and bone. Insufficiency of P and Ca or both minerals in the diet interferes with their homeostasis, results in reduced growth rate and impaired bone mineralization (Proszkowiec-Weglarz and Angel, 2013).

1.2.1. Phosphorus environmental pollution

It has been estimated that 82% of P used in agriculture is rock P, 7% of P is used in animal feed, and 11% is utilized directly for human use, such as industry, medicine and etc. (Cieślik and Konieczka, 2017). Since the human population and the demand for food and feed production is increasing (United-Nations, 2019), it can be expected that P consumption increases rapidly. Primary rock P deposits can be found in Morocco, the US, China, South Africa, and Jordan, which are non-renewable and becoming more expensive (Cieślik and Konieczka, 2017). However, literature data show that the global rock P will be exhausted soon (Tao and Huang, 2007, Li et al., 2014).

Besides the problem of limited P resources, multiple environmental impacts emerge from the intensive use of P. Phosphorus practice in agriculture activities, animal husbandry, and urbanization might lead to the accumulation of P in the soil particles, which further could also reach water bodies through subsurface flow (Ruark et al., 2014). The current P practice has been associated with P environmental pollution and eutrophication of water bodies (Amann et al., 2018),

which in turn might directly or indirectly impact human societies (Bol et al., 2018). Excessive production of sewage sludge (SS) and animal manure leads to extensive application on soils with the consequence of P accumulation (Bergfeldt et al., 2018). Recycling of P from livestock waste and SS sources could be an alternative source of P for agriculture practice and animal diets. However, there is the problem of low bioavailability of P and of heavy metals contaminating the sludge (Herzel et al., 2016).

1.2.2. Phosphorus recycling

Organic wastes such as SS contain considerable amounts of P (Cieślik and Konieczka, 2017). Sewage sludge has a high P content and has been suggested as a promising source of P (Havukainen et al., 2016), which can be used as an alternative source of P for synthetic fertilizers to provide nutrients for sustainable agriculture production (Seleiman et al., 2020). However, along with the benefits of using sludge in agriculture, it's important to consider that sludge contains contaminants such as heavy metals, pharmaceuticals, and pathogens which are harmful to the food chain (Hudcová et al., 2019, Seleiman et al., 2020). Incineration of SS leads to a considerable P content ending up in the ash with a concentration of 7-13% P (Cohen, 2009). The P concentration of the ash is comparable to P concentration in phosphate rock (12-16%) (Cohen et al., 2019). However, ash also contains certain toxic heavy metals which is unsuitable for fertiliser industry (Cohen et al., 2019). In Germany, SS regulation restricts the use of SS in agriculture and makes it obligatory to recycle at least 80% of the available P in SS or their ashes depending on the initial concentration in the wastewater (Klärschlammverordnung, 2017).

Some SSR technologies can safely remove hazardous organic pollutants from SS to produce mineral-rich recyclates, which can be used further in environmentally and ecologically sustainable agriculture (Arthurson, 2008). Until now, several kinds of P recycling technologies from SS have

been created, and depending on the recovery approaches, effective removal of heavy metals can be achieved (Egle et al., 2016).

The Ash2Phos is a wet chemical recovery process which has been developed by EasyMining Sweden AB, to recycle P from ash of mono-incinerated SS (Cohen et al., 2019). Acid digestion is the first step in the process, which extracts minerals like P or Ca from the ash (Figure 1). The desired minerals are then precipitated and separated in several processes to produce Single Superphosphate (SSP), which has a high bioavailability and a low concentration of contaminants like heavy metals. These recycled minerals could subsequently be incorporated into the production cycle, for example, by implementing them into animal feeds (Cohen et al., 2019).

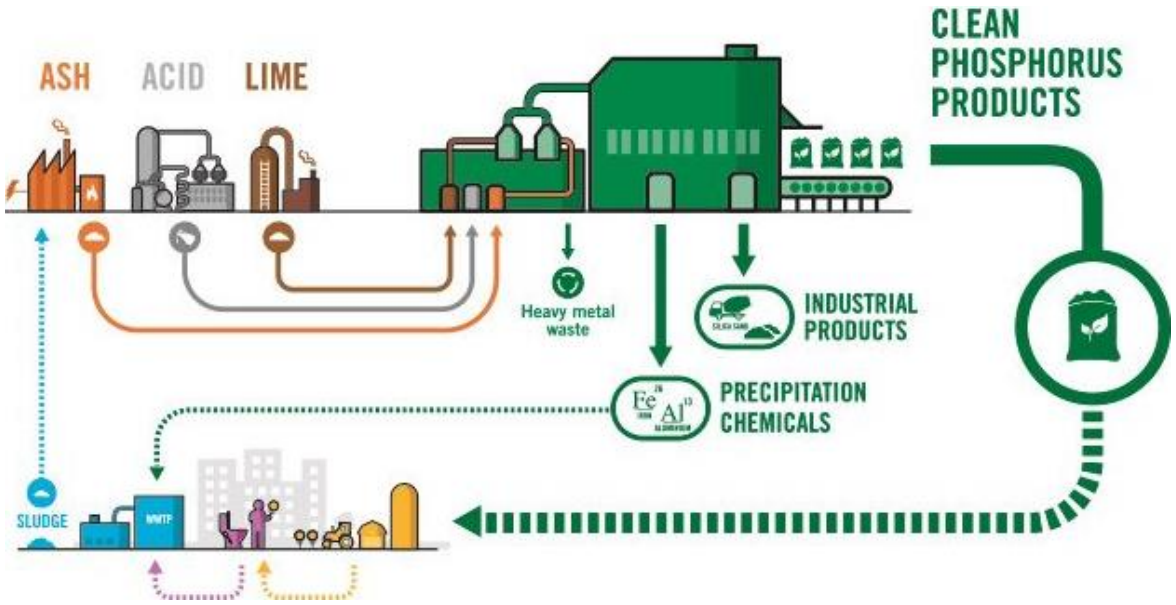


Figure 1. The Ash2Phos® process includes three steps: **1.** an acidic step, **2.** an alkaline step (where intermediate products are produced), and **3.** a conversion step where the intermediates produced in the second step are processed into final products (Adopted from EasyMining; https://www.easymining.se/technologies/ash2phos).

The PYREG® process operates according to the principle of dry carbonisation to produce biochar (BCH) (Fesharaki and Rath, 2018). In this two-step process, first, the dried SS is heated up to 650°C in the PYREG® reactor under oxygen exclusion which causes not incinerated but carbonized sludge and mineralized to SS ash (Figure 2). In the next step, the synthesis gas developing in the reactor is completely burned at about 1250°C in the combustion chamber. The produced biochar contains carbon from the organic parts and all of the minerals from the SS, including P.

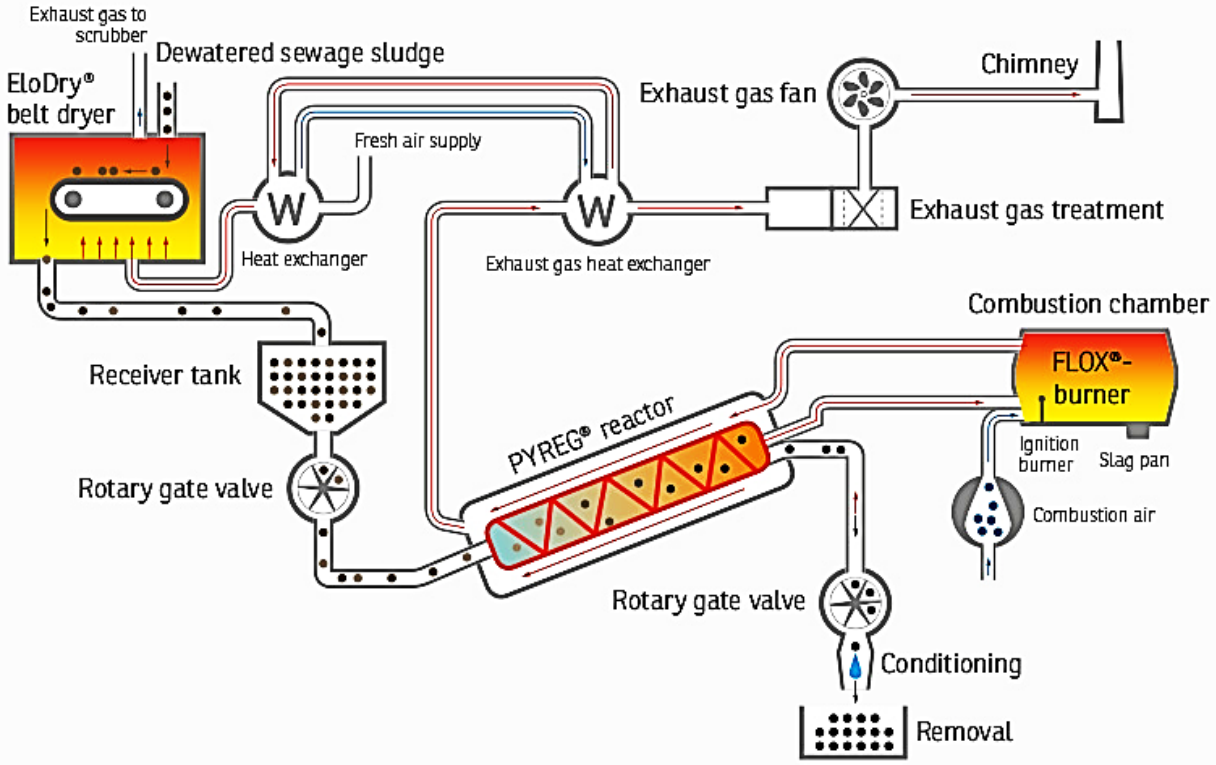


Figure 2. PYREG® is a pyrolysis process of biomass such as sewage sludge (Adopted from ELIQUO Technologies; https://www.eliquo-tech.com/en/pyreg.html).

1.3. Insects farming

Insect farming has seen a significant increase in interest over the last decade. International conferences have been held (Wuhan, 2018), and there have been an increasing number of books

and published papers on the topic (Van Huis, 2013, Van Huis et al., 2014, Surendra et al., 2016). Insect farming is a promising source of animal protein, as they have high-quality proteins, beneficial fats, and certain trace elements. Insects can be reared in high densities with small space requirements (Oonincx and De Boer, 2012). In addition, many insects can be reared on waste streams, which keeps environmental pollution low and assists in biowaste recycling (Smetana et al., 2016). According to previous studies, the ideal insect should be simple to rear, have an acceptable feed conversion rate, and be able to grow on inexpensive substrates; these features are most compatible with Dipterian species (Macombe et al., 2019).

During the recent years, there has been an increasing number of scientific publications on Black Soldier Fly (BSF) (*Hermetia illucens*). The BSF larvae (BSFL) is a wasp-like fly, originally native to South American savannah, distributed all over the tropic and temperate regions of the world with an optimum temperature range of $25^{\circ C}$ to $30^{\circ C}$, and characterized by a short life cycle of 40 to 45 days (Wang and Shelomi, 2017, Lu et al., 2022). The life cycle of BSFL consists of five stages including: egg, larvae, pupa, prepupa, and adult, with larval and pupal stages having the highest nutrient content, which largely depends on the quality of substrates (Lu et al., 2022).

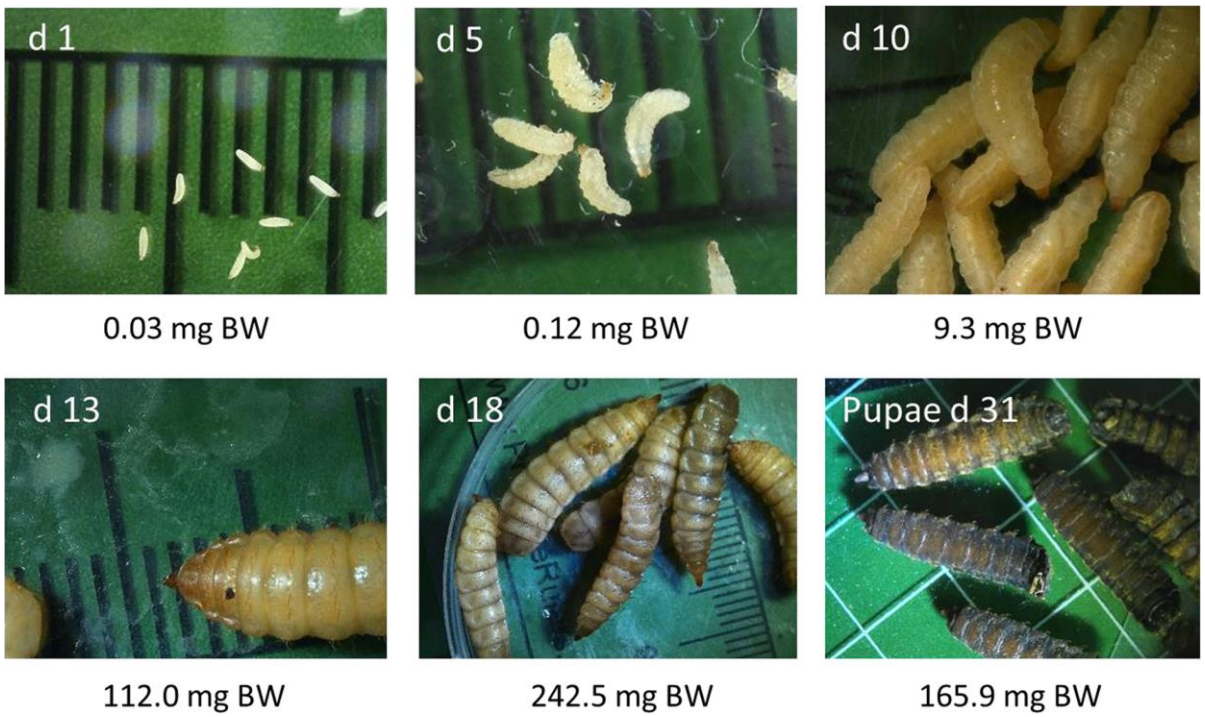


Figure 3. Development of black soldier fly larvae from d 1 after hatching up to the pupal stage (Adopted from Seyedalmoosavi et al., 2022).

Larvae of BSF are well known to feed and develop on a wide range of substrates, such as kitchen waste (Diener et al., 2011, Nguyen et al., 2015), manure (Sheppard et al., 2002, Zhou et al., 2013), fecal sludge (Lalander et al., 2018), and distillers' by-products (Tschirner and Simon, 2015). This makes BSFL of particular interest as a rich sources of proteins and fats in animal diets (Wang and Shelomi, 2017). According to EU authorities, insect larvae has been permitted to be included in poultry diets (Commission Regulation, 2019), however, the substrates for feeding larvae has been restricted to those substrates which are permitted for other farm animal. Black soldier fly larvae are capable of efficiently converting a wide variety of low-cost organic waste into high quality proteins, the content of which ranges from 38% to 46% of dry matter (DM) (Bava et al., 2019). The amino acids composition of BSFL is rich in methionine and lysine (Schiavone et al., 2017), and is reported to have similar or better protein quality than that of soybean (Maurer

et al., 2016). Larvae of BSF are capable of fat biosynthesis as well as accumulation of a large amount of fatty acids (FA) from their substrates (29% of DM), although they incorporate more saturated fats than most insects (Wang and Shelomi, 2017). As a result, BSFL can be suggested to be used as a promising feed for a variety of animals, including swine, poultry, and fish (Cammack and Tomberlin, 2017). Depending on the specific life stage, chitin content of BSFL ranges from 5.9% to 8.7% (Gariglio et al., 2019). It has been reported that chitin has antioxidant properties for both human and animal (Gariglio et al., 2019). The hypocholesterolemic effect of chitin has also been found to have a positive impact on the immune system of poultry (Swiatkiewicz et al., 2014).

1.3.1. Mineral accumulation by BSFL

Calcium, P, and K are the most abundant macro-minerals in the BSFL (Chia et al., 2020). However, previous studies reported that the feeding substrate affects the mineral concentration in the larvae. Liland et al. (2017) suggested that the concentrations of minerals in the larvae followed the concentrations of organic materials linearly. They reported that the retention of minerals in the larval body (BSFL) (as a percent of minerals added via the feeding media) declined with an increasing amount of seaweed included in the substrate for all minerals except for P. Proc et al. (2020) reported that BSFL can bioaccumulate selected elements. They observed that bioaccumulation of Cu (Copper), Fe (Iron), Hg (Mercury), Mg (Magnesium), Mo (Molybdenum), Se (Selenium), and Zn (Zinc) occurred at all stages of insect development. In addition, they found that Ca, Cd (Cadmium), Mn (Manganese), and P were bioaccumulated in the larval body. These studies suggest that BSFL can selectively change the concentration of some elements in their body composition in response to the diet.

Using BSFL fed animal manure and household organic waste as animal feed could be considered as a sustainable way to reintegrate the biowaste into the nutrient cycle (Schiavone et

al., 2017). However, in addition to high levels of nutrients, one of the potential disadvantages of using BSFL as feed component is the presence of heavy metals (e.g. Zn, Cu, Cd and Hg) (Schmitt et al., 2019). Most minerals present in the feeding substrate can be accumulated into the BSFL body (Fig. 4). However, the degree of accumulation seems to be dependent on the substrate. Phosphorus is probably one of the most interesting minerals with largest variation in accumulation rate, implying that the accumulation is heavily substrate dependent.

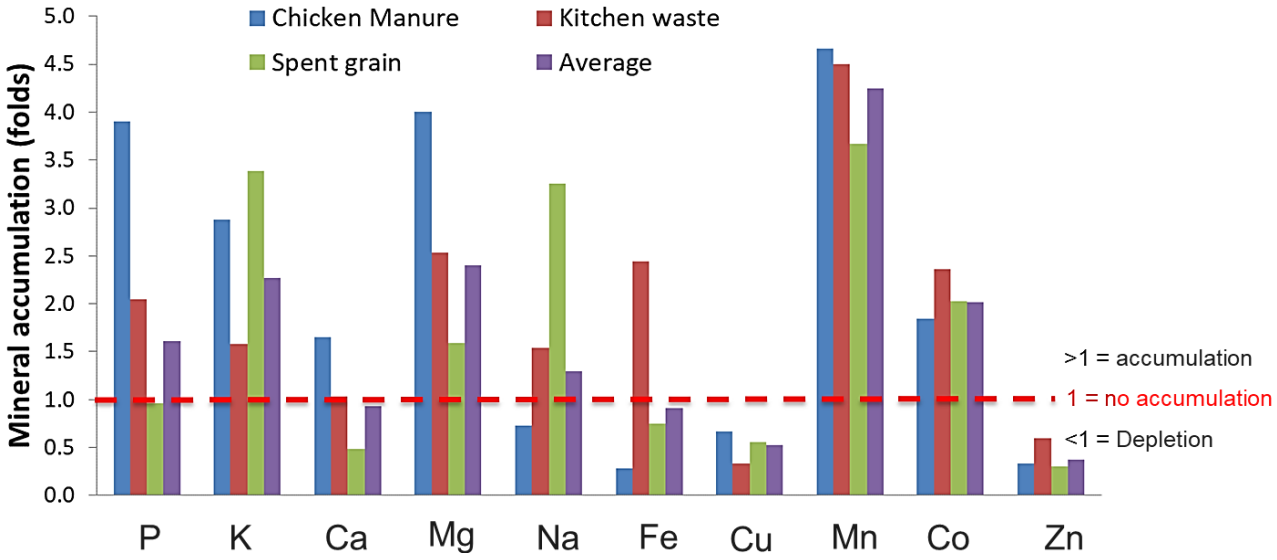


Figure 4. Mineral accumulation in BSFL from different feed sources. Data used to plot this figure have been obtained from the results of Shumo et al. (2019).

1.3.2. Black soldier fly in broiler diets

Environmental sustainability of using BSFL as feed ingredient has been discussed in different studies (Van Huis and Oonincx, 2017, Chia et al., 2019). In addition, insects, such as BSF and their larvae, have been suggested as alternative sources of protein to corn and soybean meals and hence, as potential ingredients for chicken feed (Schiavone et al., 2017, Cutrignelli et al., 2018). The fat content of BSFL is high, but it largely depends on their rearing substrate and developmental stage (Spranghers et al., 2017). Moreover, authorization of insects as animal protein is issued by

the European Commission (2021) to feed aquaculture animals, pigs, and poultry. Since BSFL seem not to accumulate pesticides or mycotoxins (Wang and Shelomi, 2017), BSFL are commercially raised on cereal by-products (Cutrignelli et al., 2018), animal manure and household organic waste and can add value by reducing organic waste biomass by 50–60% (Sheppard et al., 1994).

The biological features of BSFL suggest that it could be considered a potential source of bioactive substances, such as antimicrobial peptides (AMP), which could be highly useful in providing immediate response to the large set of various pathogens and control of other nosocomial infections, and are being currently studied for development of novel antibiotics (Casartelli et al., 2019, Zharkova et al., 2019). According to reports, BSFL have the capacity to reduce the amount of bacteria present in substrates, and this capacity is not accompanied by an increase in load of pathogens in their gut (Bruno et al., 2019). In addition, it was demonstrated that BSFL reduce pathogenic bacteria (*Escherichia coli 0157:H7* and *Salmonella enterica*) in poultry manure (Müller et al., 2017). Black solder fly larvae can partly replace conventional poultry feed ingredients, such as fishmeal and soybean meal which is expected to use less land and water, produce less GGE and has a lower environmental footprint (Dabbou et al., 2018, Ipema et al., 2022).

Little information is available about feeding whole larvae in broilers. Moula et al. (2018) observed that Ardennaise chickens fed 8% of whole de-frosted larvae (corresponding to 2% on DM basis) had significantly higher body weight than those of control chickens. In a recent study, Ipema et al. (2022) evaluated replacement of 8% of broilers diet with BSFL meal. They reported that dried larvae provided in the feeder or scattered through the pen, or live larvae scattered through the pen all increased broiler average daily gains and FCR (Ipema et al., 2022).

2. Hypotheses and objectives

Hypotheses

In the previous chapter, we overviewed the P environmental problems caused by accumulation of P in the environment. To reduce the environmental impact of P, recycling of P from livestock waste and SS sources has been suggested which could be further utilized as an alternative source of P in animal diets. However, depending on the recycling technologies, there is problem of toxic organic compounds and heavy metals contaminating the sludge and, perhaps, the recycled P products.

The advantages of BSFL in waste recycling was discussed and the role of BSFL as a new source of protein in animal diets were addressed. In addition, we reviewed literatures on the effect of using BSFL in broiler diets. Up to date, most of the literatures have been focused on using full fat or defatted BSFL, which requires expensive processing technologies. In this PhD thesis, following hypothesis were tested:

1) Provision of whole BSFL in broiler rations, causes trade-offs between the nutrients and energy obtained from conventional feed and BSFL, leading to imbalances, decreased nutritional efficiency, and poor growth performance.

2) Whole BSFL can be used in broiler diets with no adverse effects on metabolism, carcass characteristics and fatty acid compositions in different tissues of the birds.

3) Using recycled P-rich substrates in BSFL diet causes P accumulation in larvae.

4) Supplementation of broiler diets with recycled mineral-enriched BSFL beneficially affects P intake with no adverse effects on heavy metal intake.

The objectives of the experiment were included:

1) to investigate the interest of broiler chickens in consuming whole BSFL;

2) to determine the extent to which the addition of whole BSFL in broiler rations affects nutrient and energy intakes, utilization efficiency, growth performance, blood metabolites and fatty acids composition of different tissues of the birds;

3) to explore the supplementation of sewage sludge recyclates to feeding substrates on performance and mineral accumulation in BSFL;

4) to investigate the use of BSFL raised on organic materials containing sewage sludge recyclate with high P concentration in broiler diets on performance, heavy metal accumulation, metabolism, and bone status of broilers at different ages.

3. Article 1

3. Effects of increasing levels of whole Black Soldier Fly (Hermetia illucens) larvae in broiler rations on acceptance, nutrient and energy intakes and utilization, and growth performance of broilers

This chapter has been published in:

Poultry Science, 2022 101:102202

Please find the original article via the following digital object identifier:

https://doi.org/10.1016/j.psj.2022.102202

M.M. Seyedalmoosavi[†], M. Mielenz[†], S. Görs[†], P. Wolf[‡], G. Daş[†*], C.C. Metges[†]

[†]Research Institute for Farm Animal Biology (FBN), Institute of Nutritional Physiology, Dummerstorf, Germany.

[‡] University of Rostock, Agricultural and Environmental Sciences, Chair of Nutrition Physiology and Animal Nutrition, Rostock, Germany.

*Corresponding author: gdas@fbn-dummerstorf.de

Manuscript received at the Journal: 13 July 2022

Revision accepted: 15 September 2022

Available Online: 24 September 2022

ABSTRACT

Meal of Black Soldier Fly Larvae (BSFL), which requires extraction of protein and fat, is a novel protein source for poultry, while unprocessed whole BSFL could even directly be fed to chickens. Newly hatched Ross-308 chicks (n = 252) received whole BSFL at 10% (L10), 20% (L20) or 30% (L30) of voluntary feed intake (FI) of control chickens (CON) that received no BSFL but only age-specific diets (n = 63 birds / group) for 42 days (d). Acceptance and nutrient and energy intake of birds by BSFL and FI were calculated. Plasma metabolites were measured using an automatic enzymatic analyzer, and immunoglobulins with ELISA. Depending on the variable, data were analyzed using ANOVA or repeated measures ANOVA to address treatment, time and interaction effects. Birds consumed all offered larvae. With the exception of d1, time spent by birds eating their daily portion of larvae (TSL, min/pen) did not differ among the larvae supply groups ($P = 0.982$). The L10 had a higher larvae eating rate (LER) i.e., speed of larvae intake than did L20 and L30 ($P < 0.05$), implying increased competition for less available BSFL. The ratio of LER to feed eating rate (FER) was greater than 50 fold change difference (FCD), indicating a strong interest of chickens in BSFL over regular feed. Whole BSFL intake up to 30% of voluntary FI did not adversely affect broiler growth ($P > 0.05$). The L30 had lower total dry matter and metabolizable energy intakes ($P < 0.05$), although total fat intake was higher in L30 than in CON ($P < 0.05$). Compared with CON, 30% whole BSFL increased dietary protein-to-energy ratios, plasma uric acid and serum alkaline phosphatase concentrations ($P < 0.05$). We conclude that whole BSFL can be included in broiler rations up to 20% without negatively affecting growth performance and nutrient conversion efficiency, whereas a higher proportion is associated with lower protein utilization efficiency, possibly due to lower total energy intake.

Key words: Chicken, Edible environmental enrichment, Feed preference, Insect, Whole larvae.

INTRODUCTION

Black soldier fly larvae (**BSFL**; *Hermetia illucens*) meal has been suggested as a sustainable alternative protein source to soybean meal (Cutrignelli, et al., 2018; de Souza Vilela, et al., 2021; El-Hack, et al., 2020; Maurer, et al., 2016; Schiavone, et al., 2017). BSFL contain approximately 31 to 45% crude protein (**CP**) in dry matter (**DM**), and are rich in minerals such as Ca and P, implying an important ingredient for chicken diets (Bava, et al., 2019; Maurer, Holinger, Amsler, Früh, Wohlfahrt, Stamer and Leiber, 2016). The inclusion of partially defatted BSFL meal in broiler diets at 10% increased feed intake (**FI**) and average daily gain and improved feed conversion efficiency (Attivi, et al., 2020). The suitability of BSFL-meal up to 20% in broiler diets was confirmed by higher body weight (**BW**) in the grower and finisher phases (de Souza Vilela, Alvarenga, Andrew, McPhee, Kolakshyapati, Hopkins and Ruhnke, 2021). Recent studies have further revealed that the inclusion of BSFL meal in broiler diets may enhance immune functions, likely due to bioactive compounds of BSFL such as chitin and lauric acid (Dörper, et al., 2020; Fariz Zahir Ali, et al., 2019). The inclusion of BSFL meal in broiler diets however implies the extraction of protein and fat from the larvae, which requires expensive feed processing technology. In addition, drying of BSFL may result in lower availability and ileal digestibility of certain amino acids in broiler diets due to the Maillard reaction (Ruhnke, et al., 2018), and may influence the organoleptic characteristics for the birds (Moula, et al., 2018). In contrast to the meal form, less is known about the inclusion of unprocessed whole BSFL in poultry rations on acceptance, nutrient intake and utilization, performance and health of the birds. Chickens are excellent foragers of insects as these are among their natural feed sources (Star, et al., 2020). Feeding experiments indicated that diets containing insects are highly interesting for poultry species (Moula, Scippo, Douny, Degand, Dawans, Cabaraux, Hornick, Medigo, Leroy and Francis, 2018; Nascimento

Filho, et al., 2020; Star, Arsiwalla, Molist, Leushuis, Dalim and Paul, 2020; Tahamtani, et al., 2021). The use of whole BSFL as feed for poultry may be particularly important in organic farming and low-input systems (e.g. local farming with less feed processing and transportation), and where insect production could be integrated into production cycles (e.g. insects farming with locally available organic residues as feed substrate) (Nyakeri, et al., 2017). In this context, it has been reported that BW of chickens fed a standard diet supplemented with 8% whole defrozen larvae were higher than in control chickens (Moula, Scippo, Douny, Degand, Dawans, Cabaraux, Hornick, Medigo, Leroy and Francis, 2018). In addition, inclusion of 5% and 10% live BSFL in broiler rations increased activity and improved leg health without adverse effects on broiler performance (Bellezza Oddon, et al., 2021; Ipema, et al., 2020b). Recently, Tahamtani, Ivarsson, Wiklicky, Lalander, Wall, Rodenburg, Tuyttens and Hernandez (2021) fed laying hens with 0, 10, 20% of the daily DM intake or ad libitum with live BSFL and found no difference in BW of hens given BSFL up to 20%, whereas ad libitum BSFL fed hens were heavier and consumed more protein, fat and energy than control hens. However, there are no data to show how a proportion of more than 10% whole BSFL in broiler rations affects acceptance, nutrient intakes, bird performance and health. It is well known that the nutrient composition and form of broiler diets impact on their energy intake (Latshaw, 2008). Broilers can adjust their feed intake (**FI**) in response to alterations of certain dietary factors such as energy level (Hu, et al., 2021), amino acid balance (Ferket and Gernat, 2006), fiber content (Jha and Mishra, 2021) and mineral balance (Delezie, et al., 2015). Nutrient, moisture and energy contents of BSFL and standard broiler diets differ greatly (e.g. see Table 1). Therefore, we hypothesized that the provision of whole BSFL on top of balanced broiler rations, particularly at high levels, induces trade-offs in nutrient and energy intake from regular feed and BSFL, ultimately resulting in imbalances and lower nutrient

efficiency and impaired growth performance. Consequently, the objective of this study was to first investigate the interest of broiler chickens in consuming whole BSFL and then to determine the extent to which the inclusion of whole BSFL in broiler rations influences nutrient and energy intakes, utilization efficiency, as well as health statutes, growth performance, selected blood metabolites and immunity of the birds.

MATERIALS AND METHODS

A feeding experiment was conducted over 6 weeks (**wk**). Animal care and handling, stunning and slaughtering of the birds were performed by trained and authorized staff. The feeding experiment was registered under A.Z. 202022_70_A28_anz.

Animals and management

A total of 252 newly hatched chicks (Ross 308) was obtained from a commercial hatchery and housed at the experimental poultry facility at the Research Institute for Farm Animal Biology (**FBN**), Dummerstorf, Germany. The chicks were weighed at arrival and randomly allocated to one of 24 pens (n=10-11 chicks / pen) in 4 adjacent rooms of the facility. Pens in each room (n=6) were separated from each other with solid panels. Each pen was equipped with a feeder, a line of drinking nipples with cups, and a deep layer of wood shavings as litter material. Throughout the experiment, birds in different rooms were kept under the same environmental conditions. Climate conditions in the rooms were automatically controlled based on recommendations of the Aviagen Ross broiler handbook (Aviagen, 2018) by a ventilation and heating system, ensuring uniform temperature, light and aeration conditions across the pens within and between the 4 experimental rooms. Ambient temperature at the start of the experiment was 33 °C and this was gradually decreased to 21 °C at wk 6, whereas humidity was gradually increased from 37% to 70% until wk 6. The light program included a 21-hours (**h**) light (30-40 Lux) and a 3h dark period during the

first 3 days (**d**). By d4, lighting was changed to 18h of light (15-20 Lux) and 6h of darkness until the end of the experiment.

Experimental design

A completely randomized design with four treatments was used in this study. All birds received the same basal diet, designed to meet or exceed age-specific nutrient requirements of broilers in three phases, i.e. starter (d 0-14), grower (d 15-28) and finisher (d 29-42) diets (Table 1) (Aviagen, 2019). Equal number of pens (n = 6 per group) and birds (n = 63 per group, n = 10-11 per pen) were randomly allocated to each of 4 dietary treatments in four adjacent rooms. Each of the 4 treatment groups was represented in each of the 4 rooms with one or two pen-replications with further randomization for the position of the pens/treatment groups in the rooms. Broilers in the control group (**CON**, n = 63 birds in 6 pens) received the age-specific basal diet ad libitum, and had no access to BSFL. Birds in the remaining 18 pens received defrozen whole BSFL in addition to the ad libitum offered basal diet at increasing levels, i.e. 10%, 20% or 30% of the FI of CON birds (hereafter referred to as groups **L10** (n = 63), **L20** (n = 63), and **L30** (n = 63), respectively). Except for the first d of life, the daily amount of BSFL to be fed to the broilers in the L10 to L30 groups was calculated based on the FI of the CON birds on the previous day. At the first d of life, FI of broiler birds from previous experiments was used as a reference. Whole BSFL were purchased from Hermetia Deutschland GmbH & Co. KG, Baruth/Mark, Germany. All the larvae used in this experiment originated from the same rearing batch. Analyzed chemical composition of the BSFL is provided in Table 1. As soon as the live larvae were received, they were snap frozen using liquid nitrogen and stored at -20 °C until fed to broilers. Approximately 12h before feeding larvae to broilers, the larvae were stored in a refrigerator (4 °C) to thaw and then kept at room temperature for weighing of the daily portions for broilers in the L10-L30 groups. Age-specific

diets were in the form of regular feed that was coarsely ground and not pelleted. Ingredients and analyzed chemical composition of the age-specific diets are provided in Table 1. The diets were purchased from a commercial feed producer (Ceravis AG, Rendsburg, Germany).

Table 1. Ingredients and analysed chemical composition of the age-specific diets and black soldier fly larvae (BSFL) offered to broilers during the experimental period.

	Basal diets			
	Starter (d 1-14)	**Grower (d 15-28)**	**Finisher (d 29-42)**	**BSFL (d 1-42)**
Ingredients, % as fed				
Soybean meal 48%	36.0	34.0	26.5	-
Wheat	31.0	28.0	35.0	-
Maize	21.5	28.0	28.0	-
Barley	5.0	4.0	5.0	-
Linseed oil	3.0	3.0	3.0	-
Vitamin-Mineral Premix[1]	2.5	2.5	2.5	-
Oyster shells	1.0	0.5	0	-
Whole black solider fly larvae	-	-	-	100
Chemical analysis, g/kg DM				
Dry matter	893	891	892	312
Crude ash	61.6	48.3	44.8	80.4
Crude protein[2]	228	218	203	435
Crude fat	47.0	47.1	42.6	277.5
Crude fibre	24.6	25.8	34.8	72.7
Starch[3]	463.6	508.4	516.8	14.3
Total sugar (calculated as sucrose)	43.67	46.02	42.60	2.20
aNDF	118	114	110	121
ADF	41.4	43.8	38.1	77.78
ADL	n.d.	n.d.	n.d.	4.42
Chitin*	n.d.	n.d.	n.d.	73.35
ME, MJ/kg DM	13.4	14.0	13.8	16.5
Minerals, g/kg DM				
Calcium	12.1	7.6	6.1	18.1
Phosphorus	6.6	5.2	5.2	9.3
Magnesium	2.1	1.9	2.0	3.9
Sodium	1.6	1.0	1.2	1.3
Potassium	10.2	9.0	8.9	12.8
Iron	0.024	0.019	0.022	0.012
Manganese	0.011	0.084	0.011	0.021
Copper	0.017	0.014	0.019	0.010
Zinc	0.097	0.074	0.093	0.013

[1]Amount of vitamin and minerals provided through premix per kg of feed are as following; Vit. A (from vitamin A acetate), 10000 IU; Vit. D3, 2000 IU; Vit. E (from DL-α-tocopherol acetate), 20 mg; Vit. K3, 3 mg; Vit. B1, 1 mg; Vit. B2, 6 mg; Vit. B6, 3 mg; Vit. B12, 30 mcg; Niacin, 30 mg; Pantothenic acid, 10.8 mg; Folic acid, 0.4 mg; Biotin 24, mcg; Cholin, 300 mg; Fe, 55 mg; Cu, 18 mg; Zn, 80 mg; Mn, 93 mg; I, 0.66 mg; Se, 0.34 mg; Co, 0.05 mg; Phytase, 250 FTU.

[2] Crude protein content of BSFL might be overestimated due to the high non-protein content (e.g. chitin) (Shumo, et al., 2019). For amino acid composition see Supplementary Table 1.

[3]For BSFL it is glycogen

*: Calculated based on Hahn et al. (Hahn, et al., 2018) (i.e. Chitin = ADF - ADL)

Abbreviations: **NDF**: Neutral detergent fiber; **ADF**: Acid detergent fiber; **ADL**: Acid detergent lignin; **n.d.**: not determined.

Larvae provision and time records

In order to assess acceptance and interest of chickens in BSFL, we recorded the time broilers spent eating larvae and calculated a larvae eating rate. Birds in larvae supply groups, i.e. L10, L20, and L30 received defrozen BSFL at the same time each day (by 07:30 h). For this purpose, the defrozen larvae to be given to the birds of a pen were weighed and placed on a feeding plate. The plate was placed on the ground of the recipient pen, and the start time of larva eating by the birds was recorded. The pens were observed frequently, and the time when there were no more larvae left on the plate was recorded with a precision of 1 min. The time spent eating BSFL (**TSL**, min/pen) was then calculated in min (i.e. end time – start time) for each day. The BSFL eating rate (**LER**, g/min) of the birds, i.e. the amount of BSFL eaten per min was calculated. The LER was also adjusted for the BW of the birds in the pen (**LER_BW**), i.e., the amount of BSFL eaten per min and kg BW of chickens in a pen (g BSFL / kg BW^{-1} min^{-1}). The LER_BW was estimated only for the last d of each wk, where the corresponding BW for that wk was measured. Similar to LER, a theoretical feed eating rate (**FER**) was also calculated. For this purpose, the daily amount of feed consumed by the birds of a pen was divided by the duration of the light period of the day (i.e., g feed / daily light period in min). The FER was also adjusted for BW (i.e. **FER_BW**: g feed / kg BW^{-1} $min^{-1\ in\ light\ period}$), using corresponding FI and BW data on the last d of each wk. Finally, the ratio of BSFL eating rate to feed eating rate was calculated as fold change differences (**FCD**, i.e. LER:FER) without and with consideration of BW (**FCD_BW**). Throughout the study, larvae provisioning and time records were kept by the same person.

Feed intake and growth performance

Pen based daily FI from the previous day was measured in the mornings, and average daily or weekly total FI per bird was then calculated. The weekly total fresh matter intake (**FMI**; the sum

of feed and larval intake) and the resulting dry matter intake (**DMI**) per average bird of each pen were calculated. Based on the amounts of feed and BSFL intakes, and the nutrient and energy contents of the diets and BSFL, pen based nutrient (e.g. protein, fat) and metabolizable energy (**ME**) intakes were calculated for an average bird. The growth performance of the broilers was evaluated throughout the experimental period. The pen based average BW, FI, FMI, DMI, feed conversion ratio (**FCR**) with consideration of either FMI (i.e. FCR-1: g FMI per g BW gain) or DMI (i.e. FCR-2: g DMI per g BW gain), protein conversion ratio (**PCR**: g protein intake to gain 100 g BW) and energy conversion ratio (**ECR**: MJ ME intake to gain 100 g BW) of the birds were calculated on a weekly basis. To assess the homogeneity of growth of birds in a pen, a weekly coefficient of variation (**CV**) of BW was calculated for each pen.

Foot and leg health and litter moisture

Footpad dermatitis (**FPD**) and hock burn (**HB**) assessments were performed prior to slaughter on d 28 and d 42 using the Welfare Quality® assessment protocol for poultry (Welfare-Quality, 2009). If feet were dirty, they were carefully cleaned with a damp cloth before scoring; only the central plantar area was scored, and signs of foot pad lesions were recorded on a 5-point scale, where 0 indicated no lesion. Litter samples (~100 g) to measure moisture were collected from each pen on d 42. Samples were oven-dried at 103°C until they reached a constant weight.

Chemical analysis of diets and BSFL

During the experiment, feed and larval samples were collected regularly and stored at −20°C for chemical analyses. At the end of the experiment, all of the sub-samples were pooled by feed type (e.g. starter, grower, and finisher) and representative samples were analyzed for their nutrient contents. Larvae and feed samples were analyzed for DM content, crude ash, CP, crude fat, starch, crude fibre (**CF**), neutral detergent fiber (**NDF**), acid detergent fiber (**ADF**), and selected macro-

and trace minerals (Table 1) by the accredited feed laboratory of Landwirtschaftliche Untersuchungs-und Forschungsanstalt, LMS Agrarberatung GmbH (Rostock, Germany) using standard methods (Naumann, et al., 1997). The ME contents of feed and BSFL were then estimated using the equation, ME, MJ/kg DM = [(g CP × 0·01551) + (g CL × 0·03431) + (g starch × 0·01669) + (g sucrose × 0·01301)]. For BSFL, acid detergent lignin (**ADL**) content was additionally determined to estimate chitin (i.e., ADF - ADL) content (Hahn, et al., 2018). Table 1 presents the ingredients and chemical compositions of the age-specific basal diets, and summarizes the nutrient composition of BSFL. Age-specific basal diets and drinking water were provided ad libitum throughout the experimental period.

The amino acid (**AA**) compositions of the diets and larvae samples were determined using high performance liquid chromatography (**HPLC**) (1200/1260 Infinity II series, Agilent Technology, Waldbronn, Germany) (Kuhla, et al., 2010) after acidic hydrolysis of samples using a 250 × 4.6 Gemini 5 µm reversed-phase C18 110 Å column protected with a 4 × 3 pre-column (Phenomenex, Germany). Five mg of lyophilized ground sample was suspended in 2 ml of 6 M HCl. After addition of 50 µl of ascorbic acid (16 mg/ml ultrapure water), oxygen was removed from the suspension with a strong N_2 flow for 1 min, and then the sample was heated for 22 h at 110 °C. The hydrolysate was dried at 60 °C with a constant N_2 flow and then re-suspended in 2 ml of 0.1 M HCl. The suspension was then centrifuged at 1573 × g at 4°C for 20 min. For AA analysis the supernatant was diluted 1/10 with ultrapure water. The AA chromatograms were integrated with the OpenLab ChemStation software (Agilent Technologies, Waldbronn, Germany) and the AA concentrations were calculated based on a calibration with a standard AA mixture (A9906, Sigma-Aldrich/Merck, Darmstadt, Germany). The AA compositions (%) of the diets and BSFL are summarized in Supplementary Table 1.

Blood metabolites and immunoglobulin isotypes

In the end of experimental wk 4 and wk 6, two birds per pen (n = 48 / time point) were randomly chosen, weighed and slaughtered after electrical stunning. From each bird, slaughter blood was collected to obtain serum and plasma. For serum collection blood samples were kept at room temperature for approximately 1h to allow for clotting, and were then centrifuged (2,500 × g for 20 min at 4°C). Serum was harvested and stored in Eppendorf vials (Sarstedt AG & Co., Nümbrecht, Germany) at −20°C until analysis of alkaline phosphatase (**ALP**) activity using an automatic enzymatic analyzer (ABX Pentra 400, Horiba Medical, Montpellier, France) with a commercial kit (ALP Kit No. A11A01626). Plasma was harvested after centrifugation of K3-EDTA-coated evacuated tubes (Sarstedt AG & Co., Nümbrecht, Germany). Plasma albumin, total protein and uric acid (**UA**) concentrations were analyzed with ABX Pentra 400 using commercial kits [albumin: Kit No. A11A01664, total protein: Kit No. 553-412, uric acid: Kit No. LT-UR0010 (MTI diagnostics, Idstein, Germany)]. Globulin concentration was calculated as total protein minus albumin. Commercial ELISA kits (IgY: Kit No. E30-104; IgM: Kit No. E30-103; IgA: Kit No. E30-102; Bethyl Laboratories, Inc, Montgomery, TX, USA) were used to analyze immunoglobulin isotype concentration (IgY, IgM, IgA) in plasma samples. The intra-assay CV and inter-assay CV of Ig analysis ranged between 5.0 – 7.6% and 7.7 – 10.4%, respectively.

Statistical analysis

The pen was considered the experimental unit for all pen-based measurements, e.g., daily or weekly feed intake, larvae intake and nutrient intakes, time spent eating larvae, larval and feed eating rates, and BW. Weekly measured pen data for growth parameters (e.g. BW), daily nutrient and energy intake (e.g. weekly FI, protein intake, energy intake), and the corresponding weekly nutrient and energy conversion indices calculated for FCR, PCR and ECR were analyzed using a

linear mixed model (PROC MIXED) in SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). The statistical model included fixed effects of the treatment group (1-4), week (1-6) and treatment group by week interaction. The blocking effect of rooms (1-4) was also included in the model. Pen (n = 24) was considered as repeatedly measured subject over time, and was implemented in the statistical model. For daily measured or calculated variables (e.g. time spent larvae eating, larval and feed eating rates), the above mentioned model was used with day (1-42) instead of week effects. For the single-point measurements (e.g. blood metabolites and IgY isotypes) the experimental unit was a bird sampled at slaughterhouse (N = 96). Thus data related to the parameters measured on individual birds were analyzed by the general linear model procedure of SAS (PROC GLM). The statistical model included fixed effects of treatment group (1-4) and slaughter week (4, 6), interaction term, and the blocking effects of room and pens. Group differences were separated by Tukey-Kramer test at $P < 0.05$. The SLICE statement of PROC MIXED of SAS was used to conduct partitioned analyses of the LSM for interactions between treatment by day (or week) when required. The significance level was preset at $P < 0.05$, and a tendency was declared at $0.05 < P \leq 0.10$. Values are presented as LSM with their SE.

A principal component analysis (**PCA**) was conducted using JMP statistical software V.15 (SAS Institute) to investigate potential trade-offs in nutrient intakes due to different levels of BSFL in the broiler ration, and identify relevant nutrients as potential driving forces that are principally associated with differentiation among the groups in nutrient and energy intakes. For this purpose we selected 5 representative nutrients to address energy (i.e. crude fat and starch intakes), protein (CP intake), dietary fiber (NDF intake), mineral (crude ash) intakes and conducted a PCA using the weekly recorded data of the corresponding nutrients collectively.

RESULTS

All chickens appeared to be healthy, and no bird died during the experiment. The overall average FPD and HB scores were 0.0 and 0.02, respectively. Because of FPD and HB were absent or extremely low no statistical comparison of the groups was performed. The DM content of the litter ranged from 78 to 81%, and did not differ among the 4 groups ($P > 0.05$; further data not shown).

Black soldier fly larvae and feed intakes

With the exception of the first 3 d, the daily pre-determined portions of BSFL offered to the birds were consumed fully within a few min in all 3 BSFL groups (Figure 1A). For instance, on d1 L30 birds spent 509 min to consume their portion of BSFL, whereas it took only 7 min to consume BSFL equivalent to 30% of the CON FI on d42. The average TSL ranged between 11.3 and 20.5 min in L10 to L30 groups with no group difference ($P = 0.982$; Table 2) expect for the first d ($P < 0.05$; Figure 1A). On the first d, L30 birds spent more time ($P < 0.05$) to consume 30% of CON feed intake as BSFL than birds in the L10 and L20 groups. In the remaining 41 d, the TSL was similar among the 3 groups regardless of the amount of BSFL offered to the birds ($P > 0.05$). The LER depended on both the amount of BSFL offered to the birds ($P=0.010$; Table 2) and experimental d ($P < 0.001$; Figure 1B). The L10 birds consumed their portion of BSFL at a higher rate (LER = 3.97 g/min) ($P < 0.05$) than the L20 and L30 birds (Table 2). The average total LER across the 3 groups increased linearly by more than 200-fold from the first day (0.03 g/min) to the last day (6.8 g/min) of the experiment (Figure 1B). When corrected for the increasing bird BW over time (i.e. LER_BW), a linearly decreasing pattern of BSFL eating rate was observed ($P < 0.001$; Figure 1C), which nevertheless showed no dependence on the amount of BSFL offered to the birds ($P = 0.138$; Table 2). The LER_BW showed a linear decrease of two thirds from approximately 9 to 3 g/kg per min. (Figure 1C).

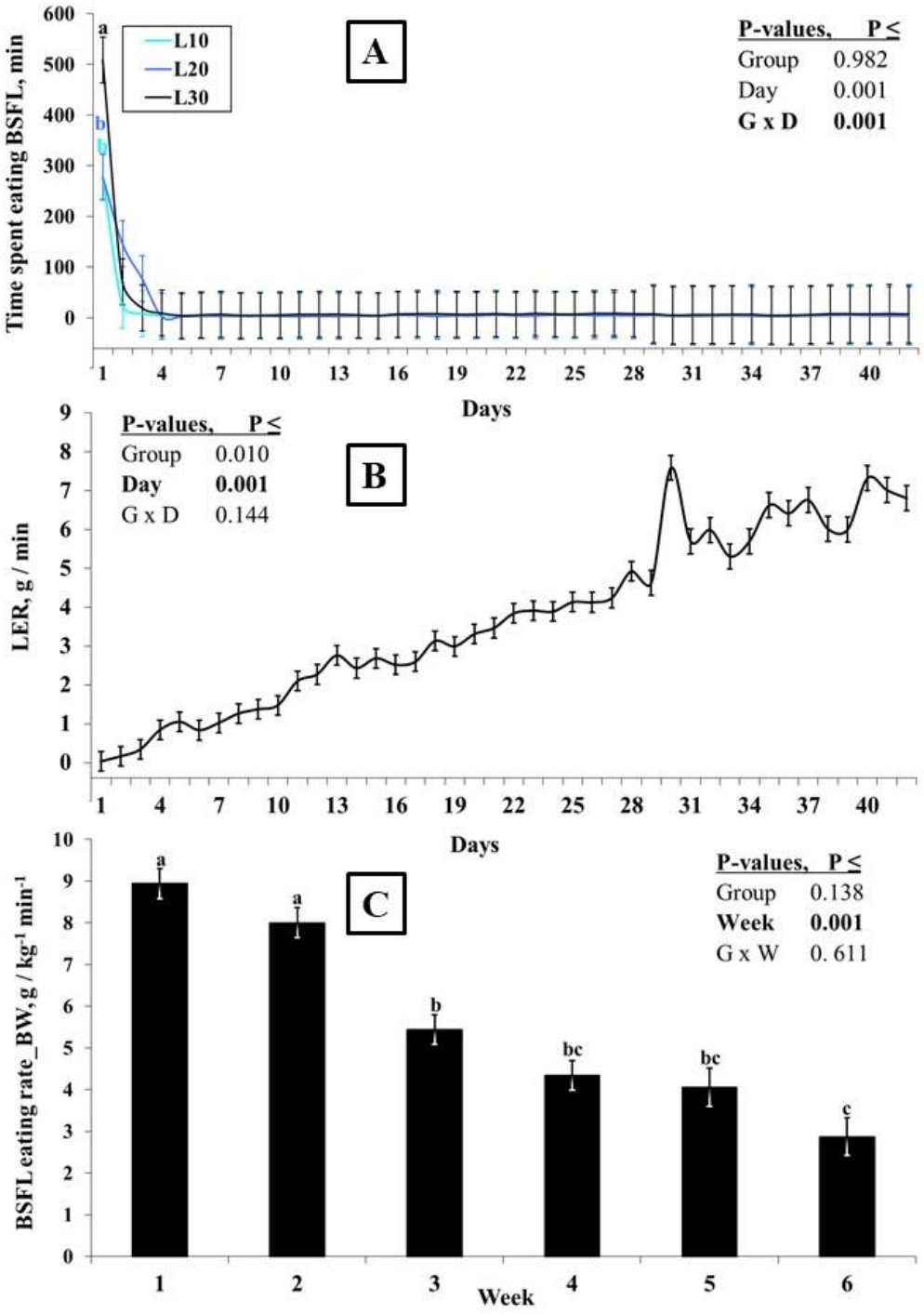


Figure 1. Time spent eating whole black soldier fly larvae (BSFL) (TSL) (**A**), larvae eating rate (LER) (**B**) and body weight adjusted LER (**C**) in broilers offered BSFL at 10% (L10), 20% (L20) or 30% (L30) of daily feed intake of control chickens. Values are LSM with their SE. a-c: Values denoted with different letters within each panel differ significantly (Tukey, $P < 0.05$).

Table 2. Impact of BSFL provision at increasing dietary levels on feed and BSFL eating rates in relation to time and body weight development of broilers.

	Dietary treatment groups[1]					P-values[2], ≤		
	CON	**L10**	**L20**	**L30**	**SE**	**G**	**W**	**G×W**
BSFL intake, g *(N=126, i.e. 3G × 42d)*	n.a.	9.1	1.2	27.3	n.a.	n.a.	n.a.	n.a.
TSL, min *(N=756, i.e. 3G × 42d × 6 pen)*	n.a.	11.3	15.6	20.5	33.65	0.982	0.001	0.001
LER, g / min *(N=756, i.e. 3G × 42d × 6 pen)*	n.a.	3.97^{a}	3.59^{b}	3.56^{b}	0.085	0.010	0.001	0.144
LER_BW, g / kg^{-1} min^{-1} *(N=108, i.e. 3G × 6wk × 6 pen)*	n.a.	6.09	5.25	5.49	0.276	0.138	0.001	0.611
Feed intake, g/day *(N=1008, i.e. 4G × 42d × 6 pen)*	94.1^{a}	84.9^{b}	84.5^{b}	71.9^{c}	1.16	0.001	0.001	0.001
FER, g / min in light period *(N=1008, i.e. 4G × 42d × 6 pen)*	0.087^{a}	0.079^{b}	0.078^{b}	0.066^{c}	0.0011	0.001	0.001	0.001
FER_BW, g / kg^{-1} min^{-1} in light period *(N=144, i.e. 4G × 6wk × 6 pen)*	$0.112^{a\dagger}$	$0.105^{ab\dagger}$	0.104^{b}	0.096^{c}	0.0018	0.001	0.001	0.001
FCD (LER:FER) *(N=108, i.e. 3G × 6wk × 6 pen)*	n.a.	55.4	50.1	56.6	2.58	0.195	0.018	0.386
FCD (LER:FER)_BW *(N=108, i.e. 3G × 6wk × 6 pen)*	n.a.	55.4	50.1	56.6	2.71	0.222	0.020	0.371

a-c: Treatment groups denoted with different letters differ significantly (Tukey, $P<0.05$). The sign (†) indicate tendency to differ (Tukey, $0.05 < P \leq 0.10$). Data are presented as LSEMANS and their SE.

[1]Dietary treatments: ad-libitum feed without access to BSFL (CON), or with BSFL amounting to 10% (L10), 20% (L20) or 30% (L30) of the feed intake of CON birds. Total number of observations used for statistical analyses, N = 144 (4 treatments each with 6 replicate pens repeatedly measured over 6 weeks). Number of observations; Number of birds, n=63 per treatment.

[2]G = Group effect, W = time effect (week or day depending on the variable), G×W = group by time interaction.

Abbreviations: **TSL**: Time spent eating BSFL; **LER**: BSFL eating rate of chickens; **LER_BW**: BSFL eating rate of chickens adjusted per kg BW; **FER**: theoretical feed eating rate; **FER_BW**: theoretical feed eating rate corrected for kg BW; **FCD**: fold change difference in ratio of LER:FER; **FCD_BW**: FCD corrected for BW.

Sample size (N) and its calculation together with further information on pen replicates and time dimension are provided beneath of each variable in *italics*.

Provision of BSFL to broilers reduced ($P < 0.001$; Table 2) voluntary FI in both L10 and L20, and did more so strongly in L30 compared with CON in a time-dependent manner ($P < 0.05$; Figure 2A). The L30 group had a lower FI compared with CON already at wk 2, and this difference remained constant until the end of the experiment ($P < 0.05$; Figure 2A). The FI in L10 and L20 was lower than that of CON at wk 3 and 4 ($P < 0.05$). Until wk 5 of the experiment, there was no difference in FMI between groups ($P > 0.05$; Figure 2B). At wk 6, both L30 and L20 birds consumed a higher amount of FM than CON and L10 birds ($P < 0.05$). In contrast to L10 and L20 groups, birds offered BSFL at 30% of the CON intake had an impaired DM intake ($P < 0.05$; Table 3), as early as wk 3 (Figure 2C).

Table 3. Effects of increasing levels of whole black soldier fly larvae in broiler rations on fresh and dry matter intakes, nutrient and energy intakes, growth performance and nutrient and energy conversion ratios of broilers.

	Dietary treatment groups[1]					P-values[2], ≤		
	CON	**L10**	**L20**	**L30**	**SE**	**G**	**W**	**G×W**
Nutrient and energy intakes								
Fresh matter, g / wk	662.3	661.2	718.3	694.3	17.95	0.124	0.001	0.001
Dry matter, g / wk	590.6^{a}	552.7^{ab}	566.9^{ab†}	508.6^{b†}	16.01	0.015	0.001	0.001
Protein, g / wk (6.25*)	124.5	120.8	128.1	120.4	3.29	0.329	0.001	0.001
Fat, g / wk	26.37^{c}	29.26^{b}	34.49^{a}	36.53^{a}	0.69	0.001	0.001	0.001
Starch, g / wk	300.1$^{a\dagger *}$	271.3^{a†}	269.0^{a*}	229.2^{b}	8.24	0.001	0.001	0.001
Crude fibre, g / wk	18.1	17.8	19.2	18.2	0.54	0.338	0.001	0.007
ADF, g / wk	23.9	23.0	24.3	22.7	0.62	0.265	0.001	0.001
NDF, g / wk	64.3^{a}	60.4^{ab}	62.2^{ab}	56.1^{b}	1.75	0.027	0.001	0.001
Chitin, g / wk	n.a.	1.46	2.92	4.37	-	-	-	-
Crude ash, g / wk	28.3	27.0	28.2	26.2	0.73	0.158	0.001	0.001
Ca, g /wk	4.29	4.19	4.47	4.30	0.103	0.303	0.001	0.001
P, g / wk	3.10	2.98	3.13	2.91	0.083	0.218	0.001	0.001
Mg, g / wk	1.18	1.14	1.20	1.13	0.032	0.314	0.001	0.001
ME, MJ / wk	8.17^{a}	7.70^{ab}	7.95^{ab}	7.19^{b}	0.22	0.035	0.001	0.001
CP:ME ratio	15.8^{d}	16.2^{c}	16.7^{b}	17.2^{a}	0.025	0.001	0.001	0.001
Growth performance								
Initial weight, g	41.9	41.7	41.5	41.7	0.157	0.488	-	-
BW, g	1118	1037	1073	981	49.2	0.280	0.001	0.033
CV of BW, %	20.0^{b†}	26.0^{ab}	27.0^{ab†}	33.9^{a}	2.34	0.006	0.001	0.129
Nutrient and energy conversion ratios								
FCR-1, FMI / BWG	1.59^{b}	1.67^{b}	1.69^{b}	1.87^{a}	0.034	0.001	0.001	0.102
FCR-2, DMI / BWG	1.414	1.398	1.332	1.364	0.024	0.132	0.001	0.022
PCR, g CP / 100 g BWG	30.5^{a}	31.2^{ab}	30.8^{ab†}	33.0^{b†}	0.578	0.030	0.001	0.046
ECR, MJ ME / 100 g BWG	1.948	1.939	1.859	1.92	0.112	0.306	0.001	0.022

a-c: Groups denoted with different letters differ significantly (Tukey, $P < 0.05$). The signs (†,*) indicate a tendency to differ (Tukey, $0.05 < P \leq 0.10$). Data are presented as LSM and their SE.

[1]Dietary treatment groups: ad-libitum feed without access to BSFL (CON), or with BSFL amounting to 10% (L10), 20% (L20) or 30% (L30) of the feed intake of CON birds. Total number of observations used for statistical analyses, N = 144 (4 treatments each with 6 replicate pens repeatedly measured over 6 weeks). Number of observations; Number of birds, n = 63 per treatment group.

[2]G = group effect, W = time effect (week), G×W = group by time interaction. **Abbreviations**: **Ca**: Calcium; **P**: Phosphorus; **BW**: body weight; **BWG**: Body weight gain; **PCR**: Protein conversion ratio (i.e. g protein needed to gain 100 g BW); **ECR**, Energy conversion ratio (i.e. MJ metabolizable energy needed to gain 100 g BW); **FCR-1**, feed conversion ratio based on FMI (i.e. g FM intake per g BW gain); **FCR-2**, feed conversion ratio corrected for DM intake (i.e. g DM intake per g BW gain).

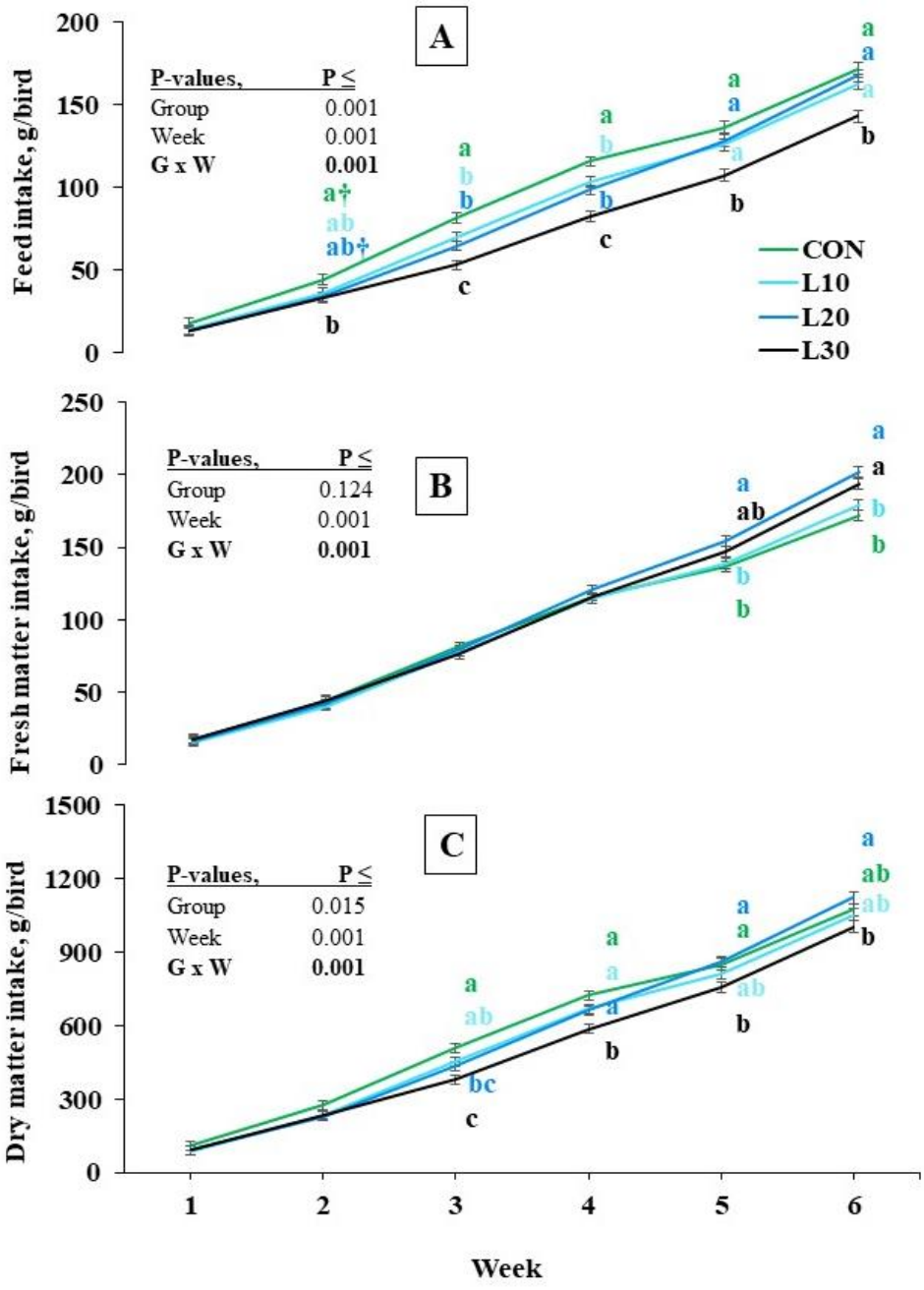


Figure 2. Effects of increasing levels of whole black soldier fly larvae in broiler rations on average feed intake (**A**), fresh matter intake (feed plus larvae) (**B**), and dry matter intake (feed plus larvae) (**C**) during the experimental weeks. Values are LSM with their SE. a-c: Values denoted with different letters at the same point within each panel differ significantly (Tukey, $P < 0.05$). The symbol † indicates a tendency of two treatments to differ (Tukey, $0.05 < P \leq 0.10$). The symbol † indicates a tendency of two treatments to differ (Tukey, $0.05 < P \leq 0.10$).

Consistent with the FI pattern, the FER of the birds (g / min) was lower in L10 to L30 than at CON ($P < 0.001$; Table 2), increased linearly in all groups, and became more clearly BSFL-dose-dependent over time (Figure 3A). The increase in FER of the birds from the first to the last d of experiment was about 25-fold. Adjusted for BW, the feed eating rate (FER_BW) showed a linear decrease in all 4 groups (Figure 3B), although differences among the groups were also strongly dependent on time. While CON birds had a higher FER_BW than L30 birds at wk 1, 3 and 4 ($P < 0.05$), there was no difference between groups by wk 5 ($P > 0.05$).

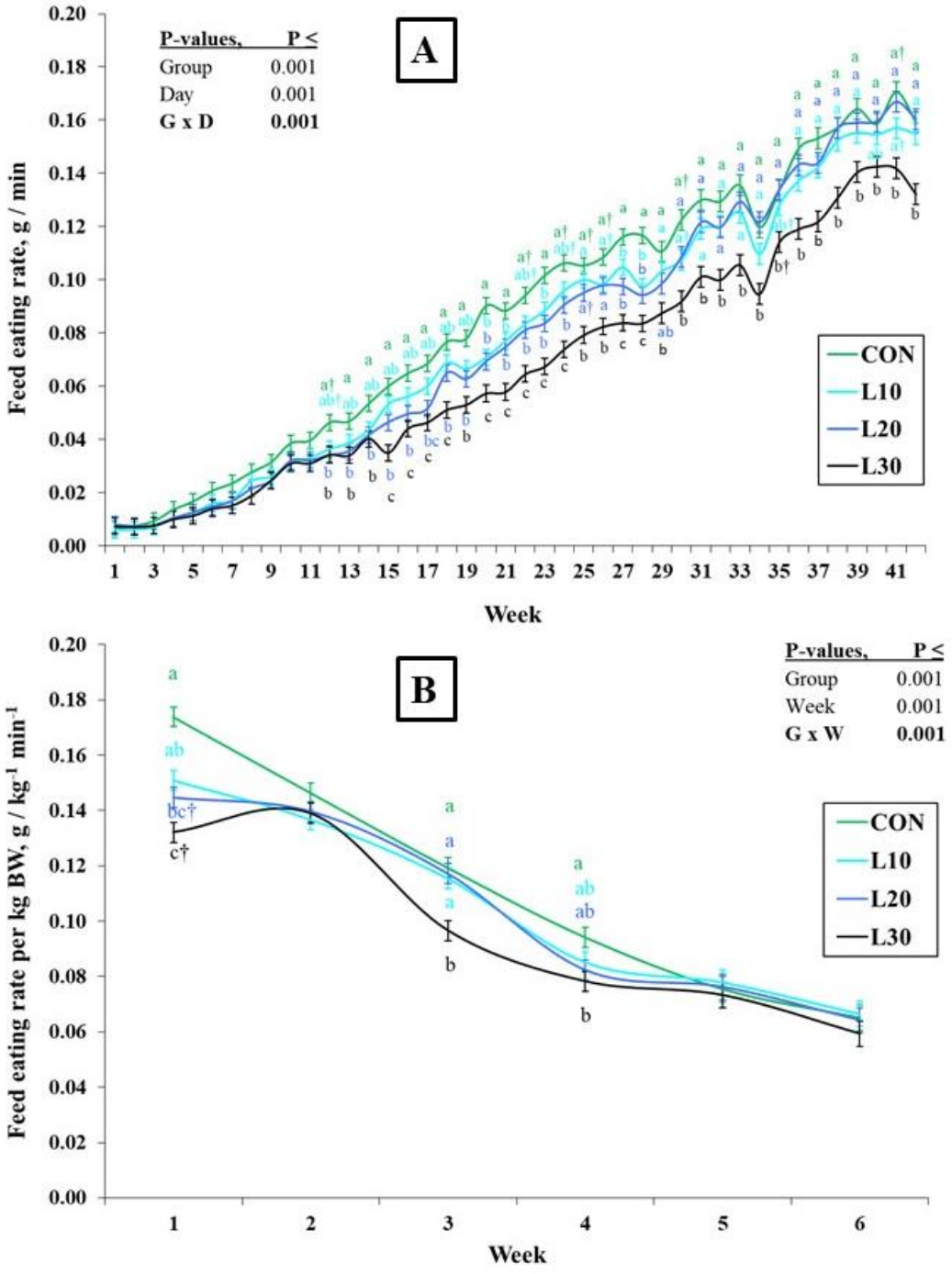


Figure 3. Effects of increasing levels of whole black soldier fly larvae in broiler rations on feed eating rates without (FER) (A) or with an adjustment per kg body weight (FER_BW) (B). Values are LSM with their SE. a-c: Values denoted with different letters at the same point within each panel differ significantly (Tukey, $P < 0.05$). The symbol † indicates a tendency of two treatments to differ (Tukey, $0.05 < P \leq 0.10$).

In order to compare time-dependent changes in larvae and feed eating rates simultaneously over 6 weeks, we calculated FCD in ratios of LER to FER only in the 3 larvae consuming groups. The FCD was not influenced by the amount of larvae offered to the birds (P = 0.195; Table 2), but slightly decreased by time with a difference only between wk 1 and wk 6 (P = 0.018; Figure 4). The FCD in BSFL to feed eating rates provided almost the same results when BSFL and feed eating rates were adjusted for BW (see Table 2).

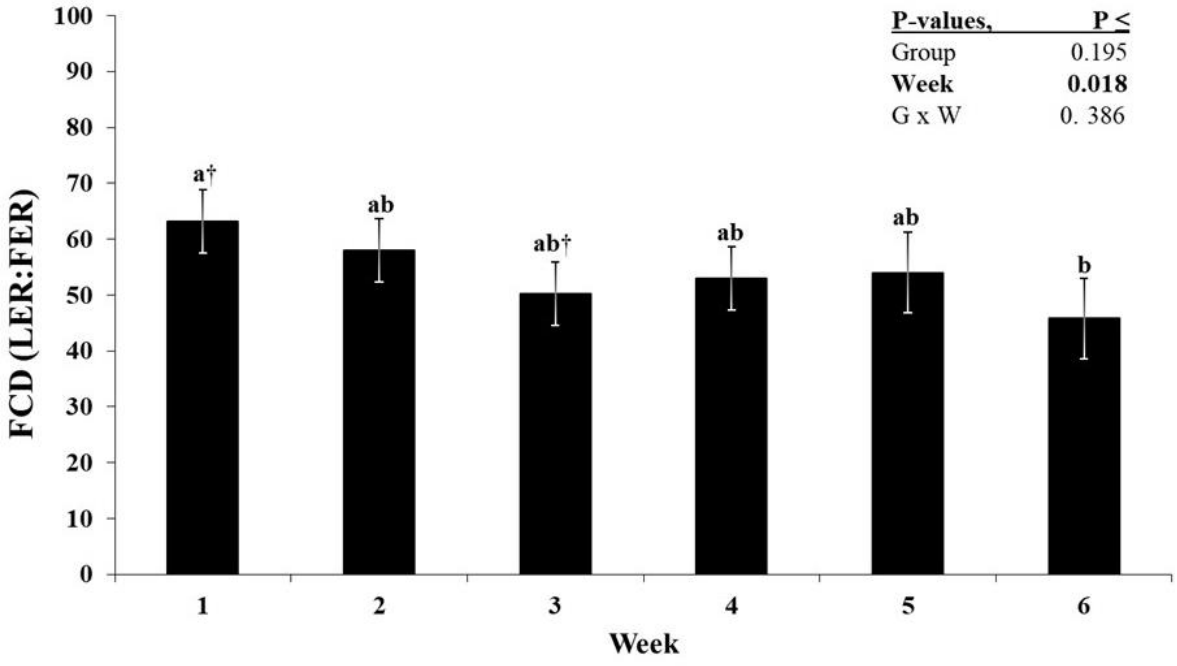


Figure 4. Time dependent changes of fold change differences (FCD) in ratios of larvae eating rate to feed eating rate (LER:FER) of broilers fed increasing levels of BSFL as part of their rations. Bars are LSM with their SE. For overall average group LSM see Table 2. a-c: Time points denoted with different letters differ significantly (Tukey, P < 0.05).

Nutrient and energy intakes, growth performance and feed conversion indices

In L30 birds the CP intake was lower than in CON birds at wk 3 (P < 0.05; Supplementary Figure 1A), while L20 birds had a higher CP intake than CON and L10 birds at wk 6 (P < 0.05). The

average fat intake was higher in L30 and L20 birds than in CON and L10 birds ($P < 0.05$, Table 3). Higher fat intake in L30 birds than in CON birds occurred for the first time at wk 2 ($P < 0.05$; Supplementary Figure 1B), which then became more pronounced in subsequent weeks. By wk 4, L10 birds also consumed a higher amount of fat than did the CON birds ($P < 0.05$).

In line with FI, the average starch and NDF consumption was lower in L30 than in CON birds ($P < 0.05$; Table 3). The average ME intake of L30 birds was lower as compared to that of CON ($P < 0.05$) with time-dependent differences at wk 3 and 4 (Supplementary Figure 1C). Also, ME intake of L30 birds was lower than that of L20 birds at wk 5 and 6 ($P < 0.05$; Supplementary Figure 1C). Provision of BSFL increased the CP:ME ratio in a linear fashion with differences among all 4 groups (Table 3), implying less energy availability per unit protein consumed with increasing levels of BSFL in the ration. The average CF and ADF intake did not differ among the groups ($P > 0.05$, Table 3), while time-dependent differences indicated higher CF and ADF intakes in L20 than in CON at wk 6 (data not shown). Significant time-dependent differences were also quantified for crude ash and mineral (e.g. Ca, P) intake. As shown in Supplementary Figure 2A, both L20 and L30 groups had higher Ca intake than L10 and CON at wk 6, whereas P and crude ash intakes were higher only in L20 at wk 6 (Supplementary Figure 2B and Supplementary Figure 2C; respectively).

Although the FMI via BSFL corresponded to the BSFL provision levels (i.e. 10, 20 and 30% of CON feed intake), due to the low DM content of BSFL (i.e. 31.2 % DM), the contribution of BSFL intake to relative DMI was smaller than the pre-set levels of BSFL provision (Figure 5). The only nutrient that was exclusively taken from feed in all 4 groups (i.e. 99.6 to 100%) was starch. With the exception of fat, relative nutrient and energy intakes via BSFL were either approximately similar to or lower than the pre-set BSFL provision levels (i.e. 10, 20 or 30%,

respectively) (Figure 5). Due to their high fat content the relative fat intake via BSFL consumption was 18.8% in L10, 32.4% in L20 and 45.3% in L30 groups, respectively, which far exceeded the pre-set BSFL provision levels. The relative intakes via feed and BSFL for selected nutrients and ME are shown in Supplementary Table 2.

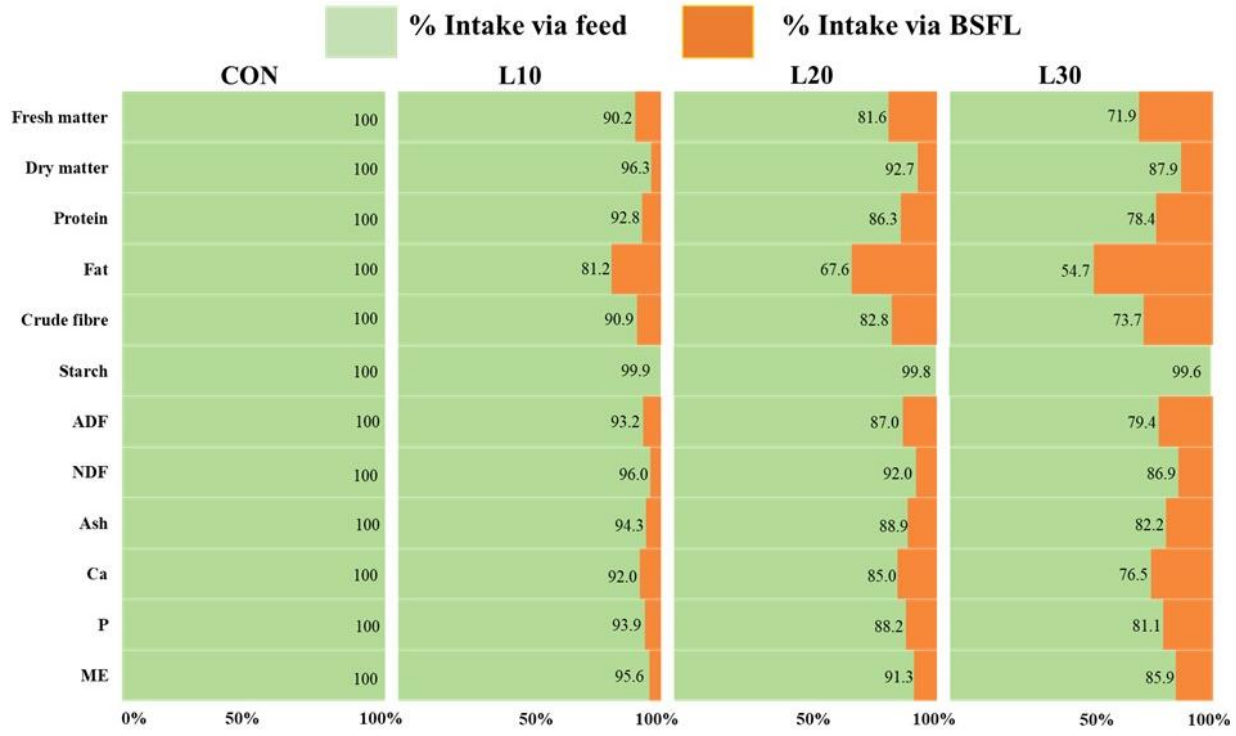


Figure 5. Effects of increasing levels of whole black soldier fly larvae in broiler rations on proportional nutrient and energy intakes via feed and larvae. For exact numbers and further details see Supplementary Table 2.

The provision of BSFL at 30% instead of 20% influenced BW in a time dependent manner. A treatment group by week interaction for BW ($P = 0.033$) indicated a lower BW in L30 than L20 birds at wk 6 ($P < 0.05$; Figure 6), whereas the larvae consuming groups did not differ from the CON group at any time point ($P > 0.05$). Although consumption of BSFL resulted in a gradual increase of heterogeneity in BW in a dose-dependent manner (Table 3), a significant increase in CV of BW was observed only in L30 as compared to CON groups ($P < 0.05$; Table 3).The L20

birds also tended to have increased CV of BW when compared with CON ($P < 0.10$), whereas CV of BW did not differ between L10 and CON groups ($P > 0.05$).

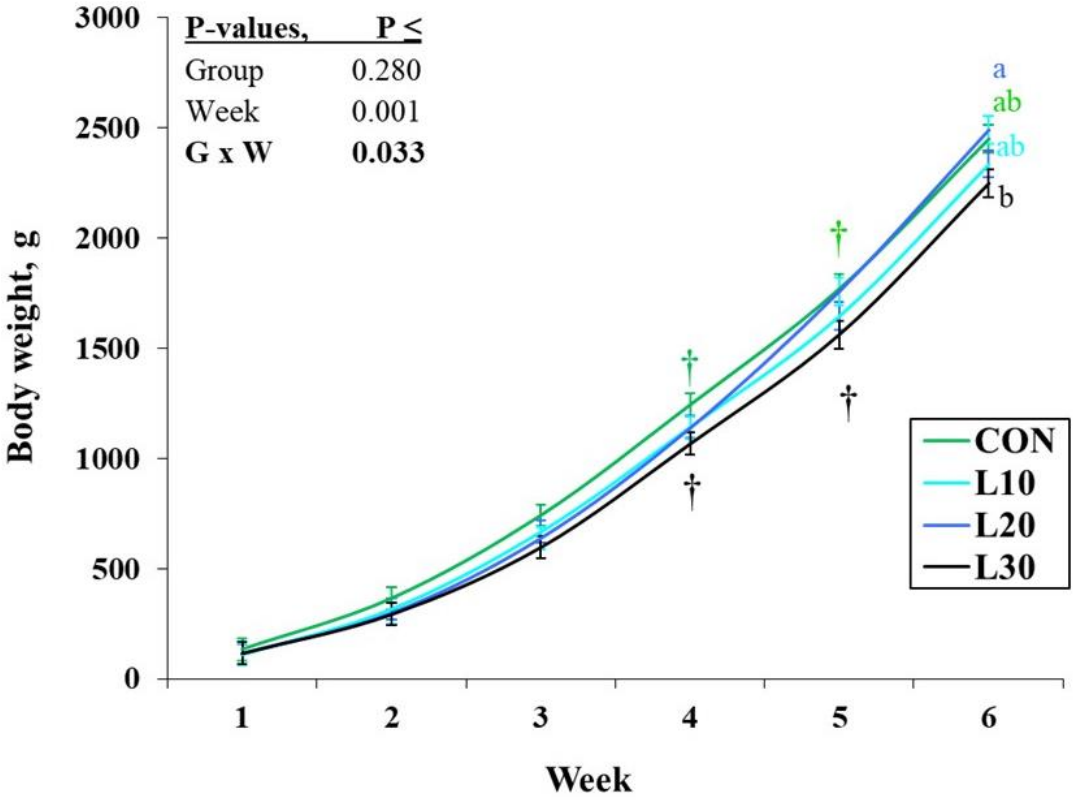


Figure 6. Time-dependent changes in body weight of broilers offered increasing levels of whole black soldier fly larvae in their rations. Values are LSM with their SE. a-: Values denoted with different letters at the same point differ significantly (Tukey, $P < 0.05$). The symbol † indicates a tendency of two treatments to differ (Tukey, $0.05 < P \leq 0.10$).

The overall feed conversion rate based on FMI (i.e. FCR-1) was higher in L30 than in the other groups ($P < 0.05$, Table 3). The overall FCR-2 based on DMI did not differ among the groups ($P = 0.132$, Table 3), whereas time-dependent differences indicated that the FCR-2 was lower in L30 than in CON birds at wk 4, but at wk 5 it was lower in L20 than those in L10 and L30 birds ($P < 0.05$, Supplementary Figure 3A). Overall, in the L30 group PCR was higher as compared with the CON group ($P < 0.05$; Table 3), which was due to time-dependent increases at wk 1, 2 and 5 ($P <$

0.05) (Supplementary Figure 3B). At wk 5, ECR was lower in L20 than in L10 and L30 groups (Supplementary Figure 3C).

Potential trade-offs in nutrient and energy intakes

The 4 treatment groups exhibited a distinct clustering of the selected nutrient intake patterns, representing energy, protein, fiber and mineral intakes (Supplementary Figure 4). Despite the slight differences from week-to-week, a gradual differentiation of the groups was repeatedly observed over 6 wk, corresponding to the increasing levels of BSFL in the ration.

The CP, NDF, starch and crude ash intakes were positively correlated with each other (see loadings in Supplementary Figure 4, right panels) and they contributed to the higher variation observed on Component 1 (Supplementary Figure 4; see X-axis in all left-panels). The main driving force of the differentiations among the 4 treatment groups was however the crude fat intake as it was less correlated with other nutrients and always corresponded well to the direction of the differentiation (see loadings in Supplementary Figure 4- right panels).

Blood metabolites and immunoglobulin isotypes

Plasma albumin, globulin and total protein concentrations were not affected by the dietary treatments ($P > 0.05$; Table 4). In the L30 group plasma UA concentration was higher than in the CON group ($P < 0.05$) although in the L20 group birds tended to show higher UA ($P = 0.052$) as well. Similar to UA, ALP activity levels also increased with increasing levels of BSFL in the ration, with L20 and L30 groups showing a higher serum ALP concentrations than in CON ($P < 0.05$; Table 4). In addition, ALP levels tended to increase in L30 birds when compared with L10 ($P < 0.10$). The level of BSFL in the ration had no effect on plasma IgY and IgM concentration of

the chickens (Table 4), whereas L30 birds tended to have higher plasma IgA concentration than did L10 birds (P = 0.053).

Table 4. Effects of increasing levels of whole black soldier fly larvae in broiler rations on selected blood metabolites and immunoglobulin concentrations of broilers.

	Dietary treatment groups[1]					P-values[2], ≤		
	CON	**L10**	**L20**	**L30**	**SE**	**G**	**W**	**G×W**
Metabolites								
Albumin, g / l	11.20	11.69	11.51	11.60	0.181	0.199	0.001	0.670
Globulin, mmol / l	14.75	14.48	14.64	14.43	0.457	0.952	0.370	0.140
Total protein, g / l	25.47	26.34	26.40	26.23	0.497	0.432	0.003	0.764
Uric acid, µmol / l	375.0 ^{b†}	410.9 ^{ab}	485.2 ^{ab†}	498.9 ^{a}	30.61	0.017	0.002	0.820
ALP[3], U / l	1757^{b}	2266^{ab†}	3629^{a}	4014^{a†}	517	0.009	0.001	0.079
Immunoglobulins								
IgY, mg / ml	1.60	1.67	1.56	1.56	0.176	0.966	0.002	0.569
IgM, mg / ml	0.145	0.153	0.139	0.163	0.010	0.288	0.001	0.560
IgA, mg / ml	0.191	0.185†	0.228	0.260†	0.021	0.053	0.155	0.148

a-c: Groups denoted with different letters differ significantly (Tukey, P<0.05). The signs (†,*) indicate tendency of two diets to differ (Tukey, 0.05 < P ≤ 0.10).
[1]Dietary treatments: ad-libitum feed without access to BSFL (CON), or with BSFL amounting to 10% (L10), 20% (L20) or 30% (L30) of the feed intake of CON birds. Total number of observations used for statistical analyses, N = 96 (i.e., 2 birds sampled from each of 6 pens allocated to each of 4 treatments at weeks 4 and 6).
[2]**G**: group effect, **W** = time effect for weeks 4 and 6, **G×W** = treatment by week interaction.
Abbreviation: **ALP**: Alkaline phosphatase ([3]measured in serum).

DISCUSSION

In this study, we first assessed apparent interest of broilers in eating BSFL when offered at either 10%, 20% or 30% of voluntary FI of CON chickens that received no BSFL. Next, nutrient and energy intakes of the birds through larvae and voluntary feed consumption were estimated, and their growth performance, nutrient conversion efficiency, plasma metabolites and immunoglobulins concentrations were then assessed. Larvae eating time and eating rates of

broilers indicated a strong preference for BSFL over regular feed. We found that chickens can consume up to 30% of their voluntary FI as BSFL in just a few minutes after a short (3 d) learning period. Apparent interest of chickens in BSFL as compared to regular feed was at least 50 times higher, implying a great potential of BSFL not only to be included in broiler rations, but also as an edible environmental enrichment tool. The nutrient and energy intakes of broilers by consumption of feed and larvae and their utilization indices for growth indicate that feeding unprocessed whole BSFL up to 20% of voluntary FI has no adverse effects. A higher level of BSFL in the daily ration (i.e. 30%) lead to a lower total DMI despite of an elevated FMI, and eventually resulted in lower ME intake that could not be compensated through the high fat content of BSFL (28% fat in DM), which, on the other hand might have impaired DMI of the birds. The lower overall energy intake in L30 birds was likely the main reason for their lower BW compared to L20 birds at wk 6. Equally important, provision of BSFL at 30% of FI in the ration leads to a lower protein utilization efficiency, likely due to a lower protein:energy intake ratio leading to higher nitrogen excretion as suggested by the higher plasma uric acid concentration in L30 birds. In addition, the higher fat intake in L20 and L30 groups might likely affect liver function and subsequently increased ALP concentration in L30.

Larvae eating time and eating rate

After a short (3 d) learning period, the birds in the larvae consuming groups were able to consume their daily BSFL portions just in a few minutes. Except for the first day, there was no difference in TSL among larvae fed groups. The lack of differences in TSL among groups consuming larvae might be largely related to within group variation (see error bars on Figure 1A). However, larvae eating rate depended on the amount of BSFL offered to the birds, with L10 birds eating more larvae per min than L20 and L30 birds. The higher LER could be due to increased

larval feeding competition in L10 birds as a result of fewer palatable nutrients such as fat and protein, which are known to be perceived by chickens (Cheled-Shoval, et al., 2017). We quantified a strong linear increase in LER with time by more than 200-fold from the first day to the last day of experiment, indicating a steadily increasing eating rate in response to time. Increasing LER over time was considered to be a result of two interrelated factors, social learning and growth, i.e. increasing body weight (Slagsvold and Wiebe, 2011; Tallentire, et al., 2018). In order to separate, at least partly, the impact of these two factors on larvae eating rate, we adjusted LER for BW of the birds and plotted it against time. After adjustment for BW, the larvae eating rate showed a completely different pattern, decreasing approximately threefold from the first to the last week, but did not depend on the amount of BSFL offered to the birds. Combined with the sharply decreasing TSL during the first 3 d, the linearly decreasing LER over time may indicate that the birds likely learn to eat BSFL already during the first week, but that their motivation to consume larvae in proportion to their body mass declines to some extent over time.

A previous study found that young birds take longer to learn foraging behaviors than adults, indicating that learning strategies change with age (Franks and Thorogood, 2018). However, there is a gap in knowledge regarding how long it takes for broiler chickens to learn foraging behavior, especially if there is competition for limited feed resources, which is usually not the case because feed is regularly offered to birds ad libitum.

The eating rate for the regular feed (**FER**) increased from wk 1 to wk 6 by about 25-fold in CON, and after adjustment for BW, FER_BW declined over time. In order to compare time-dependent changes in larvae and feed eating rates over 6 weeks in the three broiler groups offered larvae, we calculated FCD as ratios of LER to FER. The ratio of larval eating rate to foraging rate (i.e., FCD) decreased from about 60-fold to 50-fold from the first week to the last week, implying

a nearly constant preference of at least 50-fold in favor of larval eating over regular feed. The high interest, i.e. preference of chickens for BSFL compared to regular feed opens the possibility of including whole BSFL in daily rations for broilers. In addition, it also suggests a great potential of BSFL as an edible environmental enrichment when used in small amounts to stimulate the birds. Nevertheless, there is a lack of knowledge about the palatability of whole BSFL for poultry. Insect larvae are rich in various nutrients and are one of the natural feed sources of poultry, which are very motivating for consumption (Bokkers and Koene, 2002), so birds may clearly prefer larvae over regular feed. Cullere, et al. (2016) evaluated BSFL meal as a dietary supplement for quail in a feed selection trial and found that quail preferred the 15% BSFL meal diet 53.8% compared to a 44.1% preference for the control diet, suggesting that poultry favor BSFL over regular feed. Ipema, Gerrits, Bokkers, Kemp and Bolhuis (2020b) also observed a strong appetite value of live BSFL for broilers which was associated with a higher activity and increased foraging behavior. Furthermore, chickens show a clear preference for insect larvae meal (e.g. *Tenebrio molitor*) as compared to classical protein feedstuff, i.e. extruded semi-whole soybean meal (Nascimento Filho, Pereira, Oliveira, Suckeveris, Burin Junior, Mastrangelo, Costa and Menten, 2020). Previous studies suggested that providing mealworms or BSFL in broiler diets promotes animal welfare by facilitating natural behavior and reducing anxiety, increasing activity, and decreasing the incidence of leg problems (Ipema, et al., 2020a; Ipema, Gerrits, Bokkers, Kemp and Bolhuis, 2020b; Pichova, et al., 2016). When the birds in our study were offered with the predetermined BSFL portions in the morning, they stopped eating regular feed until the larvae on the plate were completely eaten, with some evidence of competitive behavior (Supplementary video 1). Accordingly, voluntary intake of regular feed was lower in larvae fed groups than in the CON

groups, and the lower voluntary FI in L30 than in L10 birds suggests a BSFL-dose-dependent nutrient and energy intake via regular feed.

Animal health, nutrient intakes, and growth performance

As reported earlier (Cullere, Tasoniero, Giaccone, Miotti-Scapin, Claeys, De Smet and Dalle Zotte, 2016; Kawasaki, et al., 2019) in our study BSFL feeding was not associated with mortality, metabolic disorders, wet litter or foot and leg problems. The nutrient composition of the BSFL used was in the range of what was previously observed (Liland, et al., 2017; Makkar, et al., 2014; Moula, Scippo, Douny, Degand, Dawans, Cabaraux, Hornick, Medigo, Leroy and Francis, 2018). In the current study, 30% of BSFL in the ration led to a lower total DMI which is in line with earlier observations (Ipema, Gerrits, Bokkers, Kemp and Bolhuis, 2020b). Despite the increased FMI of the L30 birds in the last two weeks of the experiment, their DMI was the lowest, resulting in a lower ME intake that could not be compensated by the higher fat intake via BSFL consumption. The results of the PCA analysis suggest that the higher fat intake in L30 birds was the main driving force leading to their lower DMI. It is known that high levels of dietary fat alters the response of hypothalamic appetite-related peptides (Wang, et al., 2017), which is associated with circulating insulin (Obrosova, et al., 2007). The physical constrains in digestive tract capacity of broilers might limited FMI regulation in association with energy intake (Brickett, et al., 2007; Dozier III, et al., 2006).

Previous studies showed that BSFL meal consumption at 5% of dietary DM increased broiler growth (Lee, et al., 2018). Similarly, Biasato, et al. (2020) observed positive effects of dietary inclusion of partially defatted BSFL-meal as partial replacement of soybean meal, corn gluten meal and soybean oil at low inclusion levels (i.e., 5%) on cecal microbiota or the gut mucin dynamics. However, they reported a negative influence of high inclusion levels (i.e. 15%) such as a partial

reduction of microbial complexity and reduction of potentially beneficial bacteria. Józefiak, et al. (2018) reported positive influence of using full-fat insect meals (*Hermetia illucens*, *Gryllodes sigillatus*, *Shelfordella lateralis*, *Gryllus assimilis* and *Tenebrio molitor*) in low amounts (i.e. 0.05 to 0.2%) on the top of broiler diets in terms of modulating microbial populations in the gastrointestinal tract. Results of a meta-analysis study suggested that partially substitution of conventional protein sources (i.e. less than 10%) with insects (such as black soldier fly larvae, mealworms, and maggots) in poultry diet has no adverse effect on the growth, FI, and FCR (Moula and Detilleux, 2019). Also, Moula, Scippo, Douny, Degand, Dawans, Cabaraux, Hornick, Medigo, Leroy and Francis (2018) reported that body weights of chickens fed standard feed supplemented with 8% whole defrozen larvae were higher than those of control chickens. In contrast, we observed a lower BW in L30 compared to L20 birds in wk 6, indicating higher BSFL inclusion levels may affect broiler growth, but none of the BSFL groups differed in BW from the CON group. Ipema, Gerrits, Bokkers, Kemp and Bolhuis (2020b), attributed the lower growth of BSFL-fed chickens to an imbalance in amino acid (**AA**) uptake. However, despite the large differences in crude protein contents, we did not find large differences in the composition of essential AA of age-specific diets and BSFL (Supplementary Table 1). It is likely that in our study overall protein digestibility might have been affected in the L30 group because the BSFL contained a relatively high chitin content (73 g/kg DM). It was earlier suspected by Dabbou, et al. (2018) that reduced growth of broilers fed defatted BSFL meal could be due to the chitin content of BSFL which might negatively influence the protein digestibility. It should be noted that per design of the experiment the final rations of the groups were not isocaloric or isonitrogenous after feeding different amounts of BSFL in addition to the complete diets, which might have contributed to differences in nutrient intakes and growth performance of the birds.

Blood metabolites and immunoglobulin isotypes

In chickens, UA is the main end product of nitrogen metabolism (Donsbough, et al., 2010), and excessive protein consumption can lead to an increased blood UA concentration (Musigwa, et al., 2020). We observed a linear increase of the CP:ME ratio in response to feeding increasing levels of BSFL which was accompanied by a numerical increase of plasma UA concentration, a higher plasma UA concentration in L30 birds while protein utilization was also less efficient in the same birds. Similar to UA, serum ALP activity levels increased with rising levels of BSFL in the ration, with the L20 and L30 groups having higher serum ALP concentrations than CON. The ALP enzyme is involved in active bone formation, and in chickens it is considered as a marker for skeletal health, bone disease and liver damage (Jiang, et al., 2013; Senanayake, et al., 2015). The elevated serum ALP concentration in L30 birds might be explained with the higher fat intake (see section 3.2) which was possibly associated with adverse effects on liver function (Jiang, Cheng, Cui, Zhou and Hou, 2013). The plasma IgY and IgM concentrations were not consistently affected by dietary treatment, however, the L30 birds tended to have a higher plasma IgA concentration than those in L10 group. Increased IgA concentration in response to feeding BSFL may suggest an activation of the humoral immune system (Song, et al., 2021). However, information on the effects of feeding whole BSFL to broiler chickens on blood immunoglobulin concentrations is lacking. Nevertheless, El-Hack et.al. (2020) discussed that the improved immune function in BSFL-fed hens may be due to chitin content in BSFL meal, which has the ability to stimulate the immune system.

CONCLUSION

We conclude that chickens can consume BSFL up to 30% of their voluntary FI in a few minutes after a short period of learning. Larvae eating time and eating rates of broilers suggest a strong preference for BSFL over regular feed. Whole BSFL can be included in broiler rations up to 20% without adverse effects on growth performance and nutrient conversion efficiency, whereas higher levels are associated with lower protein utilization efficiency, possibly due to lower total energy intake despite the high fat content of BSFL.

DATA AVAILABILITY

The raw data used for all analyses in this study are stored in a repository (DOI:https://doi.org/10.5281/zenodo.7110632).

DISCLOSURES All authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

CCM and GD conceived and designed the study. CCM and PW acquired funding. MMS and GD performed the experiment. MMS, GD and SG contributed to the analysis of the samples and collection of data. GD and MMS performed the statistical analysis of the data and drafted the manuscript. CCM, GD, MMS, MM, SG and PW reviewed the manuscript. All authors read and approved the submitted version of the manuscript.

ACKNOWLEDGEMENTS

This research project is part of the Leibniz ScienceCampus Phosphorus Research Rostock and co-funded by the funding line strategic networks of the Leibniz Association. The authors are grateful

to B. Mielenz, K. Kàrpàti, U. Lüdtke, E. Wünsche, K. Haß, S. Gaeth and S. Dwars and the other helpful, qualified staff at the Research Institute for Farm Animal Biology (FBN), Dummerstorf, Germany, for their assistance with laboratory analyses and sample collection and making this research project possible. We also thank Manuel Stehr (Ceravis AG, Rendsburg, Germany) for organizing the production and the transport of the age-specific broiler diets. The publication of this article was funded by the Open Access Fund of Research Institute for Farm Animal Biology (FBN).

REFERENCES

Attivi, K., K. Agboka, G. Mlaga, O. Oke, A. Teteh, O. Onagbesan, and K. Tona. 2020. Effect of black soldier fly (Hermetia Illucens) maggots meal as a substitute for fish meal on growth performance, biochemical parameters and digestibility of broiler chickens. Int. J. Poult. Sci 19:75-80. doi https://doi.org/10.3923/ijps.2020.75.80

Aviagen. 2018. Broiler Management Handbook. Pages 1–147 Newbridge, UK.

Aviagen. 2019. Ross Broiler: Nutrition Specifications. Aviagen: Huntsville, AL, USA.

Bava, L., C. Jucker, G. Gislon, D. Lupi, S. Savoldelli, M. Zucali, and S. Colombini. 2019. Rearing of Hermetia illucens on different organic by-products: Influence on growth, waste reduction, and environmental impact. Animals 9:289. doi https://doi.org/10.3390/ani9060289

Bellezza Oddon, S., I. Biasato, A. Imarisio, M. Pipan, D. Dekleva, E. Colombino, M. T. Capucchio, M. Meneguz, S. Bergagna, and R. Barbero. 2021. Black Soldier Fly and Yellow Mealworm live larvae for broiler chickens: Effects on bird performance and health status. J. Anim. Physiol. Anim. 105:10-18. doi https://doi.org/10.1111/jpn.13567

Biasato, I., I. Ferrocino, S. Dabbou, R. Evangelista, F. Gai, L. Gasco, L. Cocolin, M. T. Capucchio, and A. Schiavone. 2020. Black soldier fly and gut health in broiler chickens: insights into the relationship between cecal microbiota and intestinal mucin composition. J. Anim. Sci. Biotechnol. 11:1-12. doi https://doi.org/10.1186/s40104-019-0413-y

Bokkers, E. A., and P. Koene. 2002. Sex and type of feed effects on motivation and ability to walk for a food reward in fast growing broilers. Appl. Anim. Behav. Sci. 79:247-261. doi https://doi.org/10.1016/S0168-1591(02)00151-X

Brickett, K., J. Dahiya, H. Classen, and S. Gomis. 2007. Influence of dietary nutrient density, feed form, and lighting on growth and meat yield of broiler chickens. Poult. Sci. 86:2172-2181. doi https://doi.org/10.1093/ps/86.10.2172

Cheled-Shoval, S. L., N. Reicher, M. Y. Niv, and Z. Uni. 2017. Detecting thresholds for bitter, umami, and sweet tastants in broiler chicken using a 2-choice test method. Poult. Sci. 96:2206-2218. doi https://doi.org/10.3382/ps/pex003

Cullere, M., G. Tasoniero, V. Giaccone, R. Miotti-Scapin, E. Claeys, S. De Smet, and A. Dalle Zotte. 2016. Black soldier fly as dietary protein source for broiler quails: apparent digestibility, excreta microbial load, feed choice, performance, carcass and meat traits. Animal. 10:1923-1930. doi https://doi.org/10.1017/S1751731116001270

Cutrignelli, M. I., M. Messina, F. Tulli, B. Randazzo, I. Olivotto, L. Gasco, R. Loponte, and F. Bovera. 2018. Evaluation of an insect meal of the Black Soldier Fly (Hermetia illucens) as soybean substitute: Intestinal morphometry, enzymatic and microbial activity in laying hens. Res Vet Sci 117:209-215. doi https://doi.org/10.1016/j.rvsc.2017.12.020

Dabbou, S., F. Gai, I. Biasato, M. T. Capucchio, E. Biasibetti, D. Dezzutto, M. Meneguz, I. Plachà, L. Gasco, and A. Schiavone. 2018. Black soldier fly defatted meal as a dietary protein source for broiler chickens: Effects on growth performance, blood traits, gut morphology and histological features. J. Anim. Sci. Biotechnol. 9: 1-10 doi https://doi.org/10.1186/s40104-018-0266-9

de Souza Vilela, J., T. I. Alvarenga, N. R. Andrew, M. McPhee, M. Kolakshyapati, D. L. Hopkins, and I. Ruhnke. 2021. Technological quality, amino acid and fatty acid profile of broiler meat enhanced by dietary inclusion of black soldier fly larvae. Foods 10:297. doi https://doi.org/10.3390/foods10020297

Delezie, E., K. Bierman, L. Nollet, and L. Maertens. 2015. Impacts of calcium and phosphorus concentration, their ratio, and phytase supplementation level on growth performance, foot pad

lesions, and hock burn of broiler chickens. J. Appl. Poult. Res. 24:115-126. doi https://doi.org/10.3382/japr/pfv011

Donsbough, A., S. Powell, A. Waguespack, T. Bidner, and L. Southern. 2010. Uric acid, urea, and ammonia concentrations in serum and uric acid concentration in excreta as indicators of amino acid utilization in diets for broilers. Poult. Sci. 89:287-294. doi https://doi.org/10.3382/ps.2009-00401

Dörper, A., T. Veldkamp, and M. Dicke. 2020. Use of black soldier fly and house fly in feed to promote sustainable poultry production. J. Insects as Food Feed:1-20. doi https://doi.org/10.3920/JIFF2020.0064

Dozier III, W., C. Price, M. Kidd, A. Corzo, J. Anderson, and S. Branton. 2006. Growth performance, meat yield, and economic responses of broilers fed diets varying in metabolizable energy from thirty to fifty-nine days of age. J. Appl. Poult. Res. 15:367-382. doi https://doi.org/10.1093/japr/15.3.383

El-Hack, A., E. Mohamed, M. E. Shafi, W. Y. Alghamdi, S. A. Abdelnour, A. M. Shehata, A. E. Noreldin, E. A. Ashour, A. A. Swelum, and A. A. Al-Sagan. 2020. Black soldier fly (Hermetia illucens) meal as a promising feed ingredient for poultry: A comprehensive review. Agriculture 10:339. doi https://doi.org/10.3390/agriculture10080339

Fariz Zahir Ali, M., T. Ohta, A. Ido, C. Miura, and T. Miura. 2019. The dipterose of black soldier fly (Hermetia illucens) induces innate immune response through toll-like receptor pathway in mouse macrophage RAW264. 7 cells. Biomolecules 9:677. doi https://doi.org/10.3390/biom9110677

Ferket, P. R., and A. G. Gernat. 2006. Factors that affect feed intake of meat birds: A review. Int. J. Poult. Sci 5:905-911.

Franks, V. R., and R. Thorogood. 2018. Older and wiser? Age differences in foraging and learning by an endangered passerine. Behav. Process 148:1-9. doi https://doi.org/10.1016/j.beproc.2017.12.009

Hahn, T., A. Roth, E. Febel, M. Fijalkowska, E. Schmitt, T. Arsiwalla, and S. Zibek. 2018b. New methods for high-accuracy insect chitin measurement. J Sci Food Agric 98:5069-5073. doi https://doi.org/10.1002/jsfa.9044

Hu, X., X. Li, C. Xiao, L. Kong, Q. Zhu, and Z. Song. 2021. Effects of Dietary Energy Level on Performance, Plasma Parameters, and Central AMPK Levels in Stressed Broilers. Front. Vet. Sci 8:576. doi https://doi.org/10.3389/fvets.2021.681858

Ipema, A. F., E. A. Bokkers, W. J. Gerrits, B. Kemp, and J. E. Bolhuis. 2020a. Long-term access to live black soldier fly larvae (Hermetia illucens) stimulates activity and reduces fearfulness of broilers, without affecting health. Sci Rep 10:1-13. doi https://doi.org/10.1038/s41598-020-74514-x

Ipema, A. F., W. J. Gerrits, E. A. Bokkers, B. Kemp, and J. E. Bolhuis. 2020b. Provisioning of live black soldier fly larvae (Hermetia illucens) benefits broiler activity and leg health in a frequency-and dose-dependent manner. Appl. Anim. Behav. Sci. 230:105082. doi https://doi.org/10.1016/j.applanim.2020.105082

Jha, R., and P. Mishra. 2021. Dietary fiber in poultry nutrition and their effects on nutrient utilization, performance, gut health, and on the environment: a review. J. Anim. Sci. Biotechnol. 12:1-16. doi https://doi.org/10.1186/s40104-021-00576-0

Jiang, S., H. W. Cheng, L. Cui, Z. Zhou, and J. Hou. 2013. Changes of blood parameters associated with bone remodeling following experimentally induced fatty liver disorder in laying hens. Poult. Sci. 92:1443-1453. doi https://doi.org/10.3382/ps.2012-02800

Józefiak, A., B. Kierończyk, M. Rawski, J. Mazurkiewicz, A. Benzertiha, P. Gobbi, S. Nogales-Merida, S. Świątkiewicz, and D. Józefiak. 2018. Full-fat insect meals as feed additive–the effect on broiler chicken growth performance and gastrointestinal tract microbiota. J. Anim. Feed Sci 27:131-139. doi https://doi.org/10.22358/jafs/91967/2018

Kawasaki, K., Y. Hashimoto, A. Hori, T. Kawasaki, H. Hirayasu, S.-i. Iwase, A. Hashizume, A. Ido, C. Miura, and T. Miura. 2019. Evaluation of black soldier fly (Hermetia illucens) larvae and pre-pupae raised on household organic waste, as potential ingredients for poultry feed. Animals 9:98. doi

Kuhla, B., M. Kucia, S. Görs, D. Albrecht, M. Langhammer, S. Kuhla, and C. C. Metges. 2010. Effect of a high-protein diet on food intake and liver metabolism during pregnancy, lactation and after weaning in mice. Proteomics 10:2573-2588. doi https://doi.org/10.1002/pmic.200900789

Latshaw, J. 2008. Daily energy intake of broiler chickens is altered by proximate nutrient content and form of the diet. Poult. Sci. 87:89-95. doi https://doi.org/10.3382/ps.2007-00173

Lee, J.-A., Y.-M. Kim, Y.-K. Park, Y.-C. Yang, B.-G. Jung, and B.-J. Lee. 2018. Black soldier fly (Hermetia illucens) larvae enhances immune activities and increases survivability of broiler chicks against experimental infection of Salmonella Gallinarum. J. Vet. Med. Sci.:17-0236. doi https://doi.org/10.1292/jvms.17-0236

Liland, N. S., I. Biancarosa, P. Araujo, D. Biemans, C. G. Bruckner, R. Waagbø, B. E. Torstensen, and E.-J. Lock. 2017. Modulation of nutrient composition of black soldier fly (Hermetia illucens) larvae by feeding seaweed-enriched media. PloS One 12:e0183188. doi https://doi.org/10.1371/journal.pone.0183188

Makkar, H. P., G. Tran, V. Heuzé, and P. Ankers. 2014. State-of-the-art on use of insects as animal feed. Anim. Feed Sci. Technol. 197:1-33. doi https://doi.org/10.1016/j.anifeedsci.2014.07.008

Maurer, V., M. Holinger, Z. Amsler, B. Früh, J. Wohlfahrt, A. Stamer, and F. Leiber. 2016. Replacement of soybean cake by Hermetia illucens meal in diets for layers. J Insects Food Feed 2:83-90. doi https://doi.org/10.3920/JIFF2015.0071

Moula, N., and J. Detilleux. 2019. A meta-analysis of the effects of insects in feed on poultry growth performances. Animals 9:201. doi https://doi.org/10.3390/ani9050201

Moula, N., M.-L. Scippo, C. Douny, G. Degand, E. Dawans, J.-F. Cabaraux, J.-L. Hornick, R. C. Medigo, P. Leroy, and F. Francis. 2018. Performances of local poultry breed fed black soldier fly larvae reared on horse manure. Anim Nutr 4:73-78. doi https://doi.org/10.1016/j.aninu.2017.10.002

Musigwa, S., N. Morgan, R. A. Swick, P. Cozannet, and S.-B. Wu. 2020. Energy dynamics, nitrogen balance, and performance in broilers fed high-and reduced-CP diets. J. Appl. Poult. Res. 29:830-841. doi https://doi.org/10.1016/j.japr.2020.08.001

Nascimento Filho, M. A., R. T. Pereira, A. B. S. d. Oliveira, D. Suckeveris, A. M. Burin Junior, T. d. A. Mastrangelo, D. V. d. Costa, and J. F. M. Menten. 2020. Cafeteria-Type Feeding of Chickens Indicates a Preference for Insect (Tenebrio molitor) Larvae Meal. Animals 10:627. doi https://doi.org/10.3390/ani10040627

Naumann, C., R. Bassler, R. Seibold, and K. Barth. 1997. VDLUFA-Methodenbuch Band III, Die chemische Untersuchung von FuttermittelnVDLUFA-Verlag, Darmstadt, Germany.

Nyakeri, E., H. Ogola, M. Ayieko, and F. Amimo. 2017. Valorisation of organic waste material: growth performance of wild black soldier fly larvae (Hermetia illucens) reared on different organic wastes. J. Insects as Food Feed 3:193-202. doi https://doi.org/10.3920/JIFF2017.0004

Obrosova, I. G., O. Ilnytska, V. V. Lyzogubov, I. A. Pavlov, N. Mashtalir, J. L. Nadler, and V. R. Drel. 2007. High-fat diet–induced neuropathy of pre-diabetes and obesity: effects of "healthy" diet and aldose reductase inhibition. Diabetes 56:2598-2608. doi https://doi.org/10.2337/db06-1176

Pichova, K., J. Nordgreen, C. Leterrier, L. Kostal, and R. O. Moe. 2016. The effects of food-related environmental complexity on litter directed behaviour, fear and exploration of novel stimuli in young broiler chickens. Appl. Anim. Behav. Sci. 174:83-89. doi https://doi.org/10.1016/j.applanim.2015.11.007

Ruhnke, I., C. Normant, D. L. Campbell, Z. Iqbal, C. Lee, G. N. Hinch, and J. Roberts. 2018. Impact of on-range choice feeding with black soldier fly larvae (Hermetia illucens) on flock performance, egg quality, and range use of free-range laying hens. Anim. Nutr. 4:452-460. doi https://doi.org/10.1016/j.aninu.2018.03.005

Schiavone, A., M. Cullere, M. De Marco, M. Meneguz, I. Biasato, S. Bergagna, D. Dezzutto, F. Gai, S. Dabbou, and L. Gasco. 2017. Partial or total replacement of soybean oil by black soldier fly larvae (Hermetia illucens L.) fat in broiler diets: Effect on growth performances, feed-choice, blood traits, carcass characteristics and meat quality. Ital J Anim Sci 16:93-100. doi https://doi.org/10.1080/1828051X.2016.1249968

Senanayake, S., J. Ranasinghe, R. Waduge, K. Nizanantha, and P. Alexander. 2015. Changes in the serum enzyme levels and liver lesions of broiler birds reared under different management conditions. Trop. Agric. Res. 26:584 – 595.

Shumo, M., I. M. Osuga, F. M. Khamis, C. M. Tanga, K. K. Fiaboe, S. Subramanian, S. Ekesi, A. van Huis, and C. Borgemeister. 2019. The nutritive value of black soldier fly larvae reared on common organic waste streams in Kenya. Sci. Rep. 9:1-13. doi https://doi.org/10.1038/s41598-019-46603-z

Slagsvold, T., and K. L. Wiebe. 2011. Social learning in birds and its role in shaping a foraging niche. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 366:969-977. doi https://doi.org/10.1098/rstb.2010.0343

Song, B., D. Tang, S. Yan, H. Fan, G. Li, M. S. Shahid, T. Mahmood, and Y. Guo. 2021. Effects of age on immune function in broiler chickens. J. Anim. Sci. Biotechnol. 12:1-12. doi https://doi.org/10.1186/s40104-021-00559-1

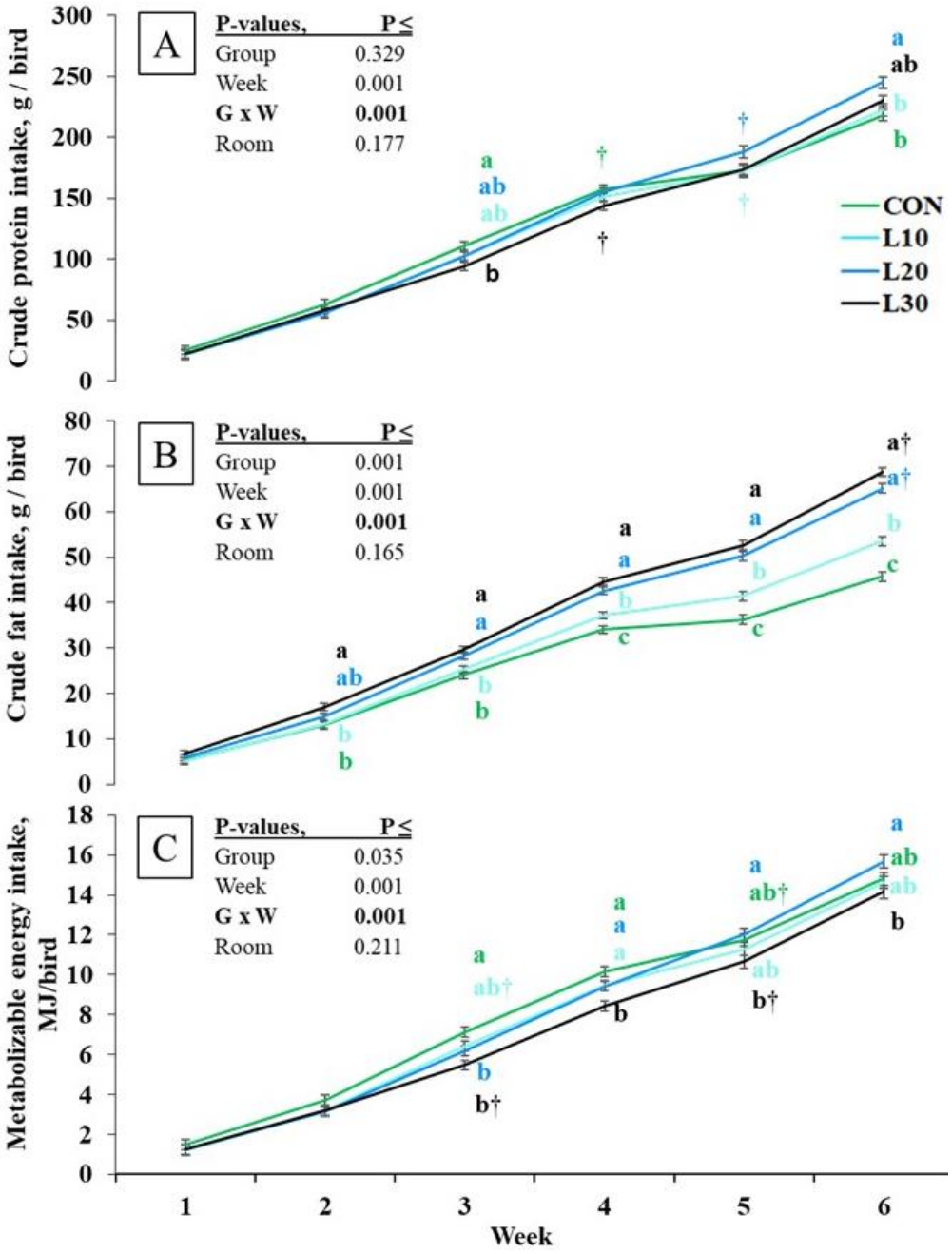
Star, L., T. Arsiwalla, F. Molist, R. Leushuis, M. Dalim, and A. Paul. 2020. Gradual provision of live black soldier fly (Hermetia illucens) larvae to older laying hens: effect on production performance, egg quality, feather condition and behavior. Animals 10:216. doi https://doi.org/10.3390/ani10020216

Tahamtani, F. M., E. Ivarsson, V. Wiklicky, C. Lalander, H. Wall, T. B. Rodenburg, F. A. Tuyttens, and C. E. Hernandez. 2021. Feeding live Black Soldier Fly larvae (Hermetia illucens) to laying hens: effects on feed consumption, hen health, hen behavior, and egg quality. Poult. Sci. 100:101400. doi https://doi.org/10.1016/j.psj.2021.101400

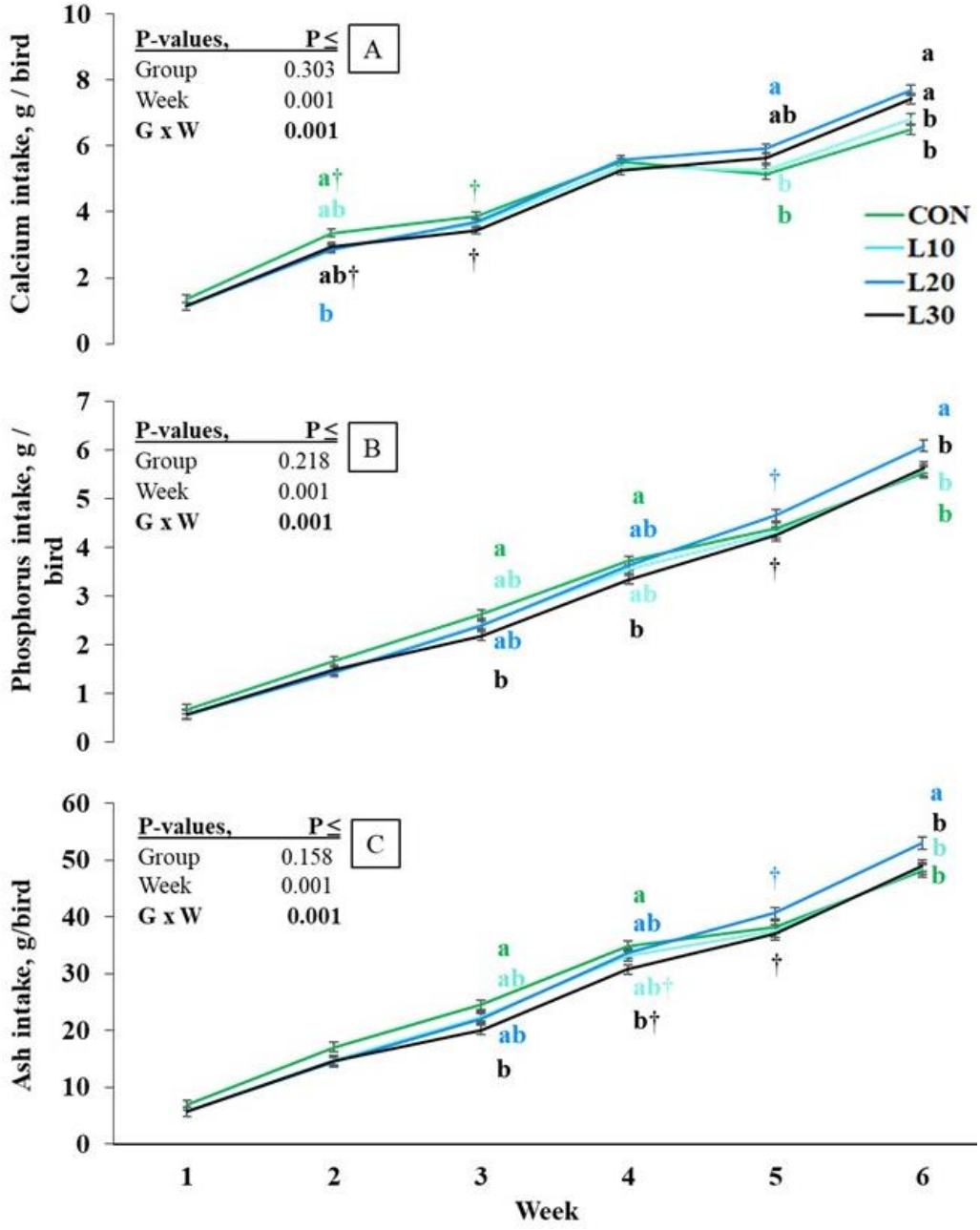
Tallentire, C., I. Leinonen, and I. Kyriazakis. 2018. Artificial selection for improved energy efficiency is reaching its limits in broiler chickens. Sci. Rep. 8:1-10. doi https://doi.org/10.1038/s41598-018-19231-2

Wang, X. J., S. H. Xu, L. Liu, Z. G. Song, H. C. Jiao, and H. Lin. 2017. Dietary fat alters the response of hypothalamic neuropeptide Y to subsequent energy intake in broiler chickens. J. Exp. Biol. 220:607-614. doi https://doi.org/10.1242/jeb.143792

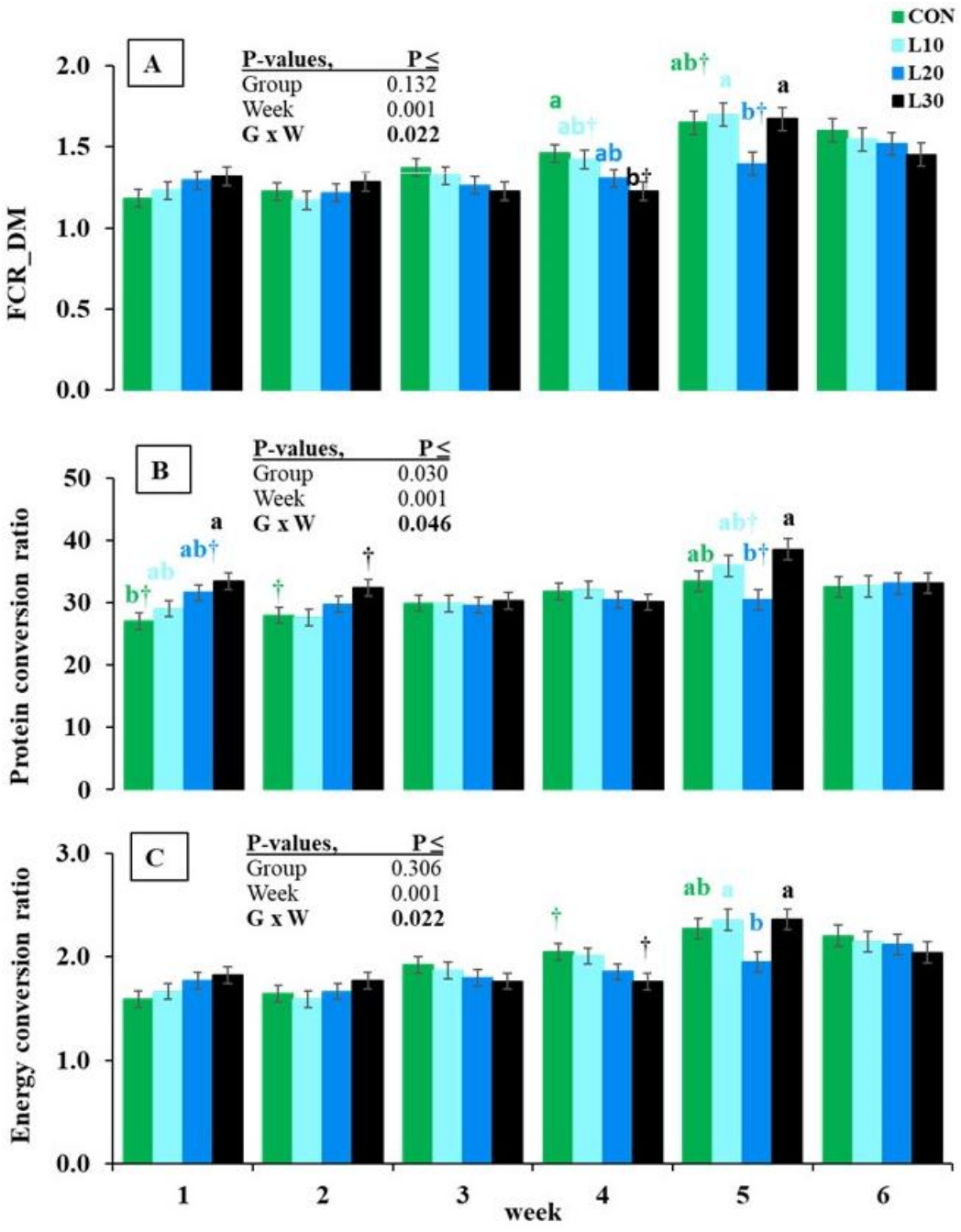
Welfare-Quality. 2009. The Welfare Quality® assessment protocol for poultry (broilers, laying hens). The Welfare Quality® Consortium, Lelystad, The Netherlands.



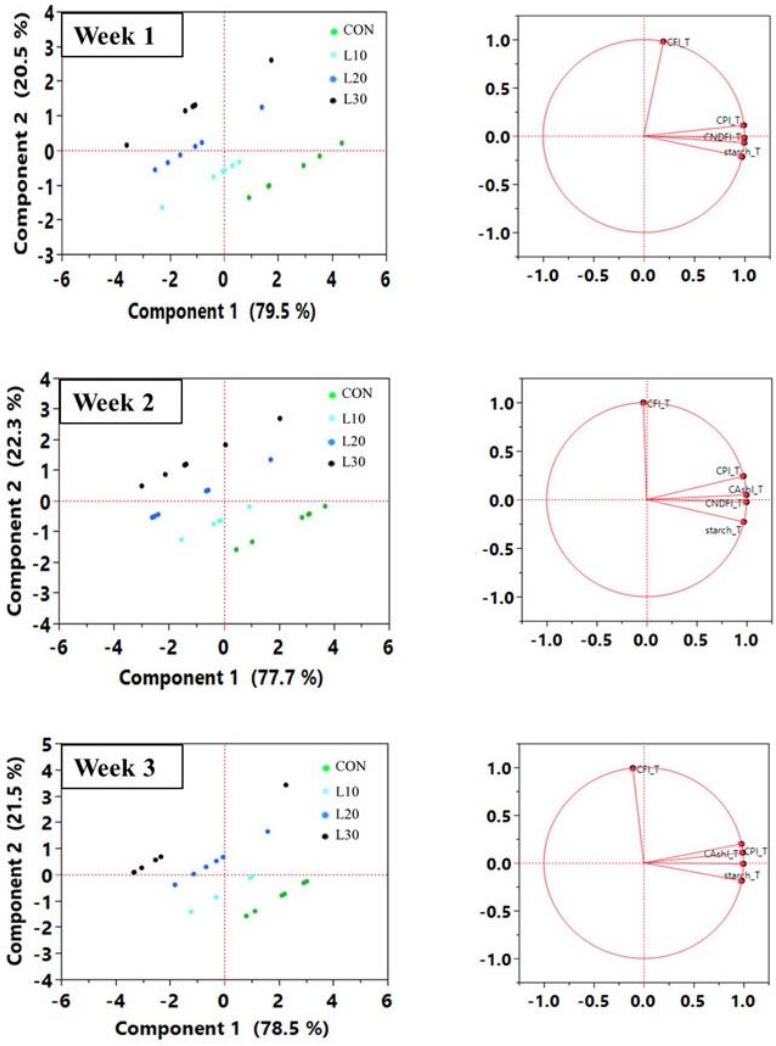
Supplementary Figure 1. Effects of increasing levels of whole black soldier fly larvae in broiler rations on crude protein intake (A), crude fat intake (B) and metabolizable energy intake (C) in broilers during the experimental weeks. Values are LSM with their SE. a-c: Values denoted with different letters at the same point within each panel differ significantly (Tukey, $P < 0.05$). The symbol † indicates a tendency of two treatments to differ (Tukey, $0.05 < P \leq 0.10$).



Supplementary Figure 2. Effects of increasing levels of whole black soldier fly larvae in broiler rations on calcium intake (A), phosphorus intake (B) and ash intake (C) in broilers during the experimental weeks. Values are LSM with their SE. a-c: Values denoted with different letters at the same point within each panel differ significantly (Tukey, $P < 0.05$). The symbol † indicates a tendency of two treatments to differ (Tukey, $0.05 < P \leq 0.10$).



Supplementary Figure 3. Effects of increasing levels of whole black soldier fly larvae in broiler rations on FCR-DM (A), protein conversion ratio (B) and energy conversion ratio (C) in broilers during the experimental weeks. Values are LSM with their SE.



Supplementary Figure 4. Principle components analysis (left panels) to identify which nutrients are the driving forces in differentiation of experimental groups over 6 experimental weeks. The component 1 and component 2 represent the samples variance. Ride side panels present the loadings of the principal components analysis. The X-axis and Y-axis values represent the contributing weights of each nutrient to the principal components 1 and 2 of the PCA component, respectively. Variables with loadings showing the same direction are highly correlated. All variables had the same units of measurement (i.e. g/wk). Abbreviations: **CFI_T**: crude fat intake; **CPI_T**: crude protein intake; **CashI_T**; crude ash intake; **CNDFI_T**; NDF intake; **starch_T**: starch intake.

Supplementary Table 1. Amino acid (AA) composition (as mg / g DM) of the age-specific basal diets and black soldier fly larvae (BSFL) offered to broilers during 42 experimental days.

AA (mg / g DM)	Basal diets			BSFL (d 1-42)
	Starter (d 1-14)	Grower (d 15-28)	Finisher (d 29-42)	
Essential				
Arginine	15.5	13.9	13.9	18.6
Histidine	6.3	6.0	6.4	12.3
Isoleucine	9.1	8.6	8.4	14.9
Leucine	18.9	18.2	17.8	25.6
Lysine	8.9	10.2	5.8	12.0
Methionine[1]	1.2	1.3	3.7	4.8
Phenylalanine	11.9	11.3	11.2	15.2
Threonine	9.3	8.6	8.5	15.1
Valine	9.8	9.4	9.1	21.0
Non- essential				
Aspartic acid + Asparagine	23.9	22.2	21.5	33.9
Glutamic acid + Glutamine	52.0	50.9	48.5	44.0
Cysteine[1]	1.3	1.3	1.2	1.4
Serine	12.4	11.5	10.9	16.7
Glycine	10.2	9.0	8.6	20.2
Alanine	10.1	9.6	9.3	27.9
Tyrosine	8.4	8.2	8.1	24.4
Proline	17.6	22.2	16.7	31.9
Sum of FAA	**4.2**	**3.8**	**4.2**	**28.0**
Sum of protein bound AA	**222.8**	**218.5**	**205.4**	**311.9**
Sum of free and protein bound AA	**227.0**	**222.3**	**209.6**	**339.8**

[1] Methionine and cysteine were partly oxidized during hydrolysis, and are thus likely underestimated.

Supplementary Table 2. Relative nutrient intakes (%) through feed and BSFL consumption in broilers offered either only regular feed (CON) or increasing levels of BSFL (10-30%) in addition to the regular feed.

Relative intakes via feed, %	**Dietary treatments[1]**					**P-values[2], ≤**		
	CON[3]	**L10**	**L20**	**L30**	SE	**G**	**W**	**G×W**
Fresh matter	100	90.1[a]	81.7[b]	72.0[c]	0.42	0.001	0.001	0.001
Dry matter	100	96.3[a]	92.7[b]	88.0[c]	0.22	0.001	0.001	0.001
Protein	100	92.8[a]	86.4[b]	78.4[c]	0.35	0.001	0.001	0.001
Fat	100	81.1[a]	67.7[b]	54.8[c]	0.53	0.001	0.001	0.001
ME MJ	100	95.6[a]	91.4[b]	85.9[c]	0.25	0.001	0.001	0.001
Crude fibre	100	90.8[a]	82.9[b]	73.8[c]	0.42	0.001	0.001	0.001
ADF	100	93.2[a]	87.1[b]	79.5[c]	0.33	0.001	0.001	0.001
NDF	100	95.9[a]	92.1[b]	86.9[c]	0.23	0.001	0.001	0.001
Ash	100	94.2[a]	89.0[b]	82.2[c]	0.29	0.001	0.001	0.001
Ca	100	92.0 [a]	85.1[b]	76.6[c]	0.35	0.001	0.001	0.001
P	100	93.8[a]	88.2[b]	81.1[c]	0.31	0.001	0.001	0.001
Mg	100	93.1[a]	86.9[b]	79.3[c]	0.34	0.001	0.001	0.001
Relative intakes via BSFL, %								
Fresh matter	0	9.8[c]	18.3[b]	28.0[a]	0.42	0.001	0.001	0.001
Dry matter	0	3.7[c]	7.3[b]	12.0[a]	0.22	0.001	0.001	0.001
Protein	0	7.2[c]	13.6[b]	21.6[a]	0.35	0.001	0.001	0.001
Fat	0	18.9[c]	32.3[b]	45.2[a]	0.53	0.001	0.001	0.001
ME MJ	0	4.4[c]	8.6[b]	14.1[a]	0.25	0.001	0.001	0.001
Crude fibre	0	9.2[c]	17.1[b]	26.2[a]	0.41	0.001	0.001	0.001
ADF	0	6.8[c]	12.9[b]	20.5[a]	0.33	0.001	0.001	0.001
NDF	0	4.1[c]	7.9[b]	13.1[a]	0.23	0.001	0.001	0.001
Chitin	0	100	100	100	-	-	-	-
Ash	0	5.8[c]	11.1[b]	17.8[a]	0.29	0.001	0.001	0.001
Ca	0	8.0[c]	14.9[b]	23.4[a]	0.35	0.001	0.001	0.001
P	0	6.2[c]	11.8[b]	18.9[a]	0.31	0.001	0.001	0.001
Mg	0	6.9[c]	13.1[b]	20.7[a]	0.34	0.001	0.001	0.001

[1]Dietary treatments: ad-libitum feed without access to BSFL (CON), or with BSFL amounting to 10% (L10), 20% (L20) or 30% (L30) of the feed intake of CON birds. Total number of observations used for statistical analyses, N = 144 (4 treatments each with 6 replicate pens repeatedly measured over 6 weeks). Number of observations; Number of birds, n=63 per treatment.

[2]G = treatment group effect, W = time effect (week), G×T = treatment group by time interaction.

[3]Note that CON intakes via feed (100%) and BSFL (0%) show no within group variation per study design, thus statistical comparisons summarized in this table exclude CON, and refer to BSFL consuming groups (L10-L30) only.

4. Article 2

---

4. Lipid metabolism, fatty acid composition and meat quality in broilers supplemented with increasing levels of defrosted black soldier fly larvae

This chapter has been published in:

Journal of Insects as Food and Feed, 2022, 1-16

Please find the original article via the following digital object identifier:

https://doi.org/10.3920/JIFF2022.0125

M.M. Seyedalmoosavi[1], D. Dannenberger[2], R. Pfuhl[2], S. Görs[1], M. Mielenz[1], S. Maak[2], P. Wolf[3], G. Daş[1*], C.C. Metges[1]

[1]Research Institute for Farm Animal Biology (FBN), Institute of Nutritional Physiology, Dummerstorf, Germany.

[2]Research Institute for Farm Animal Biology (FBN), Institute of Muscle Biology and Growth, Dummerstorf, Germany.

[3]University of Rostock, Nutrition Physiology and Animal Nutrition, Faculty of Agricultural and Environmental Sciences, Rostock, Germany.

*Corresponding author: gdas@fbn-dummerstorf.de

Manuscript received at the Journal: 27 August 2022

Revision accepted: 15 October 2022

Available Online: December 02, 2022

Abstract

A feeding experiment was conducted to investigate the impact of feeding defrosted whole black soldier fly larvae (BSFL) to broilers in increasing levels in the ration on blood metabolites, carcass characteristics (CC) and on changes in fatty acid (FA) composition in plasma, muscle and abdominal fat. Day-old chicks (Ross-308; N = 252) were assigned to one of four groups each with 6 replicate pens (10 - 11 birds/pen). The birds were fed either a demand-oriented age-specific control (CON) diet and had no access to BSFL, or fed CON plus BSFL at 10% (L10), 20% (L20) or 30% (L30) of CON feed intake. At weeks (wk) 4 and 6, birds (2 per pen) were slaughtered to collect blood, breast muscle, and abdominal fat samples and to determine CC. Plasma triglyceride concentrations increased in a dose dependent manner with increasing levels of whole BSFL compared with CON ($P<0.05$). The L30 and L20 had higher plasma non-esterified FA concentrations than CON ($P<0.05$). There were no differences in slaughter weight and CC between groups ($P>0.05$). Broilers fed 30% BSFL had the highest saturated FA proportion in plasma, muscle and abdominal fat and the lowest monounsaturated FA proportion in abdominal fat tissue ($P<0.05$). The levels of total polyunsaturated FA in plasma and abdominal fat were lower in L30 than in CON ($P<0.05$). In plasma, muscle and abdominal fat, the proportion of conjugated linoleic acid (isomer C18:2cis-9, trans-11) was highest in L30 followed by L20 and L10 compared with CON ($P<0.05$). Overall, whole BSFL could be included in broiler diets up to 20% to promote sustainability in broiler farming without adverse effects on slaughter weight, meat quality and FA compositions, whereas, the highest inclusion level (i.e. 30%) of whole BSFL in the daily ration was associated with altered FA composition in plasma, fat and meat.

Keywords: Carcass; Chicken; Fatty acid profile; Hermetia illucens; Defrosted whole larvae

1. Introduction

Heavy reliance on soybean and fishmeal as protein feed for commercial livestock is no longer considered sustainable (Dörper *et al.*, 2020). To support a more sustainable and environmentally friendly production of feed for livestock, including poultry, insects meals such as from black soldier fly larvae (BSFL; *Hermetia illucens*), have been suggested as alternative protein feed in exchange for soybean meal (Cutrignelli *et al.*, 2018; de Souza Vilela *et al.*, 2021a; Maurer *et al.*, 2016; Schiavone *et al.*, 2017). Whole insect larvae are a protein-rich source of natural feed consumed by wild birds and free-range poultry (Rumpold *et al.*, 2017). Whole BSFL contain 33 to 59% crude protein (CP), and 11 to 34% lipids in dry matter (DM), which make them an interesting ingredient for poultry feed (Bava *et al.*, 2019; Maurer *et al.*, 2016; Shumo *et al.*, 2019). In recent years, increasing attention has been paid to using BSFL in the form of meal or oil in broiler diets. Previous studies demonstrated a successful incorporation of BSFL meal in broiler diets without negative effects on key carcass traits and meat quality including carcass composition, meat pH and color, cooking loss, or lipid oxidation in broiler meat (de Souza Vilela *et al.*, 2021a; Popova *et al.*, 2020). Moreover, using full-fat BSFL meal in the diet of growing layer chickens improved growth performance, nutrient digestibility, plasma antioxidant ability and gut health (Chu *et al.*, 2020). In addition, inclusion of BSFL fat in broiler diets did not affect blood metabolites, and carcass traits and chemical composition of broiler meat were satisfactory (Dabbou *et al.*, 2021; Schiavone *et al.*, 2017). However, the industrial processing of BSFL to produce protein meal, implies additional costs which might constrain expansion of using BSFL in poultry

rations. This may be particularly important for low-input poultry farming systems particularly in developing countries. There are few reports on the successful inclusion of whole BSFL in poultry diets supporting growth performance (Ipema *et al.*, 2020a; Ipema *et al.*, 2020b; Star *et al.*, 2020; Tahamtani *et al.*, 2021) and meat quality including FA profile and protein content in meat (Moula *et al.*, 2018).

BSFL fat is rich in saturated fatty acids (SFA) and contains considerable amounts of medium chain fatty acids (MCFA), particularly lauric acid (*C12:0*) (Li *et al.*, 2022). Lauric acid, which can constitute up to 50% of BSFL fat (Franco *et al.*, 2021; Li *et al.*, 2022), can also be converted to monolaurin (or glycerol monolaurate), which has growth-promoting potential and could explain the antibacterial activity of BSFL fat (Almeida *et al.*, 2020; Franco *et al.*, 2021). Moreover, MCFA can have positive effects on energy availability without increasing lipid deposition (Li *et al.*, 2016). However, BSFL fatty acid (FA) composition is highly variable as it heavily depends on the FA composition of the feeding substrates for larvae (Ewald *et al.*, 2020). The FA composition of broiler meat is also dependent on the feed source (Cullere *et al.*, 2019; de Souza Vilela *et al.*, 2021a; Dörper *et al.*, 2020), therefore feeding BSFL to broilers has the potential to modulate FA composition of the particular meat products (e.g. breast meat), and may enrich MCFA in edible tissues (Franco *et al.*, 2021). It has been reported that BSFL fat can be used to incorporate considerable amounts of monounsaturated fatty acids (MUFA) contents in chicken breast and leg meats of broilers (Cullere *et al.*, 2019). Moreover, it has been suggested that lower proportions of polyunsaturated fatty acids (PUFA) in BSFL fat may increase antioxidant capacity in BSFL oil–fed chickens (Kim *et al.*, 2020).

Despite the reported benefits of BSFL in broiler diets, there seem to be trade-offs in the meat FA composition, characterized by increased SFA at the expense of PUFA (Cullere *et al.*, 2019; Schiavone *et al.*, 2017). There is only one study available in which whole BSFL were used in poultry diets (8% of commercial feed) but no difference in the FA composition of meat between BSFL-fed and control birds were observed (Moula *et al.*, 2018). There is a lack of knowledge on the amount of whole BSFL that can be included in broiler diets without adverse effects on metabolism and FA compositions in different tissues of chickens. Therefore, the objective of this study was to investigate the effects of feeding whole BSFL to broilers in increasing dietary levels for 42 d on carcass characteristics, blood metabolites, and particularly on changes of FA compositions in plasma, muscle and abdominal fat tissues.

2. Material and methods

Chickens and management

The feeding experiment was registered under A.Z. 202022_70_A28_anz. A total of 252 mixed-sex newly hatched chicks (Ross 308) was obtained from a commercial hatchery and housed at the experimental poultry facility at the Research Institute for Farm Animal Biology (FBN), Dummerstorf, Germany. The chicks were weighted at arrival (42 ± 0.38 g/bird) and randomly allocated to one of 24 pens (n = 10 - 11 chicks / pen) in four adjacent rooms of the facility, which resulted in 63 birds in 6 pens for each group. Pens of each room (n = 6) were separated from each other with solid panels. Each pen was equipped with one feeder, a line of drinking nipples, and a deep layer of wood shavings as litter material. Throughout the experiment, birds in different rooms were raised under the same environmental conditions. Climate conditions in the rooms were automatically controlled based on recommendations of the Aviagen Ross broiler handbook

(Aviagen, 2018) with some modification by a ventilation and heating system, ensuring uniform ambient temperature, light and ventilation conditions across the pens within and between 4 experimental rooms. Ambient temperature at the start of the experiment was 33 °C and was gradually decreased to 21 °C at week (wk) 6, whereas humidity was gradually increased from 37% to 70% until wk 6.

Experimental design, diets and BSFL provision

A completely randomized design with 4 treatments was used in this study. All birds received the same basal diet in mesh feed form. The basal diet was designed to meet or exceed age-specific nutrient recommendation of broilers (Aviagen, 2019) in three phases, i.e. starter (d 0 - 14), grower (d 15 - 28) and finisher (d 29 - 42) diets (Table 1). Equal numbers of pens (n = 6 per group) and birds (n = 63 per group) were randomly allocated to each of the 4 dietary treatments with each treatment occurring in each room at least once. Broilers in the control group (CON) received the age-specific basal diet, and had no access to BSFL. Birds in the remaining 18 pens received defrosted whole BSFL in addition to the basal diet at increasing levels, i.e. 10%, 20% or 30% of the feed intake (FI) of CON birds (hereafter referred to as groups L10 (n = 63), L20 (n = 63), and L30 (n = 63), respectively). Except for the first day (d1), the daily amount of BSFL to be fed to the broilers in L10 to L30 groups was calculated based on FI of the CON birds on the previous day. On d1, FI of broiler birds from previous experiments was used as a reference. Pen based daily FI was measured in the mornings before feeding larvae, and the average FI per bird was then calculated. The thawed whole larvae to be given to the birds of a pen were weighed and placed on

a feeding plate which was then placed on the ground of the recipient pen at the same time each day (07:30 h). Cumulative total fresh matter intake (FMI; the sum of feed and larvae intake as is) per an average bird over 42 d of each pen was calculated. Based on the amounts of cumulative feed and BSFL intakes, and the nutrient and energy contents of the diets and BSFL, pen based cumulative fat and energy intakes trough both feed and larvae consumption were calculated for an average bird over the complete fattening period (42 d). Live BSFL (a mix of 5^{th} - 6^{th} instars) were purchased from Hermetia Deutschland GmbH & Co. KG, Baruth/Mark, Germany. The larvae used in this experiment originated from the same rearing batch, and had been fed a company-specific feeding substrate in accordance with feed regulations. As soon as the live larvae were received, they were snap frozen using liquid nitrogen and stored at -20 °C until fed to broilers. Twelve hours before broilers were offered, the frozen larvae were thawed in a refrigerator (4 °C). Broilers received defrosted BSFL at approximately room temperature.

Chemical analysis of feed and BSFL

During the experiment feed and larvae sub-samples were collected at regular intervals and stored at −20°C for analysis. At the end of the experiment, all of the sub-samples were pooled by feed type (e.g. starter, grower, and finisher) and representative samples were analyzed for their nutrient contents. Analyses of nutrient composition in larvae and feed samples were performed for DM content, crude ash, CP, crude fat, starch, crude fiber, total sugar, neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL) and macro minerals by the accredited feed laboratory of Landwirtschaftliche Untersuchungs-und Forschungsanstalt der LMS Agrarberatung GmbH (LUFA, Rostock, Germany) using standard methods (Naumann and Bassler, 1997).

Metabolizable energy contents of feed and BSFL were then calculated (Naumann and Bassler, 1997). Chitin content of BSFL was calculated as ADF – ADL as described (Hahn *et al.*, 2018). Table 1 presents the ingredients and chemical compositions of the age-specific basal diets, and summarizes the nutrient composition of BSFL. The basal diet and drinking water were provided to the birds *ad libitum* throughout the experimental period. The FA composition of the feed and BSFL samples were determined as explained in next sections. The FA composition of the age-specific diets and BSFL are summarized in Table S1. BSFL fat contained 71% of SFA followed by 18% MUFA and 11% of PUFA (Table S1).

Table 1. Ingredients and analyzed chemical composition of the age-specific broiler diets and defrosted whole black soldier fly larvae (BSFL) offered to the broilers during the experimental period.

	Age-specific basal diets			
	Starter	**Grower**	**Finisher**	**BSFL**
	(d 1-14)	**(d 15-28)**	**(d 29-42)**	**(d 1-42)**
Ingredients, %				
Soybean meal 48%	36.0	34.0	26.5	-
Wheat	31.0	28.0	35.0	-
Maize	21.5	28.0	28.0	-
Barley	5.0	4.0	5.0	-
Linseed oil	3.0	3.0	3.0	-
Vit-Min. Premix[1]	2.5	2.5	2.5	-
Oyster shells	1.0	0.5	0	-
***Chemical analysis**, g/kg DM*				
Dry matter	893	891	892	312
Crude ash	61.6	48.3	44.8	80.4
Crude protein	228	218	203	435
Crude fat	47.0	47.1	42.6	278
Crude fiber	24.6	25.8	34.8	72.7
Starch[2]	464	508	517	14.3
Total sugar (calculated as sucrose)	43.7	46.0	42.6	2.2
NDF	118	114	110	121
ADF	41.4	43.8	38.1	77.8
ADL	n.d.	n.d.	n.d.	4.42
Chitin*	n.d.	n.d.	n.d.	73.4
ME, MJ/kg DM	13.4	14.0	13.8	16.5
***Minerals,** g/kg DM*				
Calcium	12.1	7.6	6.1	18.1
Phosphorus	6.6	5.2	5.2	9.3
Magnesium	2.1	1.9	2.0	3.9

[1] Amount of vitamin and minerals provided through premix per kg of feed were as following; Vit. A 10000 IU, Vit. D3 2000 IU, Vit. E 20 mg, Vit. K3 3 mg, Vit. B1 1 mg, Vit. B2 6 mg, Vit. B6 3 mg, Vit. B12 30 mcg, Niacin 30 mg, Pantothenic acid 10.8 mg, Folic acid 0.4 mg, Biotin 24 mcg, Cholin 300 mg, Fe 55 mg, Cu 18 mg, Zn 80 mg, Mn 93 mg, I 0.66 mg, Se 0.34 mg, Co 0.05 mg, Phytase 250 FTU.

[2] For BSFL its glycogen

* Calculated based on Hahn et al., 2018 (i.e. Chitin = ADF - ADL).

Abbreviations: **NDF**: neutral detergent fiber; **ADF**: acid detergent fiber; **ADL**: acid detergent lignin; **n.d.**, not determined.

Slaughtering and sample collection

In the end of wk 4 and wk 6, two birds per pen (n = 48 birds / time point) were randomly chosen from their pens, weighed and slaughtered after electrical stunning at the institute's EU approved abattoir. The stunning and slaughtering of the birds were conducted according to the German animal welfare regulations. From each slaughtered bird, blood was collected in K3-EDTA-coated vacutainers (Sarstedt AG & Co., Nümbrecht, Germany) to obtain plasma. Plasma was harvested after centrifugation at 2,500 × g for 20 min at 4°C, aliquoted in 2 mL reaction tubes, and frozen at −20°C until the determination of cholesterol, glucose, non-esterified FA (NEFA), triglycerides and FA compositions.

Carcass characteristics

Immediately after slaughter, the carcasses were labelled and weighed to determine hot carcass weight. Wings, legs, breast, and abdominal fat were dissected and weighed. In addition, breast muscle and abdominal fat were sampled from individual birds and stored at −20°C for FA analysis. The pH values of the breast muscle samples were measured at 30 min, 45 min and 24 h post mortem (p.m.) with a portable pH meter (pH-Star, Matthäus, Eckelsheim, Germany) in the thickest part of the muscle. The color of the breast muscle samples was measured at 24 h p.m. using a chromameter (Minolta CR 200, Ahrensburg, Germany) with triplicate measurements from a freshly cut surface using the parameters L* (brightness), a* (red-green) and b* (yellow-blue). Chroma ($(a^{*2}+b^{*2})^{1/2}$) and hue (arctan (b^{*}/a^{*})) indexes were calculated (Kongsup *et al.*, 2022). The drip loss procedure has been described elsewhere (Janisch *et al.*, 2015).

The breast muscle DM was determined according to AOAC methods (AOAC, 2000). The nitrogen content in breast muscle samples was measured using an elemental analyser (FlashEA 1112 NC Analyzer by Thermo Fisher Scientific, Bremen, Germany). For this, 1 to 1.5 mg of sample was weighed in tin capsules (IVA-Analysentechnik GmbH und Co. KG, Meerbusch, Germany (Size: 3.3 x 5 mm)) and sealed airtight. The samples were combusted at 900°C to carbon dioxide and nitrogen oxides; the latter were reduced to elemental nitrogen at 680°C. Combustion gases were separated via gas chromatography with helium as the carrier gas and quantified by a thermal conductivity detector. The nitrogen content was then multiplied with the factor of 6.25 to calculated the crude protein content.

Blood metabolites

Plasma NEFA, cholesterol, glucose and triglycerides concentrations of the birds were analyzed with an automatic enzymatic analyzer (ABX Pentra 400, Horiba Medical, Montpellier, France), using commercial kits [Glucose: Kit No. A11A01667, cholesterol: Kit No. A11A01634; triglyceride: Kit No. A11A01640 (Horiba ABX); NEFA: Kit No. 434-91795 (Wako Chemicals GmbH, Neuss, Germany)].

Lipid extraction and transesterification of feed, BSFL, and chicken plasma, breast muscle and abdominal fat samples

Feed and larvae

Larvae and feed samples were freeze dried and the samples were finely ground using liquid nitrogen in a mortar with a pestle. For extraction and direct FA methylation of feed and larvae samples, a modified method from Sukhija and Palmquist (1988) was used. The samples were treated with 2 mL toluene (containing 19:0 methyl ester as internal standard) and 4 mL of 5% methanolic HCl. The mixture was shaken in a water bath at 60°C for 2 h. After cooling, methyl ester (FAMEs) were extracted with 3.5 mL toluene in the presence of 8.75 mL 6% K_2CO_3 solution and vortexed. After centrifugation (ScanSpeed 40, LaboGene, Allerød, Denmark) at 1200 × g for 5 min (at 4°C), the toluene phase was separated, and 1 g Na_2SO_4 and activated charcoal were added and the mixture was stored overnight until the organic phase became colorless. After filtration, an aliquot of the toluene extract was taken and stored at –18 °C until GC analysis (Kalbe *et al.*, 2019).

Muscle, abdominal fat and plasma

The frozen breast muscle and adipose tissue samples were cut into small pieces and homogenized using a mill (Model A 11 Basic, IKA GmbH; Staufen, Germany). For lipid extraction, approx. 1 g of muscle (0.5 g adipose tissue) was weighed in a tube. Each Precellys-tube contained 20 pieces of 2.8 mm bulk beads and 2 pieces of 5 mm bulk beads (Zirconium oxide Precellys beads, Bertin Instruments, Montigny-le-Bretonneux, France). After addition of 3 mL methanol and nonadecanoic acid (19:0) as an internal standard, the extracts (in duplicate) were homogenized 3 times at 25 s intervals at 4°C and 6500 rpm using a homogenizer (Precellys Evolution, Bertin Instruments, Montigny-le-Bretonneux, France). The homogenates were vortexed and transferred to Pyrex tubes (Pyrex, Hayes, UK) containing 8 mL of chloroform. After that, the Precellys-Tubes

were washed two times with 1 mL methanol and added to the Pyrex tubes (Tönißen *et al.*, 2022). Plasma (1.5 mL) was added dropwise to a tube containing 8 mL of chloroform/methanol (2:1, v/v) with 60 μL C19:0 as an internal standard (60 mg/mL) at room temperature. The plasma sample preparation was described in detail elsewhere (Dannenberger *et al.*, 2017). All solvents used for feed, tissue and plasma lipid extraction contained 0.005% (w/v) of t-butylhydroxytoluene (BHT) to prevent oxidation of PUFA.

After filtration, the lipid extracts of tissues and plasma samples were stored at 5°C for 18 h in the dark and subsequently washed with 0.02% $CaCl_2$ solution. The organic phase was separated and dried with a mixture of Na_2SO_4 and K_2CO_3 (10:1, w/w), and the solvent was subsequently removed using a vacuum centrifuge (ScanSpeed 40; LaboGene, Allerød, Denmark) at 2000 rpm/min, 30°C, 30 min. The lipid extracts were redissolved in 300 μL of toluene, and a 25 mg aliquot was used for methyl ester preparation (Kalbe *et al.*, 2019). Briefly, for transmethylation, 2 mL of 0.5 M sodium methoxide in methanol were added to the lipid extracts, which were shaken in a 60 °C water bath for 10 min. Subsequently, 1 mL of 14% boron trifluoride in methanol was added to the mixture, which was then shaken for an additional 10 min at 60 °C. The fatty acid methyl esters (FAMEs) were extracted twice with 2 mL of *n*-hexane and stored at −18 °C until use for high-resolution gas chromatography (HR-GC) analysis.

Fatty acid analysis

The fatty acid analysis of all sample lipid extracts was performed using a capillary gas chromatograph (GC) with a CP-Sil 88 CB column (100 m × 0.25 mm, Agilent, Santa Clara, CA,

USA) that was installed in a PerkinElmer GC CLARUS 680 with a flame ionization detector and split injection (PerkinElmer Instruments, Waltham, Massachusetts, U.S.A.) as described earlier (Dannenberger *et al.*, 2012). Briefly, hydrogen was used as the carrier gas at a flow rate of 1 mL × min^{-1} while the split ratio was 1:20, with the injector and detector were set at 260 and 280 °C, respectively. The GC oven temperature program was 150 °C for 5 min; heating rate of 2°/min until 200°C and kept for 10 min; heating rate of 1°/min until 225°C and kept for 20 min. For the calibration the reference standard mixture "Sigma FAME" (Sigma-Aldrich, Deisenhofen, Germany), the methyl ester of C18:1*cis*-11, C22:5*n*-3, and C18:2*cis*-9,*trans*-11 (Matreya, State College, PA, USA), C22:4*n*-6 (Sigma-Aldrich, Deisenhofen, Germany), and C18:4*n*-3 (Larodan, Limhamn, Sweden) were used. The five-point calibration of single fatty acids ranged between 16 and 415 µg/mL and was assessed after GC analysis of five samples. Fatty acid proportions are presented as % of total fatty acids or as concentration in mg/100 g tissue. In addition to composition of FA groups (SFA, MUFA, PUFA), five nutritional indices (i.e. Nutritional Value Index, NVI; Peroxidability index, PI; Atherogenicity index, AI; Thrombogenicity Index, TI; and Hypocholesterolemic / Hypercholesterolemic ratio, HH) were calculated (Chen and Liu, 2020; Dabbou *et al.*, 2017; Dal Bosco *et al.*, 2022) to assess nutritional quality of fatty acid composition in breast meat only.

Statistical analysis and presentation of the results

The pen was considered as the experimental unit for the cumulative feed and energy intake variables (n = 6), and for the single-point measurements, birds sampled at the slaughterhouse (N

= 96) were considered as the experimental unit. Data related to the parameters measured on individual animals, i.e. blood metabolites, meat quality, carcass characteristics, and FA composition of plasma, breast meat and abdominal fat were analyzed by the general linear model (PROC GLM) of SAS. The statistical model included fixed effects of treatment (1 - 4) and slaughter week (4 and 6), interaction term, and the blocking effects of room (1 - 4) and pens (1 - 6). The Tukey test was used to separate means and identify significant differences among treatments. The significance level was preset at $P < 0.05$, and a tendency was declared at $0.05 < P \leq 0.10$. Data for the cumulative intake variables (i.e. cumulative feed, larvae, feed+larvae, fat and ME intake) up to d 42 were analyzed with the same statistical model omitting pen effect as pen replaced individual animals as the experimental unit for these variables. Similarly, week was omitted from this model as cumulative intakes corresponded to week 6 only.

A hierarchical two-way clustering analysis (HCA) was conducted using JMP statistical software v.10 (SAS Institute) to investigate patterns in the FAs compositions in three different tissues in response to the dietary treatments. Data used for this analysis comprised 43 individual FAs across plasma, fat and muscle tissues of 96 birds in two age groups (weeks 4 and 6). Furthermore, alterations in the composition of FA groups (i.e. SFA, MUFA, PUFA) in response to dietary treatments were visualized using relative differences of each treatment to the CON.

As no significant interaction effects between the two main experimental factors (treatment and time) were encountered, the results were presented in relevant tables and figures as least square

means (LSMEANS) and their standard errors (SE) as overall differences between relevant treatment groups, and additional information was provided in supplementary Tables.

3. Results

Cumulative feed, BSFL, fat and energy intakes

Birds in all BSFL groups fully consumed pre-determined proportions of offered larvae in a few min (data are not shown). Cumulative intakes over the whole fattening period (42 d) including feed, larvae, feed plus larvae (total), and fat and ME intakes trough feed and larvae consumption are presented in Table 2. The L30 group had a lower cumulative FI compared to CON ($P < 0.05$). Total intake did not differ among the other groups, while there was a tendency that L20 birds had a higher intake than CON ($P = 0.052$). Birds in the L20 and L30 groups had a higher total fat intake than in the L10 and CON groups ($P < 0.05$). Despite the high fat intake, L30 had the lowest total ME intake, even lower than CON ($P < 0.05$).

Table 2. Cumulative intakes (g/bird) over 42 days through feed and defrosted whole black soldier fly larvae (BSFL) consumption in broilers offered either only regular feed (CON) or increasing levels of BSFL (10-30%) in addition to the regular feed.

Cumulative intakes, g/bird	Dietary treatments[1]					P-value[2], ≤
	CON	L10	L20	L30	SE	T
Feed	3953^{a†}	3580^{ab}	3569^{ab†}	3034^{b}	100.0	0.001
BSFL	n.a.	382	764	1145	-	-
Feed+BSFL	3953†	3961	4332†	4180	99.6	0.052
Fat (Feed)	157.5^{a†}	142.3^{ab}	141.5^{ab†}	120.4^{b}	3.96	0.001
Fat (BSFL)	n.a.	33.1	66.2	99.3	-	-
Fat (feed+BSFL)	157.5^{c}	175.4^{b}	207.7^{a}	219.7^{a}	3.96	0.001
ME (feed), MJ	48.8^{a†}	44.2^{ab}	44.0^{ab†}	37.4^{b}	1.23	0.001
ME (BSFL), MJ	n.a.	2.0	3.9	5.9	-	-
ME (feed+BSFL), MJ	48.8^{a}	46.1^{ab}	48.0^{ab†}	43.3^{b†}	1.23	0.027

[1] Dietary treatments: ad libitum feeding without access to BSFL (CON), or with BSFL amounting to 10% (L10), 20% (L20) or 30% (L30) of the feed intake of CON birds. Total number of observations used for statistical analyses, N = 144 (4 treatments each with 6 replicate pens repeatedly measured over 6 weeks). Number of observations; Number of birds, n = 63 per treatment. Data are presented as LSEMANS and their SE. For the sake of a succinct presentation, only the most conservative (i.e. the largest) SE is presented.

[2] **T**: treatment effect. a-b: Values in a row denoted with different letters differ significantly (Tukey, $P < 0.05$). Symbol † in a row indicates a tendency of two treatments to differ (Tukey, $0.05 < P \leq 0.10$).

Abbreviation: **n.a.**, not applicable; **ME,** metabolizable energy.

Slaughter weight and carcass characteristics

There was no slaughter weight difference between treatment groups (Table 3; $P > 0.05$). Incorporation of different levels of BSFL in the rations did not affect carcass characteristics of the birds ($P > 0.05$). Dressing percentage, percentages of breast and wings significantly differed between wk 4 to wk 6 ($P < 0.001$), but no treatment or treatment by week interaction effect (not shown in Table 3) could be quantified ($P > 0.1$). Similarly, no treatment or treatment by week

interaction effect could be observed for pH and color traits of breast meat ($P > 0.1$). Dry matter contents of breast muscle were higher in L30 than in L10 and CON birds ($P < 0.05$). In line with this, the CP content of breast meat was higher in L30 than in L10 and CON ($P < 0.05$). These differences disappeared when CP contents were adjusted for DM of the meat tissue ($P = 0.133$).

Plasma metabolites concentrations

The plasma NEFA concentration was higher in L30 and L20 compared to the CON birds (Table 3; $P < 0.05$). In addition, birds in L10 tended to have a higher NEFA level than CON birds ($P < 0.10$). As compared to CON, provision of BSFL increased plasma triglyceride concentration in all BSFL groups with a significant increase from L10 to L30 (Table 3; $P < 0.05$). Plasma cholesterol concentration was not affected by the dietary treatments (Table 3; $P > 0.05$). No significant treatment × week effect was found for the plasma metabolites concentrations ($P > 0.05$). Plasma glucose concentration tended be higher in L30 than in L10 (Table 3; $P = 0.062$).

Table 3. Effects of increasing levels of defrosted whole black soldier fly larvae (BSFL) in broiler diets on carcass characteristics and breast meat quality traits, as well as on blood metabolites of broilers.

	Dietary treatments[1]					P-values[2], ≤	
	CON	L10	L20	L30	SE	T	W
Slaughter weight, g	1821	1732	1971	1817	114.5	0.602	0.001
Hot carcass weight, g	1169	1096	1254	1143	82.1	0.628	0.001
Breast weight, g	339	326	365	337	30.1	0.859	0.001
Breast DM, %	23.2^{b}	23.2^{b}	23.9^{ab}	24.2^{a}	0.22	0.006	0.077
% of Carcass weight							
Dressing percentage	63.0	62.3	62.2	62.5	0.77	0.848	0.001
Breast	27.8	28.2	27.6	29.0	0.78	0.571	0.001
Legs	33.4	33.4	33.4	32.9	0.49	0.806	0.258
Wings	10.7	10.8	10.5	10.5	0.20	0.736	0.001
Abdominal fat	2.1	2.0	2.0	2.4	0.48	0.885	0.318
Breast meat quality parameters							
24 hr Drip loss, %	40.7	39.5	39.7	38.7	1.01	0.534	0.001
pH at 30 min	6.4	6.4	6.3	6.4	0.03	0.384	0.001
pH at 45 min	6.2	6.2	6.2	6.2	0.03	0.791	0.002
pH at 24 h	5.5	5.5	5.5	5.5	0.02	0.484	0.512
L* (lightness)	55.3	54.8	54.2	53.8	0.63	0.409	0.605
a* (redness)	3.3	3.3	3.1	3.2	0.25	0.923	0.659
b* (yellowness)	4.7	4.7	4.9	4.8	0.32	0.982	0.001
C* (Chroma)	5.9	5.9	5.9	5.9	0.30	0.999	0.001
H* (Hue)	53.7	53.1	56.2	56.3	2.68	0.801	0.001
CP, % FM	20.8^{b}	20.6^{b}	21.4^{ab}	21.7^{a}	0.23	0.002	0.315
CP, % DM	89.4	88.8	89.4	90.0	0.36	0.133	0.160
Blood metabolites							

NEFA, mM	132.9^{c†}	192.8^{bc†}	271.6^{ab}	308.8^{a}	19.5	0.001	0.667
Triglyceride, mM	1.04^{c}	1.40^{b}	1.59^{ab}	1.81^{a}	0.101	0.001	0.630
Cholesterol, mM	3.04	3.17	3.26	3.33	0.109	0.272	0.002
Glucose, mM	13.86	13.76†	14.03	14.45†	0.197	0.062	0.972

[1] Dietary treatments: ad libitum feeding without access to BSFL (CON), or with BSFL amounting to 10% (L10), 20% (L20) or 30% (L30) of the feed intake of CON birds. Total number of observations used for statistical analyses, N = 96 (i.e., 2 birds sampled from each of 6 pens allocated to each of 4 treatments at weeks 4 and 6).
[2] **T**: treatment effect; **W** = time effect (weeks 4 and 6). Symbol † in a row indicates a tendency of two treatments to differ (Tukey, $0.05 < P \leq 0.10$). P-values for **T*W** = treatment by time interaction were all $P > 0.1$, and are not shown. Data are presented as LSEMANS and their SE, and the values refer to average of weeks 4 and 6 as there was no significant T*W interaction. For the sake of a succinct presentation, only the most conservative (i.e. the largest) SE is presented.
a-c: Values in a row denoted with different letters differ significantly (Tukey, $P < 0.05$).

Plasma fatty acids compositions

The composition of fatty acids (g/100 g of total fatty acids) in plasma of broilers, is presented in Table S2. The proportion of capric acid (*C10:0*) in plasma FA was higher in L30 than in CON birds (Table S2; $P < 0.05$). In addition, the L20 tended to have a higher proportion of *C10:0* than the CON group ($P < 0.05$). The plasma *C12:0* proportion was higher in L30 than those in L10 and CON birds ($P < 0.05$). Similarly, *C12:0* was higher in L20 than in CON ($P < 0.05$). The L30 and L20 birds had higher plasma myristic acid (*C14:0*) proportion than L10 ($P < 0.05$), followed by CON ($P < 0.05$). The total saturated FA level (∑SFA) was higher in L30 than in L10 and CON birds ($P < 0.05$). Accordingly, both L20 and L10 groups had higher ∑SFA levels in plasma as compared to CON ($P < 0.05$).

No significant difference was found among the 4 treatment groups for the FA proportion of MUFA in plasma ($P > 0.05$). Plasma levels of linoleic acid (*C18:2n-6)*, α-linolenic acid (*C18:3n-3*), total n-3 PUFA (∑n-3), and total n-6 PUFA (∑n-6) were lower in L30 than in CON ($P<0.05$). The

proportion of the conjugated linoleic acid (CLA) *C18:2cis-9, trans-11* FA increased in the plasma lipids of larvae fed birds in response to increasing levels of BSFL in contrast to CON ($P < 0.05$). The eicosapentaenoic acid (EPA) was lower in plasma samples of L30 an L20 than in L10 and CON ($P < 0.05$). However, proportions of docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) were not affected by the dietary groups ($P > 0.05$). Similarly, the n-6/n-3 ratio did not differ among the groups ($P > 0.05$; Table S2). No treatment × week interaction effect was found for individual or groups of FA in plasma ($P > 0.05$).

Breast muscle fatty acids compositions

The proportions of fatty acids with relatively high concentrations in the breast muscle lipids of broilers are presented in Table S3. The proportion of *C10:0* in breast muscle total FA was higher in L30 than in the other groups (Table S3; $P < 0.05$). With an increasing BSFL level in the diet, the proportions of *C12:0* and *C14:0* increased linearly ($P < 0.05$), so that higher proportions of *C12:0* and *C14:0* in the breast muscle total FA in L30 birds than in L20 followed by L10 and CON were observed ($P < 0.05$). Provision of BSFL at 30% of FI (L30) increased ∑SFA in breast muscle as compared to CON ($P < 0.05$), whereas ∑MUFA levels were not affected by the provision of BSFL ($P > 0.05$). Furthermore, there was no difference between treatment groups for total PUFA and ∑n-6 levels ($P > 0.05$), while ∑n-3 FA were reduced with increasing BSFL levels ($P > 0.05$). Although *C18:3n-6* and *C18:3n-3* proportions were reduced in response to increasing levels of BSFL, the *C18:2cis-9, trans-11* fractions in breast muscle total FA were significantly increased in treatment group L10 to L30 as opposed to CON ($P < 0.05$). The L30 had a higher n-6/n-3 ratio

compared to CON ($P < 0.05$). Supplementation with BSFL at any of the three levels did not influence NVI of breast muscle ($P > 0.05$; Table S3), whereas the L30 led to a lower PI than those in CON ($P < 0.05$). Increasing BSFL levels in the diet resulted in increasing AI and TI indices, with L30 having the highest impact ($P < 0.05$), while the opposite was the case for the HH ratio ($P < 0.05$). No treatment × week interaction was found for breast muscle FA proportions, and the nutritional indices ($P > 0.05$).

Fatty acids compositions in abdominal fat tissue

The proportions of fatty acids with relatively high concentrations in the abdominal fat tissue of broilers are presented in Table S4. The FA analysis of abdominal tissue showed that *C10:0*, *C12:0* and *C14:0* fractions increased in the BSFL-fed treatment groups in a dose dependent manner, which resulted in higher ∑SFA levels in BSFL-fed groups than those in CON (Table S4, $P < 0.05$). Broilers fed BSFL had lower *C18:1cis-9* and *C18:1cis-11* fractions in abdominal fat ($P < 0.05$), which caused a higher percentage of ∑MUFA in L30 and L20 than in CON groups ($P < 0.05$). The ∑PUFA, ∑n-3, and ∑n-6 levels were reduced in response to feeding increasing levels of BSFL ($P < 0.05$). Among PUFAs, *C18:2n-6*, γ linolenic acid (*C18:3n-6*) and *C18:3n-3* proportions decreased with increasing BSFL level ($P < 0.05$). However, the *C18:2 cis-9, trans-11* FA fraction was significantly increased in the abdominal fat in broilers which received BSFL compared to those in the CON group ($P < 0.05$). The L30 treatment had a lower proportion of DPA than in CON and L20 ($P < 0.05$), however, EPA and DHA were not affected by the dietary treatments (P

> 0.05). The n-6/n-3 ratio did not differ among the treatment groups (P > 0.05). No treatment × week interaction effect was found for abdominal fat tissue FAs (P > 0.05).

Overall changes in fatty acid compositions across different tissues in response to feeding BSFL

Changes in relative abundance of FA groups in BSFL-fed groups as compared to CON in abdominal fat (A), plasma lipids (B) and breast muscle (C) at wk 4 (Figure S1) and wk 6 (Figure 1) were linearly influenced by increasing BSFL levels in the ration. These results collectively indicate that as BSFL level increased in the ration, total SFA proportions increased at the expense of ∑MUFA, ∑PUFA, ∑n-3 and ∑n-6 in all three tissues. The alterations were more pronounced in abdominal fat tissue followed by breast muscle and plasma tissues (Figure 1).

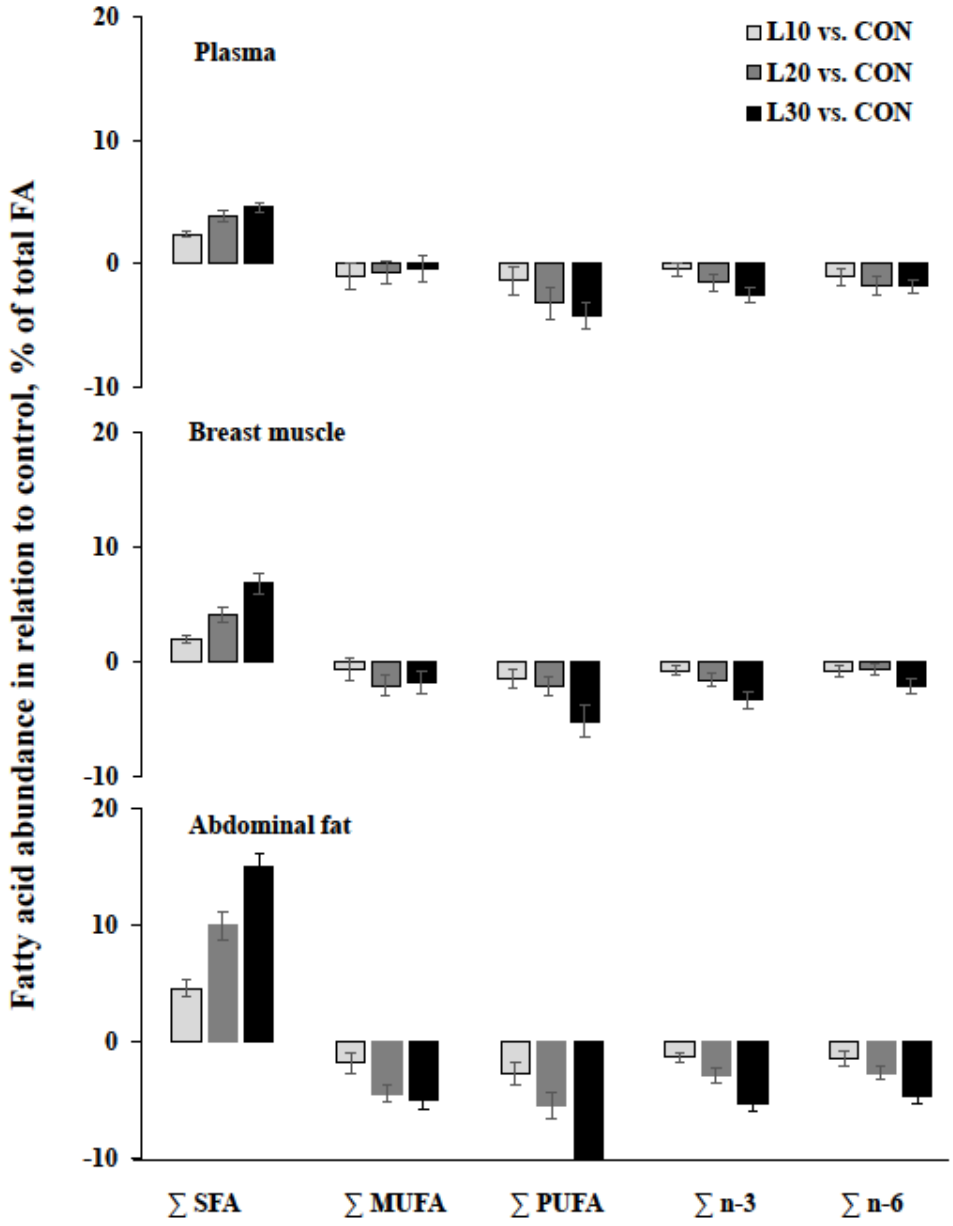


Figure 1. Relative changes in fatty acid groups abundance in plasma lipid, breast muscle and abdominal fat of broilers fed increasing dietary levels of black soldier fly larvae compared with broilers in the CON group without access to larvae. N = 48 (i.e., 2 birds sampled from each of 6 pens allocated to each of the 4 treatment groups at week 6).

A two-way hierarchical analysis was conducted to investigate the FAs compositions of different groups across different tissues (Figure 2). As shown with intensifying red and blue colors in the heatmap of the dendrogram, clustering of different variables showed dependency on the treatment groups across three tissues. Our results showed a distinct clustering of the animals from L20 and L30 groups, which had higher SFA levels, whereas CON stood either alone or in most cases clustered together with L10. On the left side of the heatmap, there is a clear clustering for SFA, indicating that L10 and CON had the lowest SFA contents, and by scrolling down the heatmap, higher SFA contents in L20 and L30 can be observed. At the bottom of the heatmap and on the right side, unsaturated fatty acids appear, and in some places they lead to a smaller clustering. Furthermore, at the mid-top of the heatmap, a clear grouping is shown in red, which indicates the presence of more unsaturated fatty acids in the CON and L10 groups, and at the bottom, this grouping can be detected in blue, which indicates lesser proportions of unsaturated fatty acids in L30 and L20 groups.

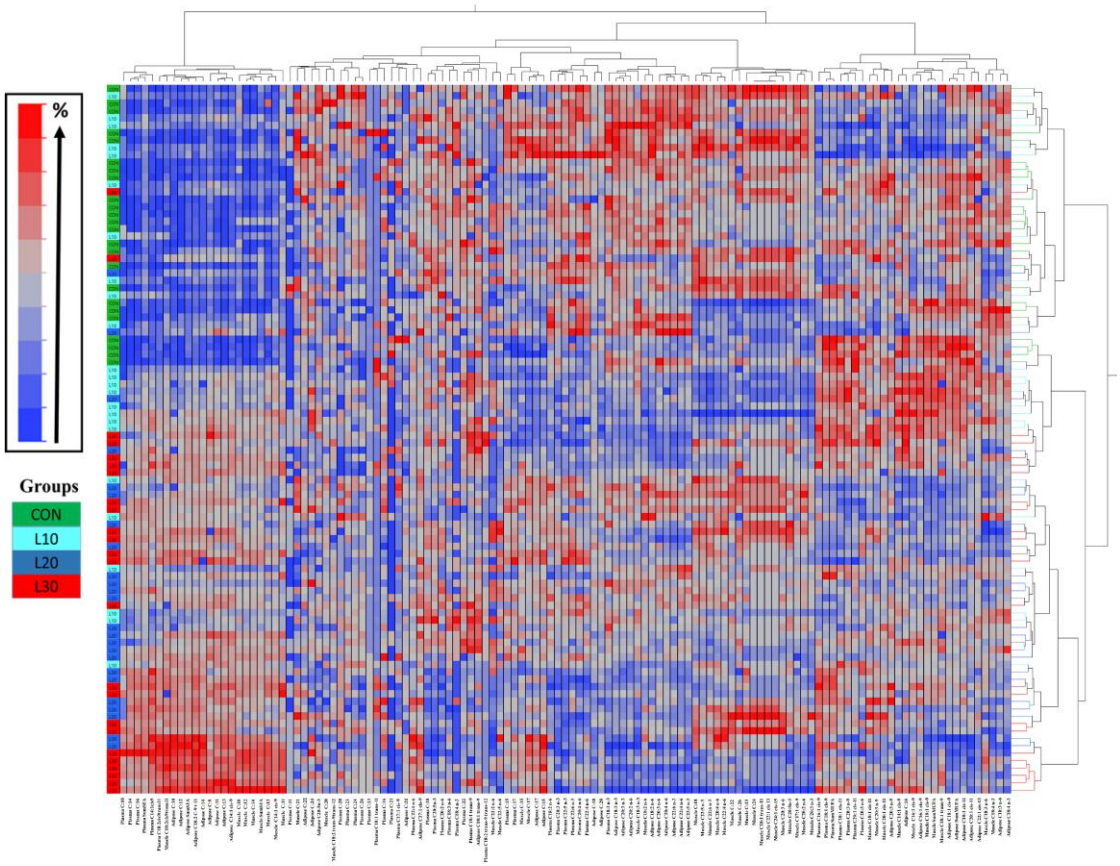


Figure 2[1]. Two-way hierarchical cluster analysis of fatty acid proportions in plasma lipid, breast muscle and abdominal fat of broilers fed increasing dietary levels of black soldier fly larvae. The heat map is based on concentrations of FAs (X-axis) in plasma, breast muscle and abdominal tissues across 4 experimental groups (Y-axis). The clusters are presented on the respective opposite axes, overlapping clusters due to similarity in diets and the resulting fatty acid proportions are then shown with similar color regions on the color-map. The deeper red represents the higher concentration and the deeper blue represents the lower concentration. The first left-column of the heatmap with 4 different colors indicates groups to which individual birds, each shown on a single line, belong to.

[1] Online version of this figure is available on:
https://www.wageningenacademic.com/doi/epdf/10.3920/JIFF2022.0125?role=tab

4. Discussion

We investigated the effect of feeding defrosted whole BSFL to broilers at increasing dietary levels on carcass characteristics, blood metabolites, and FA composition in plasma and breast muscle lipids and abdominal fat. We hypothesized that increasing levels of defrosted whole BSFL can be used in broiler diets with no adverse effects on meat quality, metabolism, and FA compositions of plasma, muscle and depot fat tissues. Our results indicated that up to 30% inclusion of defrosted whole BSFL in broiler diets did not negatively affect carcass characteristics, drip loss, meat pH and meat color of broilers. However, inclusion of 30% defrosted whole BSFL in broiler diets increased plasma NEFA and TG concentrations. In addition, the FA compositions in plasma lipids, breast muscle and abdominal fat of broilers fed 30% BSFL showed increased SFA levels at the expense of MUFA and PUFA. Increasing dietary levels of BSFL caused a dose dependent increase of the CLA fraction (C18:2 cis9, trans11) in total FA of plasma, muscle and fat tissues. However, lower proportions of ∑n-3 FA in BSFL than in the age-specific diets was reflected by an increased n-6/n-3 FA ratio in the L30 group. In the following, we discuss possible mechanisms affecting changes in carcass traits, plasma metabolites, and FA composition in plasma, muscle and fat tissues in BSFL-fed broilers.

Slaughter weight and carcass characteristics

We could not find indications that the inclusion of defrosted whole BSFL up to 30% in broiler diets affected meat quality and carcass traits. In line with our results, it has been reported that using up to 20% full-fat BSFL in the diet did not influence key meat characteristics and broiler

performance (de Souza Vilela *et al.*, 2021a; de Souza Vilela *et al.*, 2021b). Similarly, Cullere *et al.* (2019) reported that breast and leg physical meat quality and nutritional composition remained substantially unaffected in broilers that received diets in which 50% or 100% of the soybean oil was replaced with BSFL fat. However, Murawska *et al.* (2021) found that replacement of soybean meal with high levels (75% or 100%) of full-fat BSFL meal in broiler diets compromised growth performance and carcass traits (lower juiciness and taste intensity). We observed that despite high levels of fat in BSFL, abdominal fat (% of carcass weight) did not significantly differ among the groups. Despite the alterations of the fatty acid composition in abdominal fat tissue, the abdominal fat percentage in the carcass was similar among groups. However, despite the higher fat intake, L30 had a lower metabolizable energy intake. Lower ME intake in L30 birds may explain the lack of a significant difference in abdominal fat content in the carcass between the groups. The consumed fat had a high proportion of MCFA which can be utilized as a rapid energy source, improves energy availability and reduces the deposition of adipose tissue (Li *et al.*, 2016). Therefore, the lack of differences in the amount of abdominal fat depots observed in this study could be attributed to the lower ME intake in L30 birds and the high MCFA content of BSFL. Lower ME intake observed in the L30 group could be due to the overall drop in feed intake over all treatment groups which was most pronounced at the highest inclusion level (i.e. 30%). Lower feed intake in L30 could be attributed to the high dietary fat content which might affect the response of hypothalamic appetite-related peptides (Obrosova *et al.*, 2007; Wang *et al.*, 2017b), and might consequently cause the reduced feed intake. Moreover, the physical constrains in the

digestive tract of broilers might negatively affect feed intake of birds (Brickett *et al.*, 2007; Dozier III *et al.*, 2006).

Blood metabolites concentrations

Plasma triglycerides concentrations increased linearly with increasing levels of BSFL intake, whereas a higher plasma NEFA concentration in L30 compared to L10 and the CON groups was observed. High plasma NEFA concentrations in the L30 group can be explained by either a high lipolysis rate due to a low dietary energy intake or a high intake of dietary fat (Wang *et al.*, 2017a). Birds of the L30 group showed a lower ME intake which might be associated to higher plasma NEFA levels. Although we did not measure serum insulin concentrations, previous studies in chicken (Crespo and Esteve-Garcia, 2003) and humans (Lee *et al.*, 2006) showed that increased intake of n-6 FA and SFA is positively associated with insulin resistance (Sears and Perry, 2015). Plasma cholesterol concentration remained unaffected by the inclusion of BSFL in the ration. This is in line with results of Kim *et al.* (2020) that diets containing 5% of coconut oil or BSFL oil did not change the serum cholesterol levels of broilers compared to those of birds fed diets contained 5% of corn oil. It has been shown that increasing intakes of saturated fat (such as lard) can be associated with higher plasma TG and cholesterol levels in chicken (Peña-Saldarriaga *et al.*, 2020; Velasco *et al.*, 2010). This might indicate that the higher intake of dietary fat/SFA in the L30 group (219.7/92.5 g over 42d) compared to CON was not high enough to trigger an increase in plasma cholesterol. This could be attributed to the chitin content of BSFL, which might attract negatively-

charged bile acids and free FA and thus make them less available for cholesterol synthesis in the liver (Secci *et al.*, 2018).

Fatty acid compositions in different tissues

It is well known that the FA composition of muscle and adipose tissues in broilers can be modified by dietary factors (Bostami *et al.*, 2017; Kim *et al.*, 2020; Semwogerere *et al.*, 2019). In this experiment, FA analysis of BSFL revealed a high SFA (71%) and low PUFA content (i.e., approximately 12%). Plasma, breast muscle and abdominal fat FA profiles were similar to that of the FA profile of BSFL. Studies on the effect of feeding BSFL on FA composition in chicken meat are scarce. Schiavone *et al.* (2019) reported that using defatted BSFL-meal in broiler diets caused remarkable differences in the FA composition, with an increased MUFA content at the expense of PUFA. Also, de Souza Vilela *et al.* (2021a) reported that feeding up to 20% dried full-fat BSFL to broilers reduced total PUFA levels, while an increase in EPA was observed, coupled with an increase in total SFA and, in particular the *C12:0* fraction. In the present study we observed an increased SFA level in plasma, breast muscle and adipose tissue and a reduced PUFA level in plasma and adipose tissue of the broilers fed BSFL, which is probably due to the carry-over effect from BSFL fat. In addition to carry-over effects of BSFL, we assume that biochemical mechanisms may be involved in the alteration of FAs profile. In chickens, C*18:3 n-3* and C*18:2 n-6* are essential FA and precursors for long chain PUFA synthesis (Cherian, 2015) with EPA (*20:5 n–3*), docosapentaenoic acid (DPA; *22:5 n–3*), and docosahexaenoic acid (DHA; *22:6 n–3*) derived from C*18:3 n-3* and arachidonic acid (*20:4 n–6*) derived from *C18:2 n-6*, respectively (Murff and

Edwards, 2014). In our study, the lower plasma C*18:3 n-3* and *C18:2 n-6* proportions in L30 birds reflects their lower dietary intake, and might also be associated with the lower ∑PUFA level in abdominal fat. However, we did not find this effect in breast muscle tissue which could be due to the low fat content of the breast muscle. The conversion of *C18:3 n-3* and *C18:2 n-6* into long-chain PUFAs is carried out by Δ-6 desaturase, Δ-5 desaturase and elongases (Gonzalez-Soto and Mutch, 2021). It has been shown that rats fed a diet supplemented with SFA (20% w:w partially hydrogenated coconut oil) had a reduced Δ-5 desaturase activity compared with a non-purified diet (5% w:w fat) (Dang *et al.*, 1989). In addition, Valenzuela *et al.* (2017) reported that a very high fat diet (i.e. 60% fat) decreased the activity of Δ-5 and Δ-6 desaturase and PUFA accretion in liver and other tissues of mice. Whether this mechanism is also effective in poultry at a much lower fat and SFA intake needs to be clarified.

We found a higher n-6/n-3 ratio in breast muscle of L30 birds, which is likely due to the many fold higher n-6/n-3 ratio in BSFL than in age-specific diets (see Table S1). However, we could not detect this difference in plasma and abdominal fat tissues. An increased n-6/n-3 ratio larger than 5 in breast muscle is undesirable for human consumption (de Souza Vilela *et al.*, 2021a). However, the n-6/n-3 ratio of the broiler breast meat in our study was far below this threshold so that the consumption of broiler meat produced with up to 30% BSFL in the ration seems unlikely to be harmful to health in this respect. Except for the NVI, the indices assessing nutritional quality of FA composition in the breast muscle indicated significant and mostly linear changes in lipid quality as response to increasing levels of BSLF in the diet. As compared to control diet, L30 reduced PI, an index assessing the lipid peroxidation susceptibility in breast muscle tissue

(Henriques *et al.*, 2015), which may be considered as a positive effect, but increased AI and TI. The AI represents the relationship between the sum of the main SFA which favor the adhesion of lipids to cells of the immunological and circulatory system and that of the main classes of unsaturated FA inhibiting the accumulation of plaque and reduce the levels of esterified fatty acid, cholesterol, and phospholipids. The TI characterizes the thrombogenic potential of FA, showing the tendency to form clots in the blood vessels. Therefore, consumption of foods with a lower AI and TI are associated with reduced levels of circulating total cholesterol and associated cardiovascular disorders (Chen and Liu, 2020). The HH ratio is an indication of the relationship between hypocholesterolemic FA (cis-C18:1 and PUFA) and hypercholesterolemic FA (Chen and Liu, 2020). Despite the favorable PI, the higher AI and TI and lower HH in breast muscle of broilers fed with a high amount of larvae in their diets (i.e. 30%) might limit nutritional quality of the lipids in the breast meat for human consumption.

As confirmed in this study, fat of BSFL contains a high content of SFA with a considerable amount of MCFA, particularly *C12:0* (de Souza Vilela *et al.*, 2021a). The antimicrobial effects of MCFA such as *C12:0* are well studied (Kabara *et al.*, 1972). Likewise, it has been suggested that *C12:0* induces immuno-regulatory functions and alleviates inflammation during infections in broilers (Wu *et al.*, 2021). Whether an antimicrobial effect of *C12:0* is relevant in our study cannot be determined from the available data. However, it should be taken into account that a high intake of SFA is positively related to plasma cholesterol and cardiovascular disease in humans (EFSA Panel on Dietetic Products and Allergies, 2010). Considering that the SFA especially in breast muscle is

higher in the L30 treatment group, high inclusion levels of BSFL (i.e. 30%) in broiler diets seem to result in unfavorable FA composition in chicken meat for human consumption.

Our results showed that *C18:2 cis-9, trans-11* FA, a CLA isomer, was higher in BSFL (0.50% of total FAs) than in the soybean-grain based feed, which was associated to an increasing proportion of CLA in total FA of plasma, breast muscle and abdominal fat in response to increasing levels of dietary BSFL intake. The occurrence of CLA in BSFL was previously reported by Hoc et al. (Hoc *et al.*, 2020). Meat of monogastric farmed animals is a poor source of CLA (0.1 to 0.2% of total FA) (Chin *et al.*, 1992), and enrichment of poultry meat with CLA has been of interest for humans consumers (den Hartigh, 2019; Lehnen *et al.*, 2015). Although the proportion of CLA in BSFL-fed broilers' meat was not dramatically increased in our study, it indicates the potential of feeding BSFL to manipulate the CLA content in broiler meats. Dietary CLA can be readily incorporated in broiler muscle (Du and Ahn, 2002); therefore, we assume that the increased CLA level in BSFL-fed broilers is due to a carry-over effect from BSFL. The results of the current study suggest that inclusion of whole BSFL in broiler diets up to 20% of the voluntary FI did not compromise the meat quality traits including FA composition. However, higher inclusion rates (i.e. 30%) are not recommended as it might be associated with negative effects on FA composition in plasma, breast muscle and adipose tissues which reduces the nutritional quality of meat for human consumption.

5. Conclusion

The current study provides concrete information on the consequences of different levels of feeding un-processed whole BSFL to broilers. Dietary inclusion of defrosted BSFL did not influence

slaughter weight, meat quality and carcass characteristics, suggesting that BSFL can be included in broiler diets without compromising consumer acceptance. With an inclusion level of larvae (30%) we however observed pronounced alterations in the FA compositions of plasma, breast and abdominal fat resulting in an increased SFA content at the expense of MUFA and PUFA. Increasing dietary levels of BSFL resulted in a dose dependent increase in the proportion of the CLA isomer *C18:2 cis-9, trans-11* in total FA of plasma, muscle and fat tissues. Although the n-6/n-3 ratio observed in breast muscle of L30 birds was higher than in CON birds, it is still far below the threshold ratio of < 5 considered favorable for human health. The overall nutritional quality of fats in the breast meat for human consumption may decrease linearly in response to increasing dietary levels of BSFL, particularly at the highest inclusion level (i.e. 30%). In conclusion, up to 20% of defrosted whole BSFL could be used in broiler diets without considerably negative effects on the birds' metabolism, slaughter weight and carcass traits, and fatty acid profile in plasma, muscle and fat tissues.

Supplementary material

The raw data used for all analyses in this study are stored in a repository (https://doi.org/10.5281/zenodo.7347351).

Authors' contributions

CCM and GD conceived and designed the study. PW and CCM acquired funding. MMS and GD contributed to rearing and sampling of the chickens. MMS, GD, SG, and DD contributed to the analyses of the sample and collection of data. MMS and GD performed statistical analysis of the

data and drafted the manuscript. CCM, GD, MMS, MM, SG, DD, SM and PW reviewed the manuscript. GD had the final responsibility for the content of the manuscript. All authors read the article and approved the submitted version.

Conflicts of interest

All authors declare that they have no competing interest in the present work.

Acknowledgements

This research project is part of the Leibniz ScienceCampus Phosphorus Research Rostock and is co-funded by the funding line strategic networks of the Leibniz Association. The authors are deeply grateful to F. Feldt and co-workers in the FBN slaughterhouse, as well as B. Mielenz, K. Kàrpàti, U. Lüdtke, E. Wünsche, K. Wilke, R. Gaeth and S. Dwars for their support of various laboratory analyses and with sample collection. The authors wish to thank Birgit Jentz and Maria Dahm in the Institute of Muscle Biology and Growth who assisted with sample preparation and GC analysis of fatty acids. The open access publication of this article was funded by the Open Access Fund of the Research Institute for Farm Animal Biology (FBN).

References:

Almeida, C., Rijo, P. and Rosado, C., 2020. Bioactive compounds from Hermetia illucens larvae as natural ingredients for cosmetic application. Biomolecules 10: 976. https://doi.org/10.3390/biom10070976

AOAC, 2000. Official Methods of Analysis, International: Gaithersburg, MD, USA.

Aviagen, 2018. Broiler Management Handbook, Newbridge, UK, pp. 1–147

Aviagen, 2019. Ross Broiler: Nutrition Specifications. Aviagen: Huntsville, AL, USA.

Bava, L., Jucker, C., Gislon, G., Lupi, D., Savoldelli, S., Zucali, M. and Colombini, S., 2019. Rearing of Hermetia illucens on different organic by-products: Influence on growth, waste reduction, and environmental impact. Animals 9: 289. https://doi.org/10.3390/ani9060289

Bostami, A., Mun, H. and Yang, C., 2017. Breast and thigh meat chemical composition and fatty acid profile in broilers fed diet with dietary fat sources. Int J Food Process Technol 8: 1000672. https://doi.org/10.4172/2157-7110.1000672

Brickett, K., Dahiya, J., Classen, H. and Gomis, S., 2007. Influence of dietary nutrient density, feed form, and lighting on growth and meat yield of broiler chickens. Poultry Sci. 86: 2172-2181. https://doi.org/10.1093/ps/86.10.2172

Chen, J. and Liu, H., 2020. Nutritional indices for assessing fatty acids: A mini-review. Int J Mol Sci 21: 5695. https://doi.org/10.3390/ijms21165695

Cherian, G., 2015. Nutrition and metabolism in poultry: role of lipids in early diet. J Anim Sci Biotechnol 6: 1-9. https://doi.org/10.1186/s40104-015-0029-9

Chin, S., Liu, W., Storkson, J., Ha, Y. and Pariza, M., 1992. Dietary sources of conjugated dienoic isomers of linoleic acid, a newly recognized class of anticarcinogens. J Food Compos Anal 5: 185-197. https://doi.org/10.1016/0889-1575(92)90037-K

Chu, X., Li, M., Wang, G., Wang, K., Shang, R., Wang, Z. and Li, L., 2020. Evaluation of the low inclusion of full-fatted Hermetia illucens larvae meal for layer chickens: Growth performance, nutrient digestibility, and gut health. Front vet sci 7: 585843. https://doi.org/10.3389/fvets.2020.585843

Crespo, N. and Esteve-Garcia, E., 2003. Polyunsaturated fatty acids reduce insulin and very low density lipoprotein levels in broiler chickens. Poult Sci 82: 1134-1139. https://doi.org/10.1093/ps/82.7.1134

Cullere, M., Schiavone, A., Dabbou, S., Gasco, L. and Dalle Zotte, A., 2019. Meat quality and sensory traits of finisher broiler chickens fed with black soldier fly (Hermetia illucens L.) larvae fat as alternative fat source. Animals 9: 140. https://doi.org/10.3390/ani9040140

Cutrignelli, M.I., Messina, M., Tulli, F., Randazzo, B., Olivotto, I., Gasco, L., Loponte, R. and Bovera, F., 2018. Evaluation of an insect meal of the Black Soldier Fly (Hermetia illucens) as soybean substitute: Intestinal morphometry, enzymatic and microbial activity in laying hens. Res Vet Sci 117: 209-215. https://doi.org/10.1016/j.rvsc.2017.12.020

Dabbou, S., Lauwaerts, A., Ferrocino, I., Biasato, I., Sirri, F., Zampiga, M., Bergagna, S., Pagliasso, G., Gariglio, M. and Colombino, E., 2021. Modified black soldier fly larva fat in broiler diet: effects on performance, carcass traits, blood parameters, histomorphological features and gut microbiota. Animals 11: 1837. https://doi.org/10.3390/ani11061837

Dabbou, S., Renna, M., Lussiana, C., Gai, F., Rotolo, L., Kovitvadhi, A., Brugiapaglia, A., Helal, A.N., Schiavone, A. and Zoccarato, I., 2017. Bilberry pomace in growing rabbit diets: effects on quality traits of hind leg meat. Ital J Anim Sci 16: 371-379. https://doi.org/10.1080/1828051X.2017.1292413

Dal Bosco, A., Cartoni Mancinelli, A., Vaudo, G., Cavallo, M., Castellini, C. and Mattioli, S., 2022. Indexing of Fatty Acids in Poultry Meat for Its Characterization in Healthy Human Nutrition: A Comprehensive Application of the Scientific Literature and New Proposals. Nutrients 14: 3110. https://doi.org/10.3390/nu14153110

Dang, A., Kemp, K., Faas, F. and Carter, W., 1989. Effects of dietary fats on fatty acid composition and Δ5 desaturase in normal and diabetic rats. Lipids 24: 882-889. https://doi.org/10.1007/BF02535763

Dannenberger, D., Nuernberg, G., Nuernberg, K., Will, K., Schauer, N. and Schmicke, M., 2017. Effects of diets supplemented with n–3 or n–6 PUFA on pig muscle lipid metabolites measured by non-targeted LC–MS lipidomic profiling. J Food Compos Anal 56: 47-54. https://doi.org/10.1016/j.jfca.2016.11.015

Dannenberger, D., Nuernberg, K., Nuernberg, G. and Priepke, A., 2012. Different dietary protein and PUFA interventions alter the fatty acid concentrations, but not the meat quality, of porcine muscle. Nutrients 4: 1237-1246. https://doi.org/10.3390/nu4091237

de Souza Vilela, J., Alvarenga, T.I., Andrew, N.R., McPhee, M., Kolakshyapati, M., Hopkins, D.L. and Ruhnke, I., 2021a. Technological quality, amino acid and fatty acid profile of broiler meat enhanced by dietary inclusion of black soldier fly larvae. Foods 10: 297. https://doi.org/10.3390/foods10020297

de Souza Vilela, J., Andronicos, N.M., Kolakshyapati, M., Hilliar, M., Sibanda, T.Z., Andrew, N.R., Swick, R.A., Wilkinson, S. and Ruhnke, I., 2021b. Black Soldier Fly larvae in broiler diets improve broiler performance and modulate the immune system. Anim Nutr 7: 695-706. https://doi.org/10.1016/j.aninu.2020.08.014

den Hartigh, L.J., 2019. Conjugated linoleic acid effects on cancer, obesity, and atherosclerosis: A review of pre-clinical and human trials with current perspectives. Nutrients 11: 370. https://doi.org/10.3390/nu11020370

Dörper, A., Veldkamp, T. and Dicke, M., 2020. Use of black soldier fly and house fly in feed to promote sustainable poultry production. J Insects Food Feed: 1-20. https://doi.org/10.3920/JIFF2020.0064

Dozier III, W., Price, C., Kidd, M., Corzo, A., Anderson, J. and Branton, S., 2006. Growth performance, meat yield, and economic responses of broilers fed diets varying in metabolizable energy from thirty to fifty-nine days of age. J Appl Poult Res 15: 367-382. https://doi.org/10.1093/japr/15.3.383

Du, M. and Ahn, D.U., 2002. Effect of dietary conjugated linoleic acid on the growth rate of live birds and on the abdominal fat content and quality of broiler meat. Poult Sci 81: 428-433. https://doi.org/10.1093/ps/81.3.428

EFSA Panel on Dietetic Products, N. and Allergies, 2010. Scientific opinion on dietary reference values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, trans fatty acids, and cholesterol. EFSA Journal 8: 1461. https://doi.org/10.2903/j.efsa.2010.1461

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

Franco, A., Scieuzo, C., Salvia, R., Petrone, A.M., Tafi, E., Moretta, A., Schmitt, E. and Falabella, P., 2021. Lipids from Hermetia illucens, an Innovative and sustainable source. Sustainability 13: 10198. https://doi.org/10.3390/su131810198

Gonzalez-Soto, M. and Mutch, D.M., 2021. Diet regulation of long-chain PUFA synthesis: role of macronutrients, micronutrients, and polyphenols on Δ-5/Δ-6 desaturases and elongases 2/5. Adv Nutr 12: 980-994. https://doi.org/10.1093/advances/nmaa142

Hahn, T., Roth, A., Febel, E., Fijalkowska, M., Schmitt, E., Arsiwalla, T. and Zibek, S., 2018. New methods for high-accuracy insect chitin measurement. J Sci Food Agric 98: 5069-5073. https://doi.org/10.1002/jsfa.9044

Henriques, A., Blasco, H., Fleury, M.-C., Corcia, P., Echaniz-Laguna, A., Robelin, L., Rudolf, G., Lequeu, T., Bergaentzle, M. and Gachet, C., 2015. Blood cell palmitoleate-palmitate ratio is an independent prognostic factor for amyotrophic lateral sclerosis. PloS one 10: e0131512. https://doi.org/10.1371/journal.pone.0131512

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

Ipema, A.F., Bokkers, E.A., Gerrits, W.J., Kemp, B. and Bolhuis, J.E., 2020a. Long-term access to live black soldier fly larvae (Hermetia illucens) stimulates activity and reduces fearfulness of broilers, without affecting health. Sci Rep 10: 1-13. https://doi.org/10.1038/s41598-020-74514-x

Ipema, A.F., Gerrits, W.J., Bokkers, E.A., Kemp, B. and Bolhuis, J.E., 2020b. Provisioning of live black soldier fly larvae (Hermetia illucens) benefits broiler activity and leg health in a frequency-and dose-dependent manner. Appl Anim Behav Sci 230: 105082. https://doi.org/10.1016/j.applanim.2020.105082

Janisch, S., Sharifi, A., Wicke, M. and Krischek, C., 2015. Changing the incubation temperature during embryonic myogenesis influences the weight performance and meat quality of male and female broilers. Poult Sci 94: 2581-2588. https://doi.org/10.3382/ps/pev239

Kabara, J.J., Swieczkowski, D.M., Conley, A.J. and Truant, J.P., 1972. Fatty acids and derivatives as antimicrobial agents. Antimicrob Agents Chemother 2: 23-28. https://doi.org/10.1128/AAC.2.1.23

Kalbe, C., Priepke, A., Nürnberg, G. and Dannenberger, D., 2019. Effects of long-term microalgae supplementation on muscle microstructure, meat quality and fatty acid composition in growing pigs. J Anim Physiol Anim 103: 574-582. https://doi.org/10.1111/jpn.13037

Kim, Y.B., Kim, D.-H., Jeong, S.-B., Lee, J.-W., Kim, T.-H., Lee, H.-G. and Lee, K.-W., 2020. Black soldier fly larvae oil as an alternative fat source in broiler nutrition. Poult Sci 99: 3133-3143. https://doi.org/10.1016/j.psj.2020.01.018

Kongsup, P., Lertjirakul, S., Chotimanothum, B., Chundang, P. and Kovitvadhi, A., 2022. Effects of eri silkworm (Samia ricini) pupae inclusion in broiler diets on growth performances, health, carcass characteristics and meat quality. Anim Biosci 35: 711. https://doi.org/10.5713/ab.21.0323

Lee, J.S., Pinnamaneni, S.K., Eo, S.J., Cho, I.H., Pyo, J.H., Kim, C.K., Sinclair, A.J., Febbraio, M.A. and Watt, M.J., 2006. Saturated, but not n-6 polyunsaturated, fatty acids induce insulin resistance: role of intramuscular accumulation of lipid metabolites. J Appl Physiol 100: 1467-1474. https://doi.org/10.1152/japplphysiol.01438.2005

Lehnen, T.E., da Silva, M.R., Camacho, A., Marcadenti, A. and Lehnen, A.M., 2015. A review on effects of conjugated linoleic fatty acid (CLA) upon body composition and energetic metabolism. J Int Soc Sports Nutr 12: 1-11. https://doi.org/10.1186/s12970-015-0097-4

Li, S., Ji, H., Zhang, B., Tian, J., Zhou, J. and Yu, H., 2016. Influence of black soldier fly (Hermetia illucens) larvae oil on growth performance, body composition, tissue fatty acid composition and lipid deposition in juvenile Jian carp (Cyprinus carpio var. Jian). Aquaculture 465: 43-52. https://doi.org/10.1016/j.aquaculture.2016.08.020

Li, X., Dong, Y., Sun, Q., Tan, X., You, C., Huang, Y. and Zhou, M., 2022. Growth and Fatty Acid Composition of Black Soldier Fly Hermetia illucens (Diptera: Stratiomyidae) Larvae Are Influenced by Dietary Fat Sources and Levels. Animals 12: 486. https://doi.org/10.3390/ani12040486

Maurer, V., Holinger, M., Amsler, Z., Früh, B., Wohlfahrt, J., Stamer, A. and Leiber, F., 2016. Replacement of soybean cake by Hermetia illucens meal in diets for layers. J Insects Food Feed 2: 83-90. https://doi.org/10.3920/JIFF2015.0071

Moula, N., Scippo, M.-L., Douny, C., Degand, G., Dawans, E., Cabaraux, J.-F., Hornick, J.-L., Medigo, R.C., Leroy, P. and Francis, F., 2018. Performances of local poultry breed fed black soldier fly larvae reared on horse manure. Anim Nutr 4: 73-78. https://doi.org/10.1016/j.aninu.2017.10.002

Murawska, D., Daszkiewicz, T., Sobotka, W., Gesek, M., Witkowska, D., Matusevičius, P. and Bakuła, T., 2021. Partial and Total Replacement of Soybean Meal with Full-Fat Black Soldier Fly (Hermetia illucens L.) Larvae Meal in Broiler Chicken Diets: Impact on Growth Performance, Carcass Quality and Meat Quality. Animals 11: 2715. https://doi.org/10.3390/ani11092715

Murff, H.J. and Edwards, T.L., 2014. Endogenous production of long-chain polyunsaturated fatty acids and metabolic disease risk. Curr Cardiovasc Risk Rep 8: 1-9. https://doi.org/10.1007/s12170-014-0418-1

Naumann, K. and Bassler, R., 1997. VDLUFA-Methodenbuch Band III. Die chemische Untersuchung von Futtermitteln. 4. Ergänzungslieferung. Darmstadt, Germany: VDLUFA-Verlag.

Obrosova, I.G., Ilnytska, O., Lyzogubov, V.V., Pavlov, I.A., Mashtalir, N., Nadler, J.L. and Drel, V.R., 2007. High-fat diet–induced neuropathy of pre-diabetes and obesity: effects of "healthy" diet and aldose reductase inhibition. Diabetes 56: 2598-2608. https://doi.org/10.2337/db06-1176

Peña-Saldarriaga, L.M., Fernández-López, J. and Pérez-Alvarez, J.A., 2020. Quality of chicken fat by-products: Lipid profile and colour properties. Foods 9: 1046. https://doi.org/10.3390/foods9081046

Popova, T., Petkov, E. and Ignatova, M., 2020. Effect of black soldier fly (Hermetia illucens) meals in the diet on the growth performance and carcass composition in broilers. J Insects Food Feed: 1-8. https://doi.org/10.3920/JIFF2019.0050

Rumpold, B.A., Klocke, M. and Schlüter, O., 2017. Insect biodiversity: underutilized bioresource for sustainable applications in life sciences. Regional Environmental Change 17: 1445-1454. https://doi.org/10.1007/s10113-016-0967-6

Schiavone, A., Cullere, M., De Marco, M., Meneguz, M., Biasato, I., Bergagna, S., Dezzutto, D., Gai, F., Dabbou, S. and Gasco, L., 2017. Partial or total replacement of soybean oil by black soldier fly larvae (Hermetia illucens L.) fat in broiler diets: Effect on growth performances, feed-choice, blood traits, carcass characteristics and meat quality. Ital J Anim Sci 16: 93-100. https://doi.org/10.1080/1828051X.2016.1249968

Schiavone, A., Dabbou, S., Petracci, M., Zampiga, M., Sirri, F., Biasato, I., Gai, F. and Gasco, L., 2019. Black soldier fly defatted meal as a dietary protein source for broiler chickens: Effects on carcass traits, breast meat quality and safety. Animal 13: 2397-2405. https://doi.org/10.1017/S1751731119000685

Sears, B. and Perry, M., 2015. The role of fatty acids in insulin resistance. Lipids Health Dis. 14: 1-9. https://doi.org/10.1186/s12944-015-0123-1

Secci, G., Bovera, F., Nizza, S., Baronti, N., Gasco, L., Conte, G., Serra, A., Bonelli, A. and Parisi, G., 2018. Quality of eggs from Lohmann Brown Classic laying hens fed black soldier fly meal as substitute for soya bean. Animal 12: 2191-2197. https://doi.org/10.1017/S1751731117003603

Semwogerere, F., Neethling, J., Muchenje, V. and Hoffman, L.C., 2019. Meat quality, fatty acid profile, and sensory attributes of spent laying hens fed expeller press canola meal or a conventional diet. Poult Sci 98: 3557-3570. https://doi.org/10.3382/ps/pez092

Shumo, M., Osuga, I.M., Khamis, F.M., Tanga, C.M., Fiaboe, K.K., Subramanian, S., Ekesi, S., van Huis, A. and Borgemeister, C., 2019. The nutritive value of black soldier fly larvae reared on common organic waste streams in Kenya. Sci Rep 9: 1-13. https://doi.org/10.1038/s41598-019-46603-z

Star, L., Arsiwalla, T., Molist, F., Leushuis, R., Dalim, M. and Paul, A., 2020. Gradual provision of live black soldier fly (Hermetia illucens) larvae to older laying hens: effect on production performance, egg quality, feather condition and behavior. Animals 10: 216. https://doi.org/10.3390/ani10020216

Sukhija, P.S. and Palmquist, D., 1988. Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. J Agric Food Chem 36: 1202-1206.

Tahamtani, F.M., Ivarsson, E., Wiklicky, V., Lalander, C., Wall, H., Rodenburg, T.B., Tuyttens, F.A. and Hernandez, C.E., 2021. Feeding live Black Soldier Fly larvae (Hermetia illucens) to laying hens: effects on feed consumption, hen health, hen behavior, and egg quality. Poult Sci 100: 101400. https://doi.org/10.1016/j.psj.2021.101400

Tönißen, K., Pfuhl, R., Franz, G.P., Dannenberger, D., Bochert, R. and Grunow, B., 2022. Impact of spawning season on fillet quality of wild pikeperch (Sander lucioperca). Eur Food Res Technol: 1-9. https://doi.org/10.1007/s00217-022-03963-7

Valenzuela, R., Echeverria, F., Ortiz, M., Rincón-Cervera, M.Á., Espinosa, A., Hernandez-Rodas, M.C., Illesca, P., Valenzuela, A. and Videla, L.A., 2017. Hydroxytyrosol prevents reduction in liver activity of Δ-5 and Δ-6 desaturases, oxidative stress, and depletion in long chain polyunsaturated fatty acid content in different tissues of high-fat diet fed mice. Lipids Health Dis 16: 1-16. https://doi.org/10.1186/s12944-017-0450-5

Velasco, S., Ortiz, L., Alzueta, C., Rebole, A., Trevino, J. and Rodriguez, M., 2010. Effect of inulin supplementation and dietary fat source on performance, blood serum metabolites,

liver lipids, abdominal fat deposition, and tissue fatty acid composition in broiler chickens. Poult Sci 89: 1651-1662. https://doi.org/10.3382/ps.2010-00687

Wang, G., McConn, B.R., Liu, D., Cline, M.A. and Gilbert, E.R., 2017a. The effects of dietary macronutrient composition on lipid metabolism-associated factor gene expression in the adipose tissue of chickens are influenced by fasting and refeeding. BMC Obesity 4: 1-14. https://doi.org/10.1186/s40608-017-0150-8

Wang, X.J., Xu, S.H., Liu, L., Song, Z.G., Jiao, H.C. and Lin, H., 2017b. Dietary fat alters the response of hypothalamic neuropeptide Y to subsequent energy intake in broiler chickens. J Exp Biol 220: 607-614. https://doi.org/10.1242/jeb.143792

Wu, Y., Zhang, H., Zhang, R., Cao, G., Li, Q., Zhang, B., Wang, Y. and Yang, C., 2021. Serum metabolome and gut microbiome alterations in broiler chickens supplemented with lauric acid. Poult Sci 100: 101315. https://doi.org/10.1016/j.psj.2021.101315

Table S1. Fatty acids compositions of the age-specific broiler diets and defrosted whole black soldier fly larvae (BSFL) offered to broilers.

Fatty acids	Age-specific diets			BSFL (d 1-42)
	Starter (d 1-14)	Grower (d 15-28)	Finisher (d 29-42)	
SFA (% of total FA)				
Capric acid (C10:0)	<0.01	<0.01	<0.01	1.30
Undecylic acid (C11:0)	<0.01	<0.01	<0.01	0.05
Lauric acid (C12:0)	0.09	0.40	0.10	34.20
Tridecylic acid (C13:0)	<0.01	<0.01	<0.01	0.12
Myristic acid (C14:0)	0.11	0.17	0.11	14.20
Pentadecylic acid (C15:0)	0.04	0.05	0.04	0.18
Palmitic acid (C16:0)	12.8	14.0	13.0	17.8
Margaric acid (C17:0)	0.10	0.11	0.10	0.21
Stearic acid (C18:0)	4.1	3.8	4.2	2.8
Arachidic acid (C20:0)	0.28	0.27	0.28	0.09
Heneicosylic acid (C21:0)	0.02	0.01	0.02	0.01
Behenic acid (C22:0)	0.21	0.17	0.20	<0.01
Tricosylic acid (C23:0)	0.06	0.05	0.06	0.02
Lignoceric acid (C24:0)	0.22	0.15	0.22	<0.01
Cerotic acid (C26:0)	0.08	0.04	0.08	0.01
∑ SFA	18.1	19.3	18.5	70.9
MUFA (% of total FAs)				
Myristoleic acid (C14:1cis-9)	<0.01	<0.01	<0.01	0.35

Palmitoleic acid (C16:1cis-9)	0.12	0.15	0.12	3.94
Margaroleic acid (C17:1cis-9)	<0.01	<0.01	<0.01	<0.01
Elaidic acid (C18:1trans-9)	0.07	0.01	0.08	0.07
Oleic acid (C18:1cis-9)	22.1	24.2	22.9	12.4
Vaccenic acid (C18:1trans-11)	<0.01	0.04	<0.01	0.03
cis-Vaccenic acid (C18:1cis-11)	1.01	1.06	1.04	0.66
cis-11-Eicosaenoic acid (C20:1cis-11)	0.24	0.25	0.26	0.10
Docosaenoic acid (C22:1cis-13)	0.07	<0.01	0.07	<0.01
Methyl nervonate (C24:1cis-15)	<0.01	<0.01	<0.01	<0.01
∑ MUFA	23.6	25.7	24.5	17.5
PUFAs (% of total FAs)				
Linoleic acid (C18:2 n-6)	38.3	40.7	37.8	10.1
γ-Linolenic acid (C18:3 n-6)	<0.01	<0.01	<0.01	<0.01
α-Linolenic acid (C18:3 n-3)	19.8	14.3	19.1	0.9
CLA (C18:2cis-9,trans-11 isomer)	<0.01	<0.01	<0.01	0.50
Stearidonic acid (C18:4 n-3)	<0.01	<0.01	<0.01	<0.01
Eicosadienoic acid (C20:2 n-6)	0.10	<0.01	0.13	0.02
Mead acid (C20:3 n-9)	<0.01	<0.01	<0.01	0.06
Dihomo-γ-Linolenic acid (C20:3 n-6)	<0.01	<0.01	<0.01	<0.01
Eicosatrienoic (C20:3 n-3)	0.03	<0.01	0.03	0.02
Arachidonic acid (C20:4 n-6)	<0.01	<0.01	<0.01	<0.01
EPA (C20:5 n-3)	<0.01	<0.01	<0.01	<0.01

Docosadienoic acid (C22:2 n-6)	<0.01	<0.01	<0.01	0.01
Docosatetraenoic acid (C22:4 n-6)	<0.01	<0.01	<0.01	<0.01
DPA (C22:5 n-6)	<0.01	<0.01	<0.01	<0.01
DPA (C22:5 n-3)	<0.01	<0.01	<0.01	<0.01
DHA (C22:6 n-3)	<0.01	<0.01	<0.01	<0.01
∑ PUFA	**58.3**	**55.1**	**57.0**	**11.6**
∑ n-3	**19.9**	**14.3**	**19.1**	**0.96**
∑ n-6	**38.4**	**40.7**	**37.9**	**10.1**
n-6/n-3 ratio	**1.93**	**2.84**	**1.98**	**10.52**

Abbreviations: **SFA**, saturated fatty acid; **MUFA**, monounsaturated fatty acid; **PUFA**, polyunsaturated fatty acid; **CLA**, conjugated linoleic acids; **EPA**, Eicosapentaenoic acid; **DPA**, docosapentaenoic acid; **DHA**, docosahexaenoic acid.

Table S2. Effects of increasing levels of defrosted whole black soldier fly larvae (BSFL) in broiler diets on plasma fatty acid compositions of broilers.

Fatty acids	Dietary treatments[1]					P-values[2], ≤	
	CON	L10	L20	L30	SE	T	W
SFA (% of total FA)							
Capric acid (C10:0)	0.016^{b†}	0.030^{ab}	0.032^{ab†}	0.045^{a}	0.005	0.001	0.566
Undecylic acid (C11:0)	0.01	0.01	0.01	0.01	0.001	0.669	0.160
Lauric acid (C12:0)	0.298^{c}	2.03^{b}	3.31^{ab}	4.35^{a}	0.430	0.001	0.010
Tridecylic acid (C13:0)	0.02	0.01	0.02	0.02	0.001	0.548	0.069
Myristic acid (C14:0)	0.37^{c}	1.15^{b}	1.80^{a}	1.98^{a}	0.15	0.001	0.024
Pentadecylic acid (C15:0)	0.12	0.12	0.12	0.12	0.001	0.855	0.295
Palmitic acid (C16:0)	20.7	20.6	20.9	20.4	0.18	0.362	0.001
Margaric acid (C17:0)	0.16	0.16	0.18	0.17	0.001	0.419	1.000
Stearic acid (C18:0)	14.1	14.3	13.8	13.8	0.24	0.571	0.001
Arachidic acid (C20:0)	0.07	0.08	0.07	0.07	0.001	0.352	0.001
Heneicosylic acid (C21:0)	0.02†	0.02	0.01	0.01†	0.001	0.045	0.467
Behenic acid (C22:0)	0.32	0.32	0.28	0.30	0.02	0.295	0.001
Tricosylic acid (C23:0)	0.04	0.05	0.04	0.04	0.001	0.444	0.218
Lignoceric acid (C24:0)	0.05	0.06	0.04	0.04	0.001	0.188	0.155
Cerotic acid (C26:0)	0.04	0.05	0.03	0.04	0.001	0.170	0.002
∑ SFA	36.4^{c}	38.9^{b†}	40.6^{ab†}	41.5^{a}	0.42	0.001	0.065
MUFA (% of total FA)							
Myristoleic acid (C14:1cis-9)	0.04^{c}	0.08^{b†}	0.13^{ab†}	0.13^{a}	0.01	0.001	0.004
Palmitoleic acid (C16:1 cis9)	1.46†	1.77	1.75	1.93†	0.13	0.081	0.101
Margaroleic acid (C17:1cis-9)	0.08	0.08	0.11	0.08	0.01	0.354	0.139
Elaidic acid (C18:1 trans9)	0.189	0.175	0.194	0.195	0.01	0.723	0.002

Oleic acid (C18:1 cis9)	17.9	17.1	16.5	17.2	0.66	0.489	0.213
Vaccenic acid (C18:1trans-11)	0.02	0.02	0.02	0.01	0.001	0.648	0.001
cis-vaccenic acid (C18:1 cis11)	1.57	1.57	1.39	1.44	0.07	0.258	0.322
cis-11-Eicosenoic acid (C20:1cis-11)	0.19	0.18	0.16	0.17	0.001	0.101	0.144
∑ MUFA	21.4	20.9	20.3	21.2	0.84	0.764	0.236
PUFA (% of total FA)							
Linoleic acid (C18:2 n-6)	23.7^{a}	22.8^{ab}	22.3^{ab}	21.8^{b}	0.45	0.029	0.616
γ-Linolenic acid (C18:3 n-6)	0.151	0.149	0.140	0.135	0.006	0.310	1.00
α-Linolenic acid (C18:3 n-3)	$4.90^{a\dagger}$	$4.21^{ab\dagger}$	4.21^{ab}	3.56^{b}	0.21	0.001	0.796
CLA (C18:2 cis9, trans11)	0.021^{c}	0.055^{b}	0.077^{ab}	0.094^{a}	0.008	0.001	0.677
Stearidonic acid (C18:4 n-3)	0.02^{a}	0.02^{a}	0.02^{ab}	0.01^{b}	0.001	0.021	0.007
Eicosadienoic acid (C20:2 n-6)	0.27	0.26	0.24	0.25	0.001	0.184	0.001
Mead acid (C20:3 n-9)	0.35	0.39	0.37	0.39	0.04	0.860	0.055
Homo- γ –Linolenic (C20:3 n-6)	1.05	1.06	0.99	1.04	0.04	0.518	0.001
Eicosatrienoic (C20:3 n-3)	0.14^{a}	0.13^{ab}	0.11^{b}	0.11^{b}	0.001	0.014	0.037
Arachidonic (C20:4 n-6)	3.88	3.70	3.69	3.50	0.19	0.555	0.134
EPA (C20:5 n-3)	2.98^{a}	2.85^{ab}	2.45^{bc}	2.21^{c}	0.001	0.001	0.640
Adrenic acid (C22:4 n-6)	0.29	0.28	0.28	0.27	0.01	0.685	0.019
DPA (C22:5 n-3)	1.68	1.58	1.47	1.40	0.17	0.009	0.322
DPA (C22:5 n-6)	0.11	0.11	0.12	0.13	0.001	0.376	0.206
DHA (C22:6 n-3)	2.68	2.58	2.61	2.53	0.92	0.982	0.135
∑ PUFA	**42.2^{a}**	**40.2^{ab}**	**39.1^{ab}**	**37.4^{b}**	**0.94**	**0.006**	**0.062**
∑ n-3	**12.4^{a}**	**11.4^{ab}**	**10.9^{ab}**	**9.8^{b}**	**0.49**	**0.005**	**0.004**
∑ n-6	**29.4^{a}**	**28.3^{ab}**	**27.8^{ab}**	**27.1^{b}**	**0.56**	**0.036**	**0.669**
n-6/n-3 ratio	**1.45**	**1.53**	**1.69**	**1.77**	**0.11**	**0.172**	**0.485**

[1] Dietary treatments: ad-libitum feeding without access to BSFL (CON), or with BSFL amounting to 10% (L10), 20% (L20) or 30% (L30) of the feed intake of CON birds. Total number of observations used for statistical analyses, N = 96 (i.e., 2 birds sampled from each of 6 pens allocated to each of 4 treatments at weeks 4 and 6).
2 T: treatment effect; W = time effect (weeks 4 and 6). P-values for T*W = treatment by time interaction were all $P > 0.1$, and thus are not shown. In line with this, the values are presented in this table refer to average of weeks 4 and 6.
a-b: Values in a row denoted with different letters differ significantly (Tukey, $P < 0.05$). Symbols † and * in a row indicate a tendency of two treatments to differ (Tukey, $0.05 < P \leq 0.10$). For the sake of a succinct presentation, only the most conservative (i.e. the largest) SE is presented.
Abbreviations: SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; DPA, docosapentaenoic acid; CLA, conjugated linoleic acids; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Table S3. Effects of increasing levels of defrosted whole black soldier fly larvae (BSFL) in broiler diets on breast muscle fatty acid compositions of broilers.

Fatty acids	Dietary treatments[1]					P-values[2], ≤	
	CON	L10	L20	L30	SE	T	W
SFA (% of total FA)							
Capric acid (C10:0)	0.053^{b}	0.068^{b}	0.070^{b}	0.094^{a}	0.005	0.001	0.868
Undecylic acid (C11:0)	<0.01^{b†}	0.01^{b†}	0.01^{b}	0.02^{a}	0.001	0.001	0.664
Lauric acid (C12:0)	0.67^{c}	2.64^{b}	3.17^{b}	5.16^{a}	0.49	0.001	0.648
Tridecylic acid (C13:0)	0.01^{c}	0.03^{b}	0.03^{ab†}	0.05^{a†}	0.001	0.001	0.107
Myristic acid (C14:0)	0.60^{c}	1.51^{b}	1.84^{b}	2.53^{a}	0.17	0.001	0.959
Pentadecylic acid (C15:0)	0.09	0.10	0.10	0.10	0.001	0.172	0.941
Palmitic acid (C16:0)	20.5	20.2	20.1	20.0	0.21	0.368	0.001
Margaric acid (C17:0)	0.13	0.14	0.15	0.15	0.001	0.183	0.766
Stearic acid (C18:0)	9.15	8.74	9.45	8.98	0.29	0.429	0.066
Arachidic acid (C20:0)	0.06	0.06	0.06	0.06	0.001	0.180	0.965
Heneicosylic acid (C21:0)	0.02	0.02	0.02	0.02	0.001	0.995	0.001
Behenic acid (C22:0)	0.12^{ab}	0.11^{b}	0.15^{a}	0.14^{a}	0.001	0.014	0.376
Tricosylic acid (C23:0)	0.15	0.14	0.17	0.16	0.01	0.434	0.012
Lignoceric acid (C24:0)	<0.01	<0.01	<0.01	<0.01	0.001	0.461	0.721
Cerotic acid (C26:0)	0.04	0.04	0.04	0.04	0.001	0.797	0.725
∑ SFA	31.6^{b}	33.8^{b}	35.3^{b}	37.5^{a}	0.59	0.001	0.060
MUFA (% of total FA)							
Myristoleic acid (C14:1cis-9)	0.09^{c}	0.18^{b}	0.19^{ab†}	0.26^{a†}	0.02	0.001	0.638
Palmitoleic acid (C16:1 cis9)	2.73	3.00	2.55	2.85	0.24	0.636	0.426
Margaroleic acid (C17:1cis-9)	0.97†	0.76†	0.81	0.81	0.06	0.067	0.755
Elaidic acid (C18:1 trans9)	0.097	0.077	0.082	0.081	0.010	0.477	0.773
Oleic acid (C18:1 cis9)	24.4†	23.9	21.7†	21.6	0.80	0.038	0.458

Vaccenic acid (C18:1trans-11)	<0.01	<0.01	<0.01	<0.01	0.001	0.461	0.721
cis-vaccenic acid (C18:1 cis11)	2.88	2.75	3.02	3.00	0.11	0.346	0.547
cis-11-Eicosenoic acid (C20:1cis-11)	0.28	0.29	0.28	0.28	0.02	0.965	0.019
Docosenoic acid (C22:1cis-13)	<0.01	<0.01	<0.01	<0.01	0.001	0.461	0.721
Methyl nervonate (C24:1cis-15)	<0.01	<0.01	<0.01	<0.01	0.001	0.461	0.721
∑ MUFA	31.4	30.9	28.6	28.8	1.02	0.172	0.391
PUFA (% of total FA)							
Linoleic acid (C18:2 n-6)	17.6	16.9	17.3	16.4	0.42	0.165	0.006
γ-Linolenic acid (C18:3 n-6)	0.090^{ab}	0.095^{a}	0.071^{c}	0.079^{bc}	0.004	0.001	0.029
α-Linolenic acid (C18:3 n-3)	6.09^{a}	5.96^{a}	4.56^{b}	4.15^{b}	0.32	0.001	0.906
CLA (C18:2 cis9, trans11)	0.037^{c}	$0.070^{b\dagger}$	$0.097^{ab\dagger}$	0.108^{a}	0.007	0.001	0.230
Stearidonic acid (C18:4 n-3)	0.12^{a}	0.12^{a}	0.08^{b}	0.09^{b}	0.001	0.001	0.481
Eicosadienoic acid (C20:2 n-6)	0.50	0.48	0.55	0.52	0.05	0.770	0.244
Mead acid (C20:3 n-9)	0.19	$0.18^{\dagger}$	0.21	$0.23^{\dagger}$	0.02	0.084	0.340
Homo- γ –Linolenic (C20:3 n-6)	1.02	0.92	1.11	1.10	0.07	0.239	0.475
Eicosatrienoic (C20:3 n-3)	0.65	0.56	0.75	0.59	0.11	0.662	0.177
Arachidonic (C20:4 n-6)	2.98	2.80	3.46	3.07	0.20	0.219	0.525
Docosadienoic (C22:2 n-6)	0.11	0.10	0.09	0.06	0.02	0.339	0.001
Adrenic acid (C22:4 n-6)	0.57	0.54	0.65	0.61	0.04	0.335	0.538
DPA (C22:5 n-6)	0.38	0.34	0.39	0.33	0.05	0.795	0.001
DPA (C22:5 n-3)	3.10	2.76	3.17	2.84	0.23	0.575	0.033
EPA (C20:5 n-3)	1.61	1.47	1.57	1.47	0.11	0.732	0.128
DHA (C22:6 n-3)	1.86	1.77	2.03	1.92	0.18	0.824	0.678
∑ PUFA	**37.0**	**35.1**	**36.1**	**33.6**	**1.13**	**0.162**	**0.828**
∑ n-3	**13.4^{a}**	**12.7^{ab}**	**12.2^{ab}**	**11.1^{b}**	**0.57**	**0.035**	**0.270**
∑ n-6	**23.3**	**22.2**	**23.6**	**22.2**	**0.67**	**0.323**	**0.187**

n-6/n-3 ratio	**1.77^{b}**	**1.80^{ab†}**	**1.99^{ab}**	**2.08^{a†}**	**0.08**	**0.036**	**0.070**
NVI [3]	**3.77**	**3.86**	**3.35**	**3.46**	**0.17**	**0.198**	**0.124**
PI [4]	**104.3^{a}**	**99.2^{ab}**	**95.7^{ab}**	**90.7^{b}**	**3.35**	**0.041**	**0.004**
AI[5]	**0.29^{c}**	**0.35^{b}**	**0.40^{ba}**	**0.42^{a}**	**0.01**	**0.001**	**0.001**
TI [6]	**0.56^{c}**	**0.61^{bc}**	**0.65^{ba}**	**0.68^{a}**	**0.02**	**0.001**	**0.013**
HH [7]	**2.98^{a}**	**2.58^{b}**	**2.44^{bc†}**	**2.18^{c†}**	**0.08**	**0.001**	**0.203**

[1] Dietary treatments: ad libitum feeding without access to BSFL (CON), or with BSFL amounting to 10% (L10), 20% (L20) or 30% (L30) of the feed intake of CON birds. Total number of observations used for statistical analyses, N = 96 (i.e., 2 birds sampled from each of 6 pens allocated to each of 4 treatments at weeks 4 and 6).

2 T: treatment effect; W = time effect (weeks 4 and 6). P-values for T*W = treatment by time interaction were all P>0.1, and are not shown. In line with this, the values are presented in this table refer to average of weeks 4 and 6.

a-b: Values in a row denoted with different letters differ significantly (Tukey, $P < 0.05$). Symbols † and * in a row indicate a tendency of two treatments to differ (Tukey, $0.05 < P \leq 0.10$). For the sake of a succinct presentation, only the most conservative (i.e. the largest) SE is presented.

Abbreviations: SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; DPA, docosapentaenoic acid; CLA, conjugated linoleic acids; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid, nutritional value index; NVI, peroxidability index; PI, atherogenicity index; AI, thromogenicity index; TI, hypocholesterolemic / hypercholesterolemic; HH.

∑ SFA, ∑ MUFA and ∑ PUFA are the sum of 15, 10 and 16 individual FA in muscle, respectively, in these FA classes.

3 calculated as reported by (Dal Bosco et al., 2022): NVI = (C18:0 + C18:1n9 + C16:0) / (C:16).

4 calculated as reported by (Dabbou et al., 2017): PI = (% monoenoic × 0.025) + (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 4) + (% pentaenoic × 6) + (% hexaenoic × 8).

5 calculated as reported by (Dabbou et al., 2017): AI = (C12:0 × 4 + C14:0 + C16:0) / [(∑ MUFA + ∑ PUFA-n6 + ∑ PUFA-n3].

6 calculated as reported by (Dabbou et al., 2017): TI = (C14:0 + C16:0 + C18:0) / [(0.5 × ∑ MUFA) + (0.5 × ∑ PUFA-n6) + (0.5 × ∑ PUFA-n3) + (∑ PUFA-n3 / ∑ PUFA-n6)].

7 calculated as reported by (Chen and Liu, 2020): HH = (cis- C18:1 + ∑ PUFA) / (C12:0 + C14:0 + C16:0).

Table S4. Effects of increasing levels of defrosted whole black soldier fly larvae (BSFL) in broiler diets on abdominal tissue fatty acid compositions of broilers.

Fatty acids	Dietary treatments[1]					P-values[2], ≤	
	CON	L10	L20	L30	SE	T	W
SFA (% of total FA)							
Caprylic acid (C8:0)	0.01	0.02	0.02	0.02	0.001	0.001	0.413
Capric acid (C10:0)	$0.026^{c\dagger}$	$0.067^{bc\dagger *}$	0.117^{b*}	0.162^{a}	0.013	0.001	0.955
Undecylic acid (C11:0)	0.01^{c}	0.02^{b}	$0.02^{a\dagger}$	$0.03^{a\dagger}$	0.001	0.001	0.840
Lauric acid (C12:0)	0.941^{c}	4.60^{b}	8.13^{a}	10.38^{a}	0.84	0.001	0.561
Tridecylic acid (C13:0)	0.01^{c}	0.06^{b}	$0.09^{a\dagger}$	$0.11^{a\dagger}$	0.001	0.001	0.613
Myristic acid (C14:0)	0.779^{c}	2.06^{b}	3.05^{ab}	3.61^{a}	0.28	0.001	0.483
Pentadecylic acid (C15:0)	0.10^{b}	0.10^{ab}	0.11^{a}	0.11^{a}	0.001	0.008	0.143
Palmitic acid (C16:0)	20.3	20.3	20.0	20.3	0.36	0.935	0.009
Margaric acid (C17:0)	0.13	0.14	0.14	0.14	0.001	0.449	0.275
Stearic acid (C18:0)	5.23	5.33	5.00	4.78	0.17	0.118	0.029
Arachidic acid (C20:0)	0.06	0.06	0.05	0.05	0.001	0.464	0.258
Heneicosylic acid (C21:0)	0.01	0.01	0.01	0.01	0.001	0.884	0.380
Behenic acid (C22:0)	0.01	0.01	0.01	0.01	0.001	0.497	0.065
Lignoceric acid (C24:0)	0.01	0.01	0.01	0.01	0.001	0.776	0.338
∑ SFA	27.6^{c}	$32.8^{b\dagger}$	$36.8^{ab\dagger}$	39.7^{a}	1.02	0.001	0.519
MUFA (% of total FA)							
Myristoleic acid (C14:1cis-9)	0.17^{d}	0.34^{c}	0.48^{b}	0.57^{a}	0.03	0.001	0.820
Palmitoleic acid (C16:1 cis9)	5.21	5.18	5.07	5.33	0.24	0.868	0.971
Margaroleic acid (C17:1cis-9)	$0.07^{b\dagger}$	0.08^{ab}	0.09^{a}	$0.08^{ab\dagger}$	0.001	0.019	0.288
Elaidic acid (C18:1 trans9)	0.141	0.124	0.139	0.141	0.009	0.451	0.004
Oleic acid (C18:1 cis9)	35.2^{a}	32.5^{b}	30.5^{bc}	29.8^{c}	0.70	0.001	0.498
cis-vaccenic acid (C18:1 cis11)	2.02^{a}	1.87^{ab}	1.72^{b}	1.72^{b}	0.070	0.010	0.278
cis-11-Eicosenoic acid (C20:1cis-11)	0.24^{a}	0.21^{b}	0.21^{b}	0.19^{b}	0.001	0.001	0.124
Docosenoic acid (C22:1cis-13)	0.01^{a}	0.01^{ab}	0.01^{ab}	0.01^{b}	0.001	0.035	0.762

∑ MUFA	43.1[a]	40.3[ab]	38.2[b]	37.9[b]	0.92	0.001	0.655
PUFA (% of total FA)							
Linoleic acid (C18:2 n-6)	16.49[a†]	15.56[a]	14.58[ab†]	13.41[b]	0.57	0.003	0.106
γ-Linolenic acid (C18:3 n-6)	0.118[a]	0.111[ab]	0.100[b]	0.100[b]	0.003	0.001	0.282
α-Linolenic acid (C18:3 n3)	11.29[a]	9.97[ab]	9.11[bc]	7.74[c]	0.54	0.001	0.948
CLA (C18:2 cis9, trans11)	0.040[a]	0.076[b]	0.106[c†]	0.126[c†]	0.006	0.001	0.142
Stearidonic acid (C18:4 n-3)	0.21[a]	0.19[ab]	0.16[b]	0.16[b]	0.01	0.004	0.976
Table S4 continues							
Eicosadienoic acid (C20:2 n-6)	0.09[a]	0.09[ab]	0.08[ab]	0.07[b]	0.001	0.014	0.334
Mead acid (C20:3 n-9)	0.04	0.04	0.04	0.04	0.001	0.278	0.027
Homo-γ-Linolenic (C20:3 n-6)	0.12[a]	0.11[b]	0.11[b]	0.09[b]	0.001	0.001	0.013
Eicosatrienoic (C20:3 n-3)	0.08[a†]	0.07[a]	0.07[ab†]	0.05[b]	0.001	0.001	0.779
Arachidonic (C20:4 n-6)	0.204[a]	0.168[ab]	0.182[ab]	0.147[b]	0.015	0.046	0.011
EPA (C20:5 n-3)	0.238	0.214	0.198	0.248	0.024	0.339	0.031
Adrenic acid (C22:4 n-6)	0.03[†]	0.03	0.03[±]	0.02[†±]	0.001	0.052	0.742
DPA (C22:5 n-3)	0.212[a]	0.179[ab]	0.187[a]	0.133[b]	0.014	0.002	0.360
DHA (C22:6 n-3)	0.085	0.075	0.079	0.064	0.007	0.238	0.001
∑ PUFA	**29.3[a]**	**26.9[ab]**	**25.0[bc]**	**22.4[c]**	**1.09**	**0.001**	**0.329**
∑ n-3	**12.11[a]**	**10.70[ab]**	**9.80[bc]**	**8.40[c]**	**0.58**	**0.001**	**0.912**
∑ n-6	**17.1[a†]**	**16.1[a]**	**15.1[ab†]**	**13.9[b]**	**0.59**	**0.002**	**0.096**
n-6/n-3 ratio	**1.45**	**1.53**	**1.69**	**1.77**	**0.11**	**0.172**	**0.485**

[1]Dietary treatments: ad-libitum feeding without access to BSFL (CON), or with BSFL amounting to 10% (L10), 20% (L20) or 30% (L30) of the feed intake of CON birds. Total number of observations used for statistical analyses, N = 96 (i.e., 2 birds sampled from each of 6 pens allocated to each of 4 treatments at weeks 4 and 6).

[2]**T**: treatment effect; **W** = time effect (weeks 4 and 6). P-values for **T*W** = treatment by time interaction were all P>0.1, and are not shown. In line with this, the values are presented in this table refer to average of weeks 4 and 6.

a-b: Values in a row denoted with different letters differ significantly (Tukey, $P < 0.05$). Symbols † and * in a row indicate a tendency of two treatments to differ (Tukey, $0.05 < P \leq 0.10$). For the sake of a succinct presentation, only the most conservative (i.e. the largest) SE is presented.

Abbreviations: **SFA**, saturated fatty acid; **MUFA**, monounsaturated fatty acid; **PUFA**, polyunsaturated fatty acid; **CLA**, conjugated linoleic acids; **EPA**, eicosapentaenoic acid; **DPA**, docosapentaenoic acid; **DHA**, docosahexaenoic acid.

∑ SFA, ∑ MUFA and ∑ PUFA are the sum of 16, 10 and 16 individual FA in abdominal tissue, respectively, in theses FA classes. Cerotic acid (C26:0), docosenoic acid (C22:1cis-13), methyl nervonate (C24:1cis-15), methyl nervonate (C24:1cis-15), docosadienoic acid (C22:2n-6) and docosapentaenoic acid (C22:5n-6) were below the detection limit.

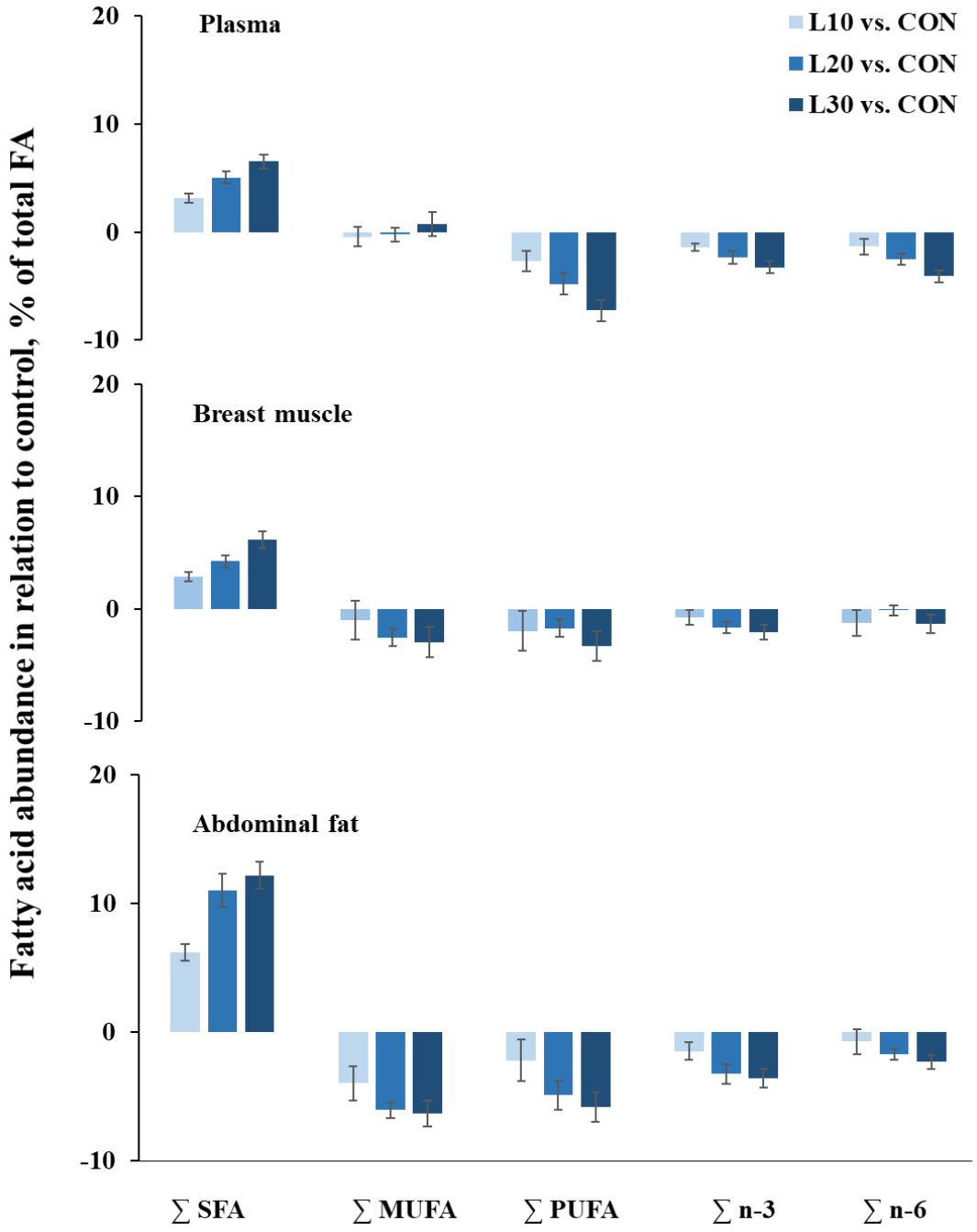


Figure S1. Relative changes in fatty acid abundance in plasma lipid, breast muscle and abdominal fat of broilers fed increasing dietary levels of black soldier fly larvae compared with broilers in the CON group without access to larvae. N = 48 (i.e., 2 birds sampled from each of 6 pens allocated to each of the 4 treatment groups at week 4).

5. Upcycling of recycled minerals from sewage sludge through black soldier fly larvae (Hermetia Illucens): impact on growth and mineral accumulation

This chapter has been published in:

Journal of Environmental Management, 2023, 344, 118695

Please find the original article via the following digital object identifier:

https://doi.org/10.1016/j.jenvman.2023.118695

Mohammad M. Seyedalmoosavi[a], Manfred Mielenz[a], Kai Schleifer[a], Solvig Görs[a], Petra Wolf[b], Jens Tränckner[c], Liane Hüther[d], Sven Dänicke[d], Gürbüz Daş[a], Cornelia C. Metges[a]*

[a] Research Institute for Farm Animal Biology (FBN), Institute of Nutritional Physiology, Dummerstorf, Germany

[b] University of Rostock, Nutrition Physiology and Animal Nutrition, Faculty of Agricultural and Environmental Sciences, Rostock, Germany

[c] University of Rostock, Water Management, Faculty of Agricultural and Environmental Sciences, Rostock, Germany

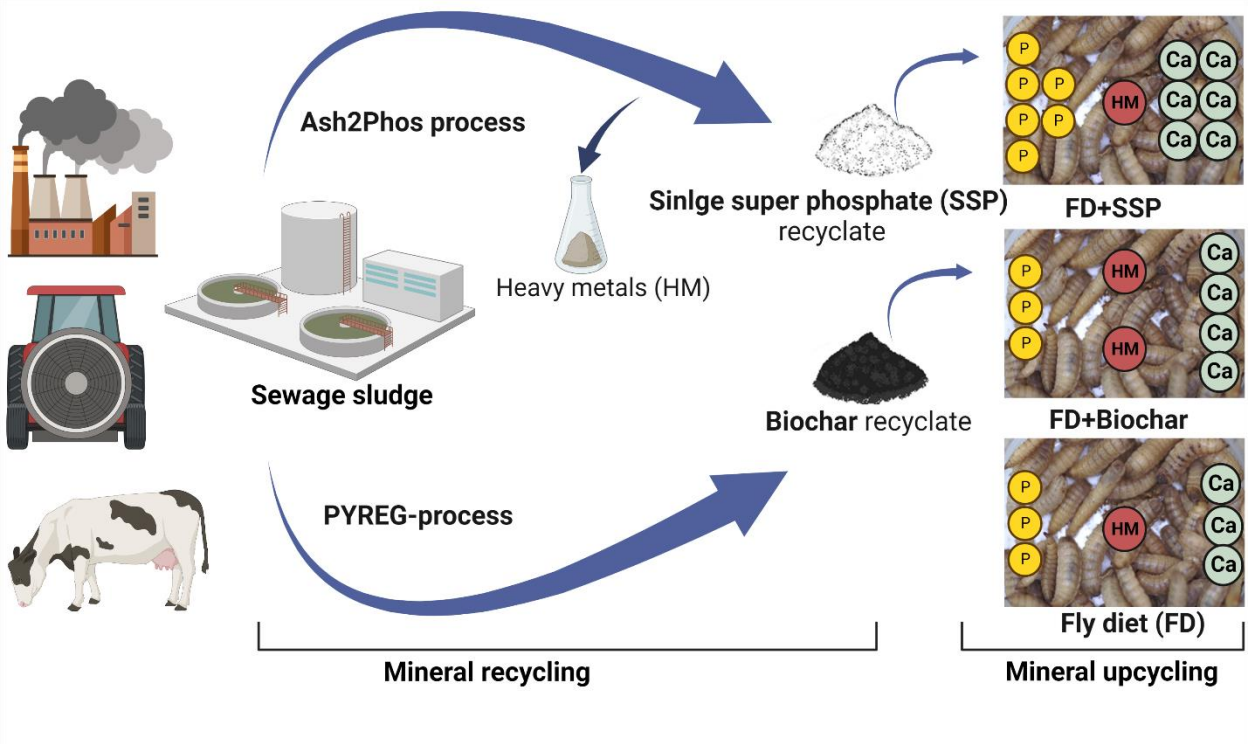
[d] Institute of Animal Nutrition, Federal Research Institute for Animal Health, Braunschweig, Germany

*Corresponding author: metges@fbn-dummerstorf.de

HIGHLIGHTS

- Larvae production can be used as a tool for upcycling and recovery of minerals.
- Single superphosphate recyclate increased larval Ca and P content more than Biochar
- Single superphosphate resulted in lower heavy metal content in larvae and frass
- Among heavy metals in larvae, just cadmium and manganese exceed EU feed legislation

Graphical abstract



ABSTRACT

Phosphorous (P) resources are finite. Sewage sludge recyclates (SSR) are not only of interest as plant fertilizer but also as potential source of minerals in animal nutrition. However, besides P and calcium (Ca), SSR contain heavy metals. Under EU legislation, the use of SSR derivatives in animal feed is not permitted, but given the need to improve nutrient recycling, it could be an environmentally sound future mineral source. Black soldier fly larvae (BSFL) convert low-grade biomass into valuable proteins and lipids, and accumulate minerals in their body. It was hypothesized that BSFL modify and increase their mineral content in response to feeding on SSR containing substrates. The objective was to evaluate the upcycling of minerals from SSR into agri-food nutrient cycles through BSFL. Growth, nutrient and mineral composition were compared in BSFL reared either on a modified Gainesville fly diet (FD) or on FD supplemented with either 4% of biochar (FD + BCH) or 3.6% of single-superphosphate (FD + SSP) recyclate (n = 6 BSFL rearing units/group). Larval mass, mineral and nutrient concentrations and yields were determined, and the bioaccumulation factor (BAF) was calculated. The FD + SSP substrate decreased specific growth rate and crude fat of BSFL ($P < 0.05$) compared to FD. The FD + SSP larvae had higher Ca and P contents and yields but the BAF for Ca was lowest. The FD + BCH larvae increased Ca, iron, cadmium and lead contents compared to FD. Larvae produced on FD + SSP showed lower lead and higher arsenic concentration than on FD + BCH. Frass of FD + BCH had higher heavy metal concentration than FD + SSP and FD ($P < 0.05$). Except for cadmium and manganese, the larval heavy metal concentration was below the legally permitted upper concentrations for feed. In conclusion, the SSR used could enrich BSFL with Ca and P but at the expense of growth. Due

to the accumulation of Cd and Mn, BSFL or products thereof can only be a component of farmed animal feed whereas in BSFL frass heavy metal concentrations remained below the upper limit authorized by EU.

Keywords: Bioaccumulation; Black soldier fly larvae; Mineral recycling; Nutrient cycle; Resource sustainability; Waste recycling

1. Introduction

The major source of phosphorous (P) is rock phosphate, which is a finite resource. Thus, excessive use of P in agri-food systems must be avoided (Van der Kooij et al., 2020). In addition, oversupply of P in animal feed and fertilizers, and P accumulation in the environment increase the risk of P leaching and leads to P losses from the agri-food systems, contributing to environmental pollution (Siddique and Robinson, 2003; Van der Kooij et al., 2020). Thus, P recycling is urgently needed, and part of the solution could be P recovery from sewage sludge as it is a promising source of P (Egle et al., 2016).

Besides its use in plant fertilizers, P is an essential mineral in animal diets (Algren et al., 2022), and the implementation of recycled P in animal feed could reintegrate it into the production cycle. However, due to safety considerations, it is currently prohibited to use waste streams (e.g. sewage sludge recyclates (SSR)) in animal feed (European Commission, 2009). Furthermore, sewage sludge contains heavy metals and organic contaminants, but some of the P recovery technologies extract P from the sewage sludge ash produced by incineration, such as e.g. the Ash2Phos process, which reaches a P recovery of ~90% (Cohen et al., 2019), and achieve significant reductions in pollutants and heavy metals in the recyclate products (Egle et al., 2016; Van der Kooij et al., 2020).

Recently, carbonization products, known as Biochar (BCH), derived from the thermal processing of sewage sludge has received attention as an efficient P-recovery option (Buss et al., 2022). It was suggested that BCH application in soil mitigates emission of ammonia and nitrite oxide from soil, and improve N use efficiency and crop productivity (Dawar et al., 2021). Besides P, BCH contains other minerals essential to animals, among them Ca, iron (Fe), potassium (K) and magnesium (Mg) (Zhao et al., 2018).

In recent years, there has been increasing interest in black soldier fly (Hermetia illucens) larvae (BSFL) as a sustainable protein source in animal feed to replace in part soybean meal and particularly fishmeal, which may contribute to meet the increasing demand for food and feed of a growing global population (Hunter et al., 2017; Raman et al., 2022; United Nations, 2019). It is known that the nutrient content of BSFL can be modified by the nutrient content of their feeding substrates (Diener et al., 2015; Van der Fels-Klerx et al., 2016). Furthermore, concentrations of minerals in the BSFL reflect the concentrations in their feeding substrates (Liland et al., 2017). Many elements can accumulate in BSFL, with Ca, P, and K being the most abundant macro-minerals in the BSFL (Chia et al., 2020; Schmitt et al., 2019). Concurrently, accumulation of certain heavy metals such as cadmium (Cd) in BSFL has been reported, which must be considered from a safety perspective (Proc et al., 2020). In general, the uptake of undesirable substances in animals depends on their concentrations in feed, the duration of exposure, diet composition, and the nutritional and ontogenetic status of the animal. Thus, it is necessary to assess the risk of potential accumulation of hazardous substances in BSFL which ultimately contributes to further development of legislation for safe feeds (Lievens et al., 2021).

Therefore, it was hypothesized that: 1) BSFL modify their mineral content in response to the mineral and heavy metal composition in SSR; and 2) SSR added to BSFL feeding substrates lead to mineral enriched larvae suitable for the implementation in animal feed. Therefore, the objective of this study was to evaluate upcycling of minerals from SSR into agri-food nutrient cycles through BSFL. The results presented in this paper provide novel information on a new sustainable approach to reintegrating recycled P and other minerals from sewage sludge into the agri-food nutrient cycle to reduce P release to the environment.

2. Material and Methods

2.1. Animals, sewage sludge recyclates and feeding substrate

A BSFL stock was obtained from Hermetia Baruth GmbH (Baruth, Germany) in the year 2019. Black soldier fly eggs were collected from the 12th and 13th generations of the established colony in the insect facility of the Research Institute for Farm Animal Biology (FBN) in Dummerstorf, Germany. Adult flies were kept in an indoor cage at 27.5 °C and 70% relative humidity with 12 h of artificial light per day (d) from a LED source (daylight spectrum). The flies were provided with 3% (w:v) sugar solution and water. Eggs were harvested daily from the oviposition site made of perforated plastic balls (Bioball, Berlan GmbH, Klingenthal, Germany) and placed above a 200 mL box filled with dead adult flies from the previous generation surrounded by boxes with fermenting chicken starter feed (Trede & von Pein GmbH, Itzehoe, Germany) to stimulate egg laying (Dortmans et al., 2017). Larvae hatched from the egg-laying balls in new hatching boxes with closed lids. Three days after placement of the bioballs in the hatching box, most larvae hatched and were transferred to an open plastic container (30 × 20 × 15 cm) provided with 300 g

of chicken starter feed (30% feed: 70% water) on which the larvae were reared up to the fifth d post-hatch.

Two different sources of mineral rich SSR, namely BCH and Single Superphosphate (SSP) were used. The BCH recyclate was derived from pyrolysis of sewage sludge produced by the PYREG-process (PYREG GmbH, Dörth, Germany) as described (Fesharaki and Rath, 2018). The SSP originated from the recycling of sewage sludge produced by incineration using the Ash2Phos process developed by EasyMining Sweden AB (Cohen et al., 2019). The SSP was enriched in P and Ca but had lower heavy metal contents compared to the carbon-rich BCH (Table S1 in the supplementary data). The Ash2Phos process used the residue ash from the incineration of the sewage sludge, while in the PYREG process, the drained and dried sewage sludge was carbonised at around 500–700 °C under oxygen exclusion (Cohen et al., 2019).

A standard fly diet (Hogsette, 1992) with reduced P content was formulated using corn meal, wheat bran, lucerne, and sugar beet pulp (i.e. modified Gainesville fly diet; FD; Table S1 in the supplementary data). The dietary components were analysed by the accredited laboratory LUFA (Landwirtschaftliche Untersuchungs-und Forschungsanstalt der LMS Agrarberatung GmbH), Rostock, Germany. The FD diet was supplemented either with 4% of BCH (FD + BCH) or 3.6% of SSP (FD + SSP) normalized for dry matter (DM) at the expense of wheat bran and corn meal (Table S1 in the supplementary data). Final feeding substrates were prepared with addition of water to obtain a 30% feed: 70% water ratio.

2.2. Experimental setup

On the 5th day after hatching, the larvae were sieved, and weighed, and a larval mass of approximately 8000 individuals was placed in each of 18 pre-weighed containers (40 × 60 × 22 cm). The containers seeded with 5-day-old larvae were allocated to one of the three experimental feeding substrates in each of the two runs (i.e. N = 18 containers/run; total N = 36) (Fig. 1). Six replicate containers were used per each of the feeding substrates per experimental run. The larvae were batch-fed on d 5 (2500 g), 9 (4000 g) and 13 (3500 g) after hatching. The position of each container within the racks was randomized (Mersenne Twister algorithm (Matsumoto and Nishimura, 1998)) and the position within each rack was changed daily. Harvesting took place when approximately 10% of the larvae became prepupae, at which point the feeding experiment was terminated. After an experimental feeding period of 13–15 d, harvesting of larvae took place 18–20 d after hatching. For larvae and frass (i.e. mixture of insect feces, cuticles, parts of dead insects and feeding substrate residuals) sampling, we selected randomly 18 containers from a total of 36 containers (i.e. n = 6 containers per substrate).

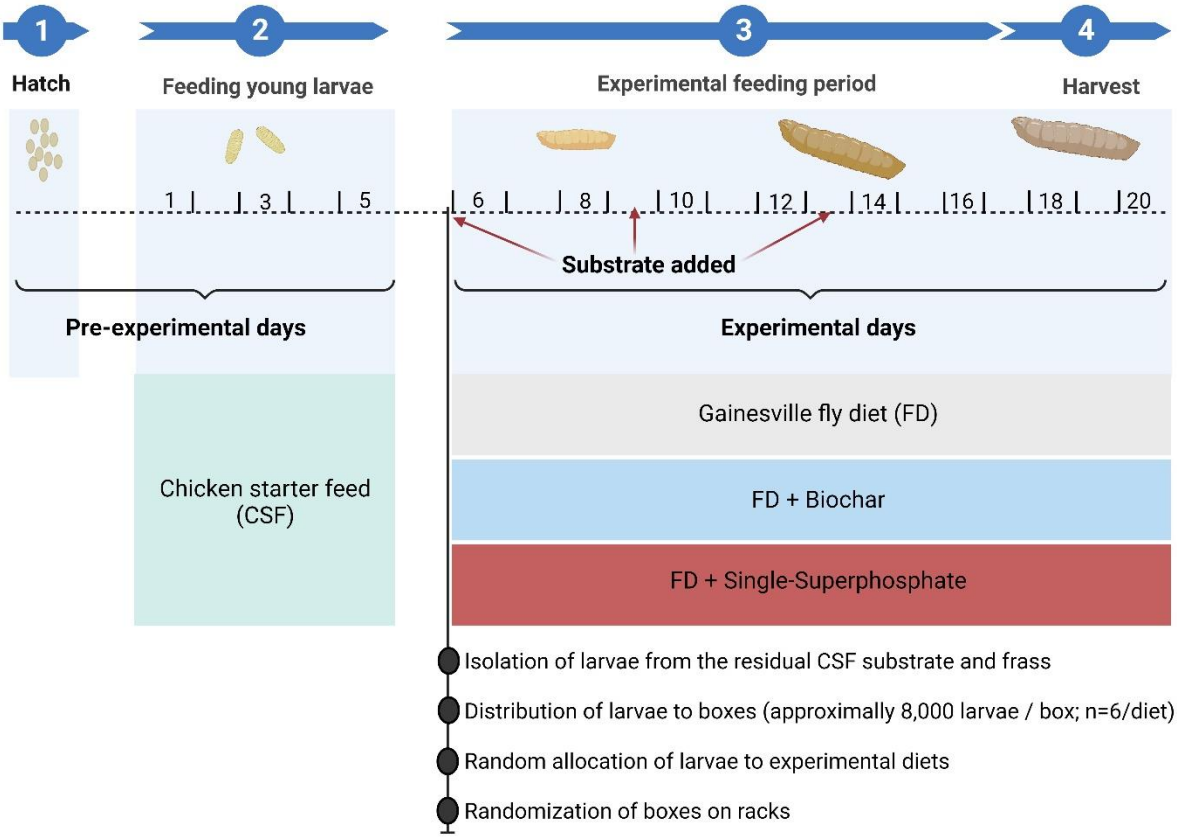


Fig. 1. Schematic diagram representing the pre-experimental and experimental days with the corresponding feeding substrates used after hatch until harvest.

At the end of the experiment, larvae were separated from the feed residue and all of the containers were weighed again to calculate the total amount of frass. The isolated larvae were washed and then dried with paper towels. The total fresh larval mass (LM) was measured for each container. In addition, the average LM (mg per larvae) per each container was calculated by counting and weighing of at least 100 harvested larvae. The larvae were then devitalized by being transferred into liquid nitrogen (N2); the frozen larvae of each container were mixed, and representative larvae collected in a 200 mL vessel were used for the analysis of heavy metal, mineral and nutrient concentrations. The heavy metal classification was based on atomic weights ranging between 63.5 and 200.6 (Srivastava and Majumder, 2008). Samples were collected from the freshly sieved frass of each container (n = 6 containers per feeding substrate) into 1-L buckets for heavy metal, mineral and nutrient analysis. All samples were stored at −20 °C for further analyses.

2.3. Proximate composition

The contents of Ca, P, Mg, K, sodium (Na) (method of Verband Deutscher Landwirtschaftlicher Untersuchungs-und Forschungsanstalten (VDLUFA) III 10.8.2), Mn, Fe, zinc (Zn), copper (Cu), arsenic (As), Cd, lead (Pb) (method VDLUFA III 17.9.1) and mercury (Hg) (VDLUFA III 17.4.3) in the feeding substrate, larvae and the frass were analysed by the accredited feed laboratory LUFA Rostock (Naumann et al., 1997). Additionally, an extended Weende analysis (Van Soest et al., 1991) was performed in frass and the feeding substrates by LUFA Rostock to determine DM, crude ash, crude protein (CP), crude fat, starch, metabolizable energy (ME), aNDFom (neutral detergent fibre after amylase treatment based on organic matter) and ADFom (acid detergent fibre) (Naumann et al., 1997). Gross energy (GE) of the substrates was calculated by multiplying

metabolizable energy (ME) with the conversion factor of 1.379 (NRC, 1994). For the larvae, a Weende analysis was performed at the Institute of Animal Nutrition of the Federal Research Institute of Animal Health (Friedrich-Loeffler-Institute) in Braunschweig, Germany. The DM of larvae and frass was analysed using a moisture analyser (Satorius, MA37; Göttingen, Germany). Frozen larvae and frass samples were weighed (2.5 g) and heated gradually to 130 °C until a constant mass was reached. Minerals, nutrients and heavy metal contents of the substrates, larvae and frass were expressed as concentrations (g/kg DM), and absolute amounts (mg or g) per each container. Yield per container was then calculated as the nutrient or mineral concentration in larvae multiplied by the total larval mass on DM basis (i.e. mg or g/container). The N and C contents of dried larvae and substrates were measured as previously described (Seyedalmoosavi et al., 2022a). Content of CP was calculated by N content × 6.25.

2.4. Calculations

Substrate conversion ratio (SCR) (i.e. g substrate provided per g LM gain by BSFL), protein conversion ratio (PCR = g protein supply to gain 100 g LM) and GE conversion ratio (GECR = ME (Mega Joule (MJ)) supply to gain 100 g LM) of the larvae were calculated for each rearing container based on fresh matter. The LM gain was calculated as the difference between the final LM at harvest and the initial LM at start of the experiment. Accounting for developmental time, the specific growth rate (SGR) was calculated (Lugert et al., 2016).

The bioaccumulation factor (BAF) of minerals and heavy metals was calculated according to Eq. 1:

$$BAF = \frac{MClarvae\ at\ harvest}{MCsubstrate} \quad (1)$$

where, MC_{larvae} is the mineral concentration [g / kg DM] in larvae at harvest and $MC_{substrate}$ is the mineral concentration in the substrates fed [g / kg DM]. The BAF is the ratio between the mineral/heavy metal concentration in BSFL and the concentration in the substrate (Walker, 1990); a BAF above 1 indicates mineral enrichment in the larvae, whereas values below 1 imply depletion.

In addition, retention (%) of minerals and heavy metals per container is the total mass [g] of minerals or heavy metals in larvae divided by the total mass [g] of minerals or heavy metals in the feeding substrate and was calculated according to Eq. 2:

$$\text{Retention (\%)} = \frac{Total\ mass\ of\ mineral\ in\ larvae\ at\ harvest}{Total\ mass\ of\ mineral\ in\ substrate} \times 100 \qquad (2)$$

2.5. Statistical Analysis

The experimental unit was the rearing container (n = 6 replicate rearing containers per treatment group; N = 18). Data were analysed by analysis of variance using the general linear model (PROC GLM) of SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). The statistical model included the fixed effects of substrate treatments (FD, FD + BCH, FD + SSP) and the blocking effect of the runs (1 and 2). Since the interaction treatment × block was not significant, it was omitted from the model. Treatment group differences were separated by Tukey-Kramer test at $P < 0.05$. The significance level was $P < 0.05$, and a tendency was declared at $0.05 < P \leq 0.10$. All data are presented as LSMeans and their SE. In order to investigate overall changes in mineral and heavy metal concentrations of BSFL due to differences in feeding substrates, a linear discriminant analysis was performed using JMP 15 (SAS Institute Inc.). For this purpose, minerals (Ca, P, Mg, Na, K) or heavy metal (Mn, Fe, Zn, Cu, As, Cd and Pb) concentrations of BSFL were used as

covariates and the feeding substrate as the categorical variable in two discriminant analyses separately. Measurement units of all the covariates were normalized to mg/kg DM.

3. Results and discussion

3.1. Growth, nutrient utilization efficiencies and body composition

The LM did not differ among the substrate groups, however the LM of FD + SSP larvae tended to be lower than in larvae grown on FD substrate (Table 1; $P = 0.066$). The LM observed in the FD group was of the same magnitude as reported by Tomberlin et al. (2002), who used Gainesville diet to feed BSFL (average LM; 104 mg). Broeckx et al. (2021) even reported lower LM in BSFL fed Gainesville diet (83.8 mg). In contrast, Miranda et al. (2020) observed a higher LM (173 mg) for BSFL fed Gainesville diet. Because in these studies a similar substrate composition for feeding the larvae was used, the differences in LM across the studies might be largely related to different rearing conditions such as larval density, humidity and temperature, as well as BSFL genetic variation (Barragan-Fonseca et al., 2018; Chia et al., 2018; Sandrock et al., 2022). The SGR in the FD + SSP larvae was lower compared with those in FD and FD + BCH (Table 1; $P < 0.05$), which was due to a longer development time of FD + SSP larvae compared to FD and FD + BCH larvae (20 d vs. 18 d, respectively). The SCR, PCR, and GECR values did not differ among the groups (Table 1; $P > 0.10$), and the SCR ranged between 3.38 and 3.64 which is similar to that reported by Broeckx et al. (2021) for larvae fed Gainesville diet (SCR = 3.43).

Table 1

Growth, efficiency indicators and carbon/nitrogen ratio in black soldier fly (*Hermetia illucens*) larvae fed on a fly diet (FD) supplemented with or without two different sewage sludge recyclates (FD+BCH, FD+SSP)

	Feeding substrates[1]				P-values[2], ≤	
	FD	**FD+BCH**	**FD+SSP**	**SE**	**T**	**B**
LM, mg	103.6†	99.5	89.7†	4.0	0.066	0.044
LDM/container, g	254.5	250.0	230.9	9.6	0.207	0.595
SGR, %	50.22[a]	50.02[a]	45.32[b]	1.24	0.021	0.908
SCR	3.38	3.58	3.64	0.16	0.514	0.849
PCR	44.33	45.14	45.88	2.07	0.868	0.835
GECR	3.14	3.11	3.16	0.14	0.968	0.826
C, %	49.12[a]	48.34[a]	45.45[b]	0.39	0.001	0.001
N, %	7.37[a]	7.28[ab†]	7.07[b†]	0.06	0.013	0.003
C/N	6.66[a]	6.64[a]	6.43[b]	0.05	0.004	0.837

a-c: Values in a row that are marked with different letters differ significantly (Tukey, $P < 0.05$). The symbol † in a row indicates a tendency of two treatments to differ (Tukey, $0.05 < P \leq 0.10$).

[1]**FD:** modified Gainesville fly diet**; FD+BCH:** Pyrolysis product of sewage sludge (Biochar; BCH) added to FD (4%); **FD+SSP:** Single Superphosphate (SSP) added to FD (3.6%).

Total number of observations used for statistical analyses, N = 18 (i.e., n = 6 replicate rearing containers per treatment group over 2 experimental runs).

[2]**T**: treatment effect; **B** = block effect (experimental run (2 runs) considered as block).

Abbreviations: LM: larval mass; LDM/container: larval dry mass per container; SGR: specific growth rate; SCR: substrate conversion ratio; PCR: protein conversion ratio; GECR: gross energy conversion ratio; C: carbon; N: nitrogen.

SGR = (ln (harvest weight in g) – ln (initial weight at d5 in g)) / fattening period (d) × 100 (Lugert et al., 2016)

SCR = Substrate supply (g) / body weight gain (g)

PCR = Crude protein supply (g) / 100 g body weight gain
GECR = Gross energy supply (MJ GE) / 100 g body weight gain

The FD + SSP larvae had a lower C and N content and a lower C/N ratio than those larvae fed on FD and FD + BCH (Table 1; $P < 0.05$). This could be explained by the fact that in FD + SSP larvae the C content was 3.7% lower while the N content was only 0.3% lower compared to the FD fed larvae. In a review, Makkar et al. (2014) reported the chemical composition and mineral content of BSFL reared on different substrates and showed that depending on the lipid and energy content of substrates, the lipid content of BSFL varies between 11% and 58% of DM. In the current study, the lipid content of FD larvae was similar to that reported in previous studies (17–28% DM), where Gainesville diet was fed to BSFL (Arabzadeh et al., 2022; Arnone et al., 2022; Pliantiangtam et al., 2021). Although energy and crude fat content of the substrates in the present study were approximately similar (Table S1 in the supplementary data), the FD + SSP larvae had a lower crude fat content (Table 2; $P < 0.05$) and yield (Table 3; $P < 0.05$) (by 33% and 27%, respectively) than FD + BCH and FD larvae, presumably reflecting the lower C content of FD + SSP larvae (Table 1). The lower crude fat content in FD + SSP larvae might be linked to a higher accumulation of heavy metals such as Fe, As, Cd, and Pb than in FD and FD + BCH larvae (Fig. 4, Fig. 5). Schmitt et al. (2019) discussed that a decreased fat content in BSFL might be related to high accumulation of heavy metals such as Cd, Cu and Zn in the larvae. Also a high concentration of Cd (20–40 mg) in the diet of Galleria mellonella larvae reduced lipid content in the larval body (Emre et al., 2013). The authors suggested that due to the high heavy metal content, a large proportion of larval lipids could be metabolized to provide the required energy under the stress

caused by heavy metals (Emre et al., 2013). In addition, the lower intake of heavy metal-contaminated substrates by Folsomia candida larvae may indicate an attempt by the larvae to counteract an oversupply of heavy metals, resulting in lower energy intake and thus lower body fat (Fountain and Hopkin, 2001).

Table 2

Nutrient, macro-mineral and heavy metal concentrations and bioaccumulation factor (BAF) of macro-minerals in black soldier fly (*Hermetia illucens)* larvae fed on a fly diet (FD) supplemented with or without two different sewage sludge recyclates (FD+BCH, FD+SSP).

	Feeding substrates[1]				**P-values[2], ≤**	
	FD	**FD+BCH**	**FD+SSP**	**SE**	**T**	**B**
Dry matter, g / kg FM	285.7	292.6	278.5	0.66	0.339	0.095
Nutrients, g / kg DM						
Crude protein[3]	469.9[†]	459.1	455.5[†]	4.0	0.057	0.141
Crude fat	224.8[a]	215.7[a]	172.5[b]	7.0	0.001	0.007
Crude fibre	65.7	68.1	74.0	3.8	0.300	0.126
aNDF	119.8	128.4	135.1	7.7	0.385	0.702
ADF	86.5	90.1	95.5	5.1	0.471	0.629
Crude ash	147.3[c]	165.7[b]	192.1[a]	1.8	0.001	0.001
Macrominerals, g / kg DM						
Ca	45.77[c]	53.24[b]	64.59[a]	1.22	0.001	0.022
P	7.14[b]	7.24[b]	10.89[a]	0.39	0.001	0.035
Mg	3.31[b]	3.63[ab]	3.96[a]	0.12	0.005	0.015

Na	0.94[b]	0.94[b]	1.27[a]	0.03	0.001	0.212
K	10.89[b]	10.01[b]	14.46[a]	0.74	0.005	0.071
Heavy metals, mg / kg DM						
Mn	285	304	303	7.46	0.167	0.108
Fe	150[b]	557[a]	339[ab]	74.4	0.006	0.248
Zn	95.8[†]	107.4[†]	98.5	3.6	0.087	0.047
Cu	9.92[†]	12.22[†]	10.14	0.67	0.052	0.085
As	0.047[b]	0.066[b]	0.115[a]	0.006	0.001	0.340
Cd	0.63[b]	0.77[a]	0.74[ab]	0.037	0.045	0.147
Pb	0.23[c]	1.16[a]	0.54[b]	0.05	0.001	0.201
Hg	0.000	0.000	0.001	-	-	-
BAF[4]						
Ca	5.70[a†]	5.31[a†]	3.81[b]	0.11	0.001	0.010
P	1.48[a]	1.03[b]	1.32[ab]	0.06	0.001	0.067
Mg	1.26[b]	1.31[b]	1.54[a]	0.04	0.001	0.015
Na	1.99[a]	1.70[b]	1.16[c]	0.06	0.001	0.409
K	0.730[b]	0.754[b]	0.995[a]	0.050	0.001	0.135

a-c: Values in a row that are marked with different letters differ significantly (Tukey, $P < 0.05$). The symbol † in a row indicates a tendency of two treatments to differ (Tukey, $0.05 < P \leq 0.10$).

[1]**FD:** modified Gainesville fly diet**; FD+BCH:** Pyrolysis product of sewage sludge (Biochar; BCH) added to FD (4%); **FD+SSP:** Single Superphosphate (SSP) added to FD (3.6%).

Total number of observations used for statistical analyses, N = 18 (i.e., n=6 replicate rearing containers per treatment group within 2 experimental runs).

[2]**T**: treatment effect; **B** = block effect (experimental run (2 runs) considered as block).

[3]Calculated as N × 6.25.

[4]BAF values > 1 refer to an accumulation of minerals in BSFL and values < 1 refer to a relative depletion of minerals in BSFL during the experimental period.

Abbreviations: **DM**: Dry matter; a**NDF**: Neutral detergent fibre after amylase treatment; **ADF**: Acid detergent fibre.

Table 3

Nutrient, macro-mineral and heavy metal yield (per container) in black soldier fly (*Hermetia illucens*) larvae fed on a fly diet supplemented with or without two different sewage sludge recyclates (BCH, SSP)

	Feeding substrates[1]				P-values[2], ≤	
	FD	**FD+BCH**	**FD+SSP**	**SE**	**T**	**B**
Nutrients, g						
Crude protein[3]	120	115	105	4.67	0.117	0.419
Crude fat	57.35[a]	53.93[a]	39.92[b]	2.75	0.001	0.032
Crude fibre	16.69	16.98	16.97	0.93	0.969	0.287
aNDF	30.38	32.14	31.00	1.87	0.795	0.963
ADF	21.98	22.50	21.93	1.30	0.941	0.992
Crude ash	37.42[b]	41.40[ab]	44.34[a]	1.65	0.031	0.585
Macrominerals, g						
Ca	11.63[b]	13.32[ab]	14.93[a]	0.68	0.013	0.441
P	1.81[b]	1.80[b]	2.52[a]	0.12	<0.001	0.158
Mg	0.84	0.91	0.91	0.04	0.436	0.177
Na	0.24[b]	0.23[b]	0.29[a]	0.01	0.007	0.717
K	2.76	2.74	3.34	0.21	0.107	0.173

Heavy metals, µg						
Mn	72,459	75,721	69,970	3,446	0.501	0.637
Fe	36,968[b]	137,745[a†]	78,472[ab†]	18,451	0.006	0.257
Zn	24,356	26,759	22,759	1,283	0.113	0.278
Cu	2,525	3,043[†]	2,346[†]	202	0.066	0.231
As	12.0[b†]	16.3[b†]	26.4[a]	1.35	<0.001	0.474
Cd	161	192	170	11.23	0.154	0.391
Pb	58.7[c]	288.6[a]	123.6[b]	15.4	<0.001	0.318
Hg	0.10	0.04	0.19	0.14	0.744	0.172

a-c: Values in a row that are marked with different letters differ significantly (Tukey, $P < 0.05$). The symbol † in a row indicates a trend for a difference between two treatments (Tukey, $0.05 < P \leq 0.10$).
[1]**FD:** modified Gainesville fly diet**; FD+BCH:** Pyrolysis product of sewage sludge (Biochar; BCH) added to FD (4%); **FD+SSP:** Single Superphosphate (SSP) added to FD (3.6%).
Total number of observations used for statistical analyses, N = 18 (i.e., n=6 replicate rearing containers per treatment group within 2 experimental runs).
[2]**T**: treatment effect; **B** = block effect (experimental run (2 runs) considered as block).
[3]Calculated as N × 6.25.
Abbreviations: **DM**: Dry matter; a**NDF**: Neutral detergent fibre after amylase treatment; **ADF**: Acid detergent fibre.

The CP content of the BSFL ranged between 46 and 47% of DM in the larval body with no difference among the three groups (Table 2). However, FD + SSP fed BSFL tended to have a lower CP content than BSFL fed on FD substrate (Table 2; P = 0.055). In previous studies, larval CP values ranging from 38 to 51% DM were found in BSFL fed Gainesville diet (Arabzadeh et al., 2022; Arnone et al., 2022; Pliantiangtam et al., 2021), which was the same order of magnitude as the values found in our study.

There was no difference among the groups for BSFL crude fibre, aNDFom, and ADFom concentrations or for their yields (Table 2, Table 3; $P > 0.10$). In plant material, aNDFom is composed of cellulose, lignin, and hemicellulose while ADFom is composed of cellulose and lignin (Van Soest and Robertson, 1977). However, the components of ADFom and aNDFom in insects are not fully known. Fibre content in the body of edible insects such as BSFL is mainly composed of chitin (linear polymer of b-(1–4) N-acetyl-D-glucosamine units) (Soetemans et al., 2020). Besides chitin, ADF could contain also amino acids probably representing cuticular proteins (Finke, 2007).

Larvae fed substrates containing either SSR had a higher crude ash content than larvae grown on FD substrate (Table 2; $P < 0.05$). The crude ash concentration of FD + SSP larvae was even higher than that of FD + BCH larvae, whereas the crude ash yield was higher in the FD + SSP larvae than in the FD control (Table 2, Table 3; $P < 0.05$). Depending on the substrate, the larvae in the present study contained 14–19% DM of crude ash which was similar to that reported earlier (Pliantiangtam et al., 2021) for BSFL fed Gainesville diet (∼15% DM) or various other diets (9–28% DM) as reviewed by Makkar et al. (2014).

Overall, the inclusion of BCH and SSP recyclates in the BSFL feeding substrates was associated with differences in larval body nutrient contents in terms of CP, fat and crude ash, but did not affect larval feed, protein and nutrient utilization efficiencies.

3.2. Macro-mineral contents, bioaccumulation, yield and retention

Recycled minerals in both SSP and BCH may be reintegrated into the nutrient cycle through the use as feed components for BSFL, implying a potential upcycling of valuable minerals. The SSP recyclate contained higher Ca, P and Na levels, but was lower in Mg and K concentration than BCH (Table S1 in the supplementary data). In order to evaluate the efficiency of mineral incorporation in BSFL, concentration, yield, BAF, and retention were calculated as the most relevant parameters (Daş et al., 2023) for each mineral and heavy metal. The BAF is an estimate of the efficiency of larvae to accumulate certain minerals from substrate per body mass unit (i.e. on concentration basis) without considering larval growth (Walker, 1990). Yield and retention represent the efficiency of total substrate minerals accumulated in total larval body mass in absolute (i.e. g) or relative (%) terms, respectively. Yield and retention can reflect element enrichment in the larvae not only due to concentration changes in the larvae as compared to the feeding substrate, but additionally due to larval growth (i.e. total larval mass).

According to Makkar et al. (2014) the Ca content of BSFL ranges between 50.0 and 86.3 g/kg DM which is similar to what was observed in our study (46–64 g/kg DM). Likely, as a consequence of the mineralized exoskeleton in BSFL which is high in Ca (Finke, 2013), Ca BAF values were greater than 1 in all groups, indicating a Ca enrichment in the larvae relative to the Ca content in the substrate (Table 2). The FD + SSP and FD + BCH larvae had a higher Ca concentration than those in the FD group (Table 2; $P < 0.05$), whereas among the SSR-fed groups, the FD + SSP larvae had the highest Ca concentration ($P < 0.05$). This reflects the highest Ca concentration among the three feeding substrates, followed by FD + BCH and FD substrates (Table S1 in the

supplementary data). Nonetheless, FD + SSP larvae had a lower Ca BAF than those of the FD + BCH and FD groups (Table 2; $P < 0.05$), which may suggest a less efficient utilization of Ca by BSFL in response to the higher Ca availability in the FD + SSP substrate. In line with the lower Ca BAF, FD + SSP larvae had a lower Ca retention than those in the FD + BCH and FD groups (Table 4; $P < 0.05$). Nevertheless total Ca yield per container was higher in FD + SSP than in FD larvae (Table 3; $P < 0.05$), because of the higher Ca concentration in FD + SSP larvae, even though larval size tended to be higher in FD larvae.

Table 4

Macro-mineral and heavy metal retention (%) in black soldier fly (*Hermetia illucens*) larvae fed on a fly diet supplemented with or without two different sewage sludge recyclates (BCH, SSP)

	Feeding substrates[1]				P-values[2], ≤	
	FD	FD+BCH	FD+SSP	SE	T	B
Macrominerals, %						
Ca	55.4[a]	50.9[a]	33.9[b]	2.35	0.001	0.379
P	14.7[a]	10.0[b]	11.9[b]	0.69	0.001	0.241
Mg	12.5	12.7	13.9	0.64	0.296	0.176
Na	19.8[a]	16.6[b]	10.5[c]	0.83	0.001	0.927
K	7.2	7.3	9.0	0.57	0.080	0.171
Heavy metals, %						
Mn	42.9[a]	35.8[b]	41.5[ab]	1.84	0.028	0.737
Fe	4.0[b]	0.9[c]	8.7[a]	0.43	0.001	0.329

Zn	24.2[a]	10.3[b]	23.4[a]	0.87	0.001	0.608
Cu	10.1[a]	4.3[b]	9.5[a]	0.47	0.001	0.391
As	3.9[b†]	2.7[b†]	6.0[a]	0.32	0.001	0.673
Cd	48.2	54.6	51.7	3.3	0.400	0.410
Pb	8.4[b]	3.1[c]	14.7[a]	0.91	0.001	0.303
Hg	0.2	0.1	0.3	0.24	0.743	0.173

a-c: Values in a row that are marked with different letters differ significantly (Tukey, $P < 0.05$). The symbol † in a row indicates a trend for a difference between two treatments (Tukey, $0.05 < P \leq 0.10$).
[1]**FD:** modified Gainesville fly diet**; FD+BCH:** Pyrolysis product of sewage sludge (Biochar; BCH) added to FD (4%); **FD+SSP:** Single Superphosphate (SSP) added to FD (3.6%).
Total number of observations used for statistical analyses, N = 18 (i.e., n=6 replicate rearing containers per group within 2 experimental runs).
[2]**T**: treatment effect; **B** = block effect (experimental run (2 runs) considered as block).

The P concentration in larvae ranged between 7 and 11 g/kg DM, which was similar to previous reports summarized by Makkar et al. (2014) (6–15 g/kg DM). The P concentration was higher in FD + SSP larvae than that in FD + BCH and FD larvae (Table 2; $P < 0.05$), which was linked to a higher P concentration in the FD + SSP substrate than in FD + BCH and FD (Table S1 in the supplementary data). Phosphorus accumulated in all groups (BAF >1), however, the FD + BCH larvae had a lower P accumulation than those in the FD group (Table 2; $P < 0.05$), suggesting a lower P accumulation efficiency from the substrate with BCH recyclate. The FD + SSP larvae had a higher P yield than those larvae fed on FD + BCH and FD (Table 3; $P < 0.05$). Nevertheless, both FD + SSP and FD + BCH larvae had a lower P retention than those in FD (Table 4; $P < 0.05$), suggesting a lower efficiency of total P retention.

A linear discriminant analysis of the larval mineral concentration was performed (Fig. 2) to visualize the overall differentiation in mineral composition of the BSFL induced by different feeding substrates, and to identify minerals most responsible for the differentiation. The overall mineral composition of BSFL showed three distinct and non-overlapping clusters that are clearly attributable to the feeding substrates. Phosphorous and Mg associated most strongly with the separation of groups on both the first and second canonical axes, while Ca and Na were more associated with the separation of groups on the second canonical axis (Fig. 2). As shown in Fig. 2, the FD and the FD + BCH groups are closer together on the first canonical axis as compared to the FD + SSP group. The main reason for the separation of the FD + SSP larvae from the other two groups were their higher Ca, P and Na concentrations. In summary, our study shows that the Ca and P content of FD + SSP larvae increased in response to a higher Ca and P content of the SSP recyclate. In line with our results, previous studies reported plasticity of mineral composition in BSFL depending on the mineral contents in the substrate (Liland et al., 2017; Spranghers et al., 2017). To date, knowledge on the regulation of Ca and P homeostasis in BSFL is limited but the involvement of the Malpighian tubules in homeostasis and storage of minerals in insects such as Drosophila melanogaster (Browne and O'Donnell, 2016; Rose et al., 2019) is likely to be present in BSFL, too.

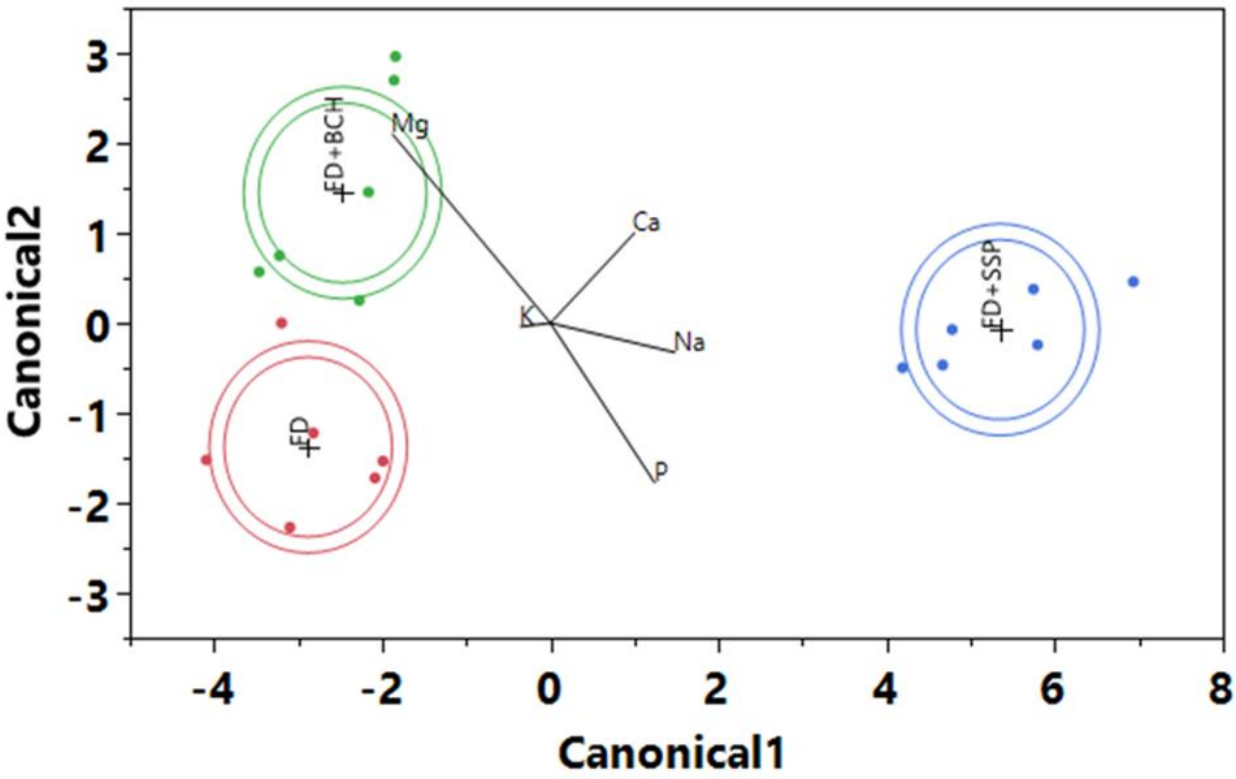


Figure 2. Canonical plot of points and means (+) from linear discriminant analysis of larval concentrations of the macro-minerals calcium (Ca), phosphorus (P), magnesium (Mg), sodium (Na) and potassium (K) with substrate groups defined as FD: modified Gainesville diet; FD + BCH: pyrolysis product of sewage sludge (BCH) added to FD (4%); FD + SSP: single superphosphate (SSP) added to FD (3.6%). All variables had the same units of measurement (i.e. mg/kg DM). Each dot represents the overall response of one container of BSFL fed on a specific substrate. The inner circles represent the 95% confidence region for containing the true overall mean of the group, and the outer circles are the 50% contours. Rays with covariates names show the coordinate directions in the canonical space.

Compared to BSFL fed on FD substrate, FD + SSP larvae had a higher Mg BAF in spite of similar Mg concentrations in the feeding substrates (i.e. 2.6–28 g/kg DM), suggesting a greater bioaccumulation efficiency of Mg due to recyclate supplementation.

With a concentration range of 0.9–1.3 g/kg, larvae in the current study contained less Na than reported by Zulkifli et al. (2022) and Shumo et al. (2019) at 2–5 g/kg for BSFL fed various residues. The FD + SSP larvae had a higher Na concentration and Na yield than larvae in the FD + BCH and FD groups (Table 2; $P < 0.05$, Table 3; $P < 0.01$). The Na BAF showed that larvae fed on both FD + SSP or FD + BCH substrates had a lower efficiency for Na accumulation than when fed FD substrate only (Table 2; $P < 0.05$). Larvae of the FD + SSP group had the lowest BAF and retention for Na (Table 2; $P < 0.05$; Table 4; $P < 0.001$), which could be due to the higher Na content in the SSP recyclate (Table S1 in the supplementary data).

The BAF of K shows that this mineral was not accumulated (Table 2; BAF <1) in the larval body on any of the substrates. Nevertheless, FD + SSP larvae had a higher K BAF than those in the FD + BCH and FD groups ($P < 0.05$), suggesting that K was less depleted when larvae were fed on FD + SSP than on FD or FD + BCH substrates. No difference was found among the groups for K concentration, yield and retention ($P > 0.10$).

Overall, our results suggest differences in mineral accumulation patterns in BSFL that are strongly dependent on the source of minerals (SSP or BCH). The responsible mechanisms for mineral incorporation in BSFL are poorly understood (Seyedalmoosavi et al., 2022b), but it is possibly related to the chemical form, ionization and oxidation status of the mineral, and the mineral tolerance of the intestinal microbiota (Wu et al., 2021).

3.3. Heavy metal content, bioaccumulation, yield and retention

Transition-metal ions (iron, copper, manganese, and zinc) are important in various physiological processes in insects, including immunity and interactions with microbes (Hrdina and Iatsenko, 2022). However, little is known on mechanisms of metal metabolism in Hermetia illucens. Thus, we focus here on the accumulation of metal ions in BSFL due to its environmental relevance. The SSP recyclate had a lower heavy metal content (Mn, Fe, Zn, Cu, As, and Pb) than BCH, resulting in different mineral and heavy metal contents in the feeding substrates. The concentration of both Cd and Hg in SSR were lower than the lower limit of quantification of 0.02 mg/kg DM (Table S1 in the supplementary data).

The linear discriminant analysis of the heavy metal (Fig. 3) concentration showed a full separation of the three feeding substrates in their overall effects on heavy metal composition of BSFL. Copper and Fe were mainly responsible for the separation of groups along the second canonical axis, i.e. particularly separating FD and FD + SSP diets from each other. On the other hand, Pb and Mn had the highest association with the separation of groups on the first canonical axis, i.e. leading to differentiation of FD + BCH from the other two substrates in their overall effects on heavy metal concentration of BSFL (Fig. 3). Indeed, particularly the Pb content in the FD + BCH larvae (Table 2) was highest among the three substrates.

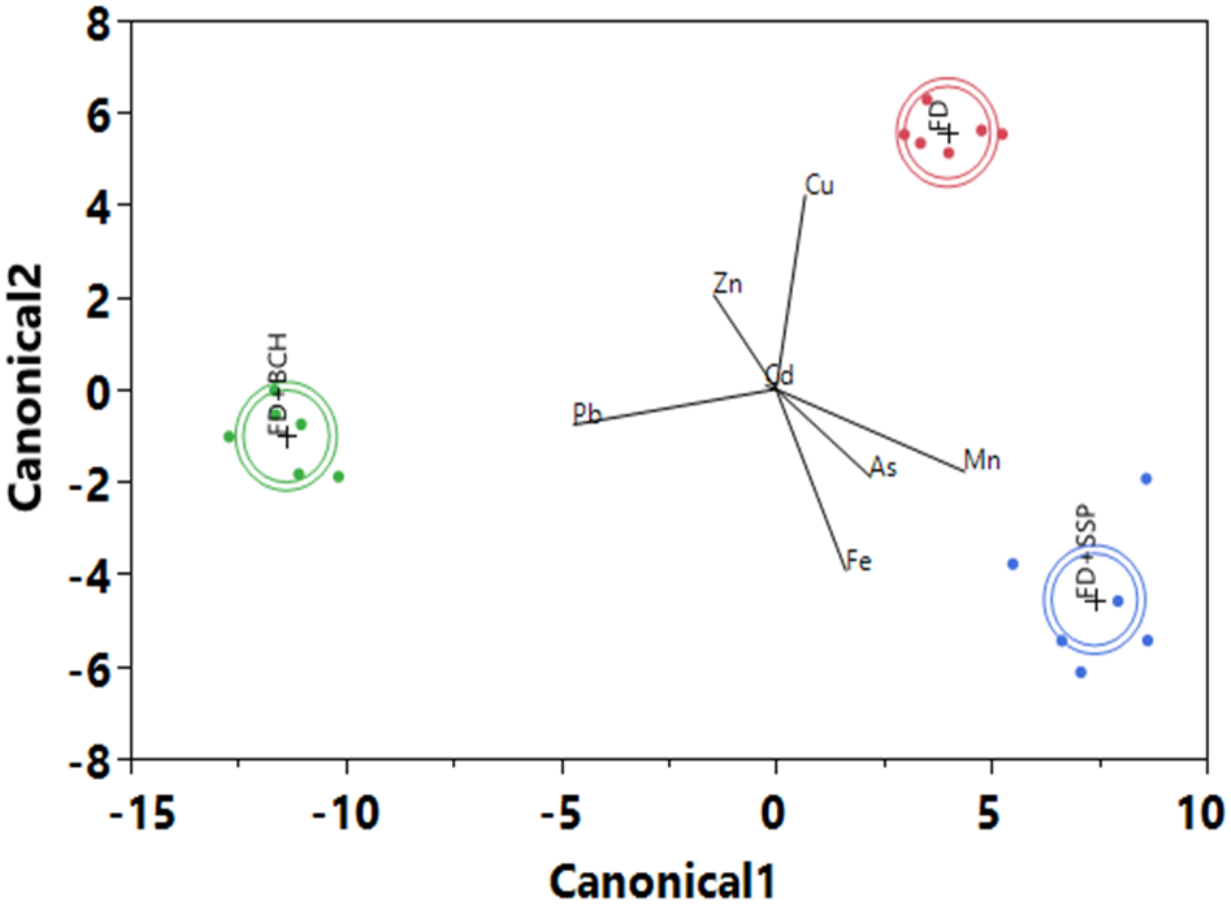


Figure 3. Canonical plot of points and means (+) from linear discriminant analysis of larval concentrations of the heavy metals manganese (Mn), iron (Fe), zinc (Zn), copper (Cu), arsenic (As), cadmium (Cd) and lead (Pb) with substrate groups defined as FD: modified Gainesville diet; FD + BCH: pyrolysis product of sewage sludge (BCH) added to FD (4%); FD + SSP: single superphosphate (SSP) added to FD (3.6%). All variables had the same units of measurement (i.e. mg/kg DM). Each dot represents the overall response of one container of BSFL fed on a specific substrate. The inner circles represent the 95% confidence region for containing the true overall

mean of the group, and the outer circles are the 50% contours. Rays with covariates names show the coordinate directions in the canonical space.

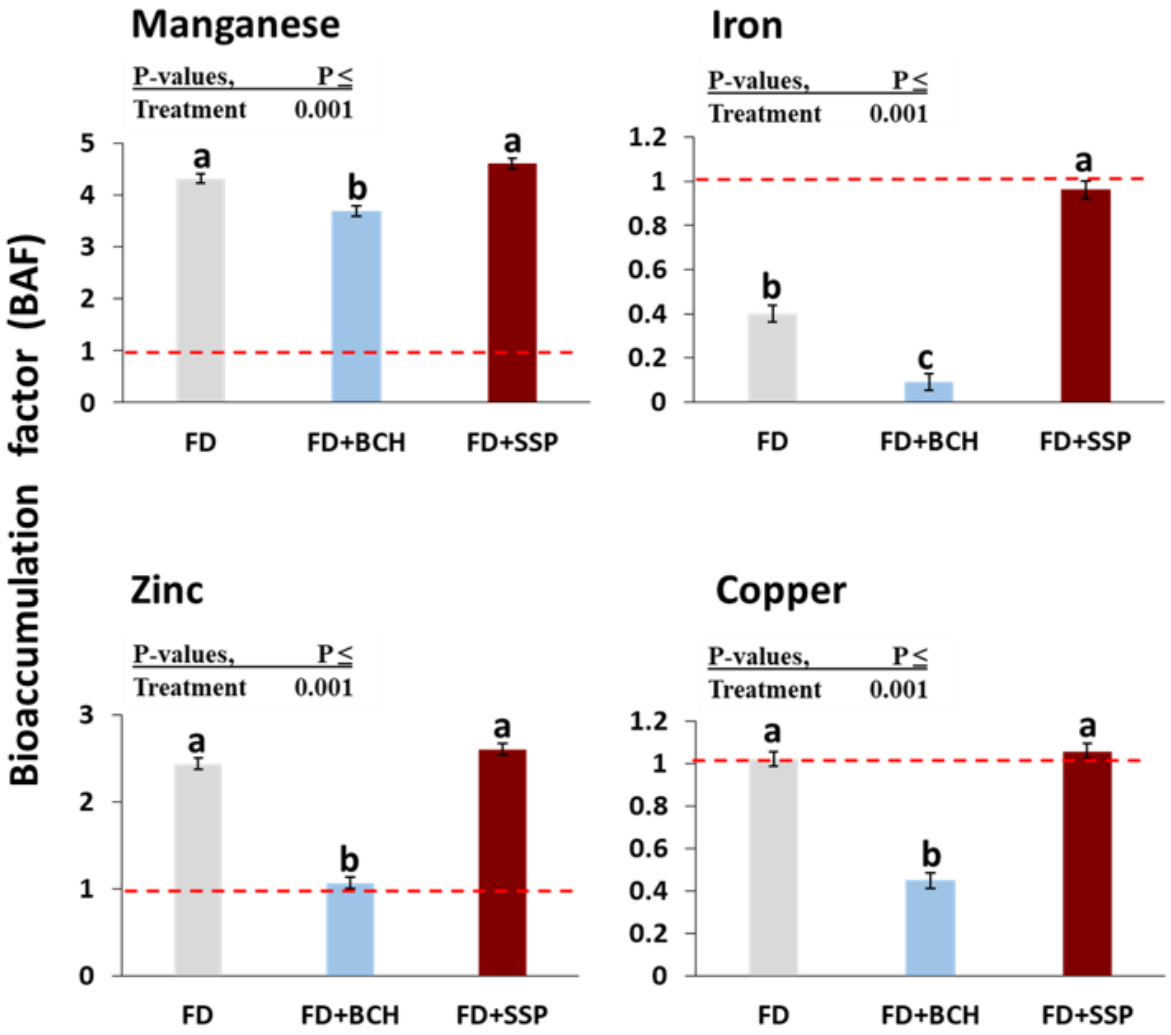


Fig. 4. Bioaccumulation factor (BAF) of manganese, iron, zinc and copper in black soldier fly (Hermetia illucens) larvae fed on a fly diet (FD) supplemented with or without two different sewage sludge recyclates (FD + BCH and FD + SSP). Values are LSM with their SE. a–c: Values denoted with different letters within each panel differ significantly (Tukey, $P < 0.05$). BAF >1 refers to accumulation of minerals in BSFL and BAF <1 refers to depletion of minerals from larval

body during the experimental period. FD: modified Gainesville diet; FD + BCH: pyrolysis product of sewage sludge (BCH) added to FD (4%); FD + SSP: single superphosphate (SSP) added to FD (3.6%). Total number of observations used for statistical analysis, N = 18 (i.e. n = 6 replicate rearing containers per group over 2 experiment runs).

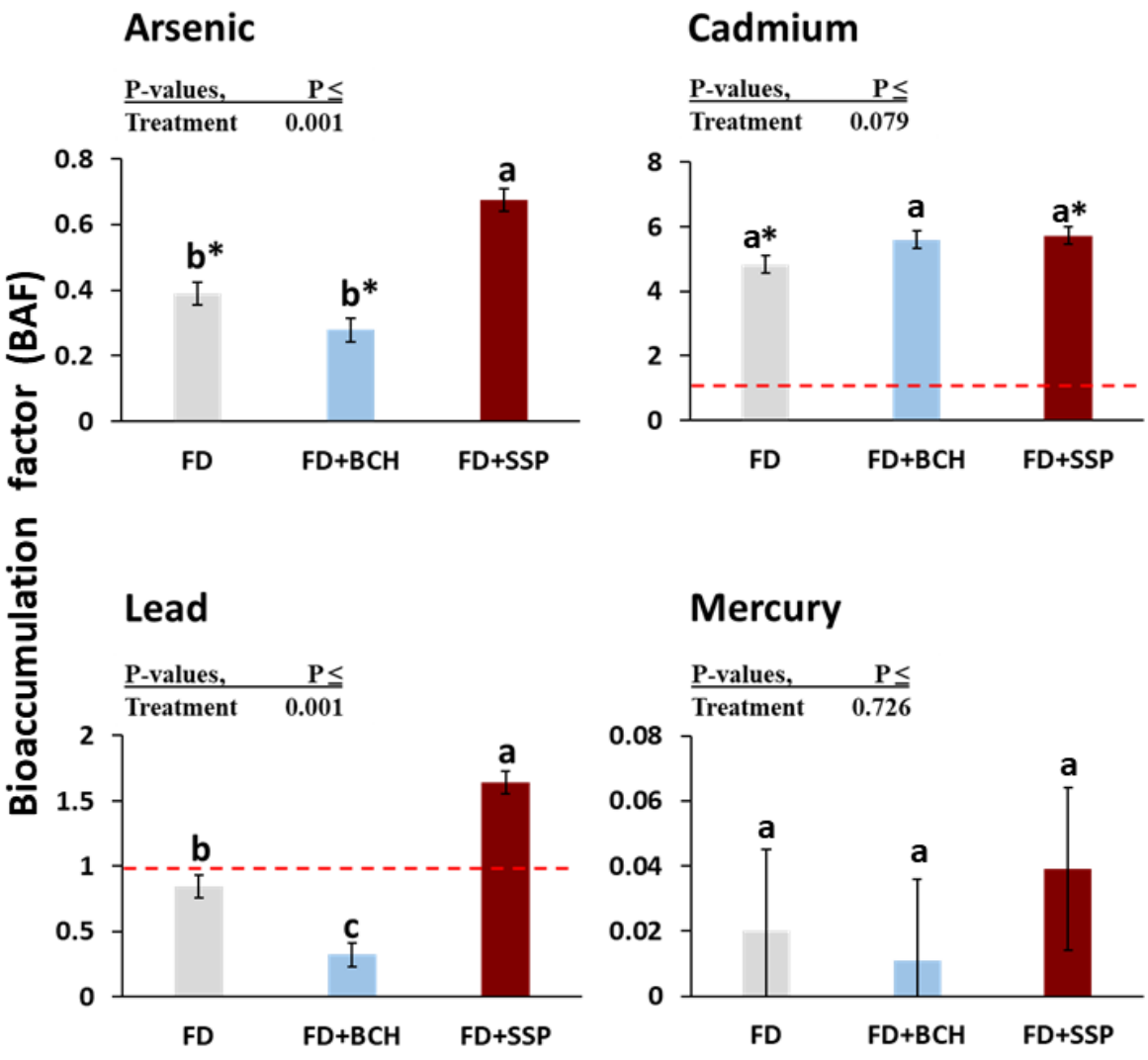


Fig. 5. Bioaccumulation factor (BAF) of arsenic, cadmium, lead and mercury in black soldier fly (Hermetia illucens) larvae fed on a fly diet (FD) supplemented with or without two different sewage sludge recyclates (FD + BCH and FD + SSP). Values are LSM with their SE. a–c: Values denoted with different letters within each panel differ significantly (Tukey, $P < 0.05$). The symbol * indicates a tendency of two treatments to differ (Tukey, $0.05 < P \leq 0.10$). BAF >1 refers to accumulation of minerals in BSFL and BAF <1 refers to depletion of minerals from larval body during the experimental period. FD: modified Gainesville diet; FD + BCH: pyrolysis product of sewage sludge (BCH) added to FD (4%); FD + SSP: single superphosphate (SSP) added to FD (3.6%). Total number of observations used for statistical analysis, N = 18 (i.e. n = 6 replicate rearing containers per group over 2 experiment runs).

In the current experiment, the range of larval Mn concentrations (285–304 mg/kg DM) did not differ among the groups (Table 2; $P < 0.05$), and was similar to the values reported by Spranghers et al. (2017) (220–380 mg/kg DM) in BSFL fed different substrates (chicken feed, digestate and vegetable waste). However, the larval Mn concentration observed, even in the group not supplemented by SSR, exceeded the maximal Mn concentration in complete feed legally prescribed by the EU Commission (150 mg/kg DM; Table S2 in the supplementary data) (European Commission, 2003, 2006). The Mn BAF value was lower in FD + BCH larvae than in those fed on FD + SSP and FD substrates (Fig. 4; $P < 0.05$), which was possibly linked to the high Mn concentration found in the FD + BCH feeding substrate (Table S1 in the supplementary data). The yield for larval Mn did not differ among the groups (Table 3; $P < 0.05$), whereas retention of Mn was lower in FD + BCH larvae than of those in the FD group (Table 4; $P < 0.05$). Carbon-

coated minerals on the surface of BCH effectively reduce the bioavailability of heavy metals in soil (Joseph et al., 2021). Moreover, mineral bioavailability in BCH depends on several factors such as pH of the environment, feedstock source and pyrolytic temperature (Ding et al., 2016). In addition, bioavailability of minerals and heavy metals for BSFL may depend on the pH in the substrate and their gastrointestinal tract. The pH (acidic to alkaline) values vary in different intestiBonelli et al., 2019nal compartments of BSFL () which may affect mineral uptake. Therefore, although the concentration of certain minerals such as Mn was higher in BCH than in the SSP recyclate, it does not mean that all of it was bioavailable to the larvae.

Insects such as Drosophila melanogaster are highly sensitive to excessive Mn concentrations, which negatively affect feeding behaviour and brain functions (Ben-Shahar, 2018; Søvik et al., 2017). This is comparable to neurotoxic effects of Mn in certain animal models and humans (Ben-Shahar, 2018), as discussed by Bessa et al. (2021). Therefore, when BSFL should be used as food or feed, care must be taken, not to exceed the recommendations for Mn in human food or animal feed (Broom et al., 2021; DGE, 2021).

The iron concentration was higher in FD + BCH larvae than those fed FD (Table 2; $P < 0.05$) which is linked to the exceedingly high Fe concentrations in the BCH recyclate (328 times higher than in SSP) and consequently also in the respective feeding substrate (Table S1 in the supplementary data). Larval Fe concentration in the present study was comparable to BSFL fed on chicken manure and brewer's spent grain (Shumo et al., 2019). The iron content in BSFL fed BCH supplemented substrates, along with Mn concentration, was the highest heavy metal content of all elements measured. Interestingly, the BAF results show that BSFL did not accumulate Fe from

any of the feeding substrates (i.e. BAF <1; Fig. 4). However, FD + SSP larvae had the highest BAF for Fe, which was several times higher than in FD + BCH and FD larvae, respectively (Fig. 4; $P < 0.05$). The lowest BAF in FD + BCH larvae suggests depletion of Fe in BSFL to prevent excessively high and perhaps toxic Fe concentrations. As a function of Fe concentration and larval mass, the Fe yield was higher in FD + BCH larvae than in those of the FD group (Table 3; $P < 0.05$), while the Fe retention was lowest in FD + BCH larvae (Table 4; $P < 0.05$). Knowledge regarding the Fe homeostasis in BSFL is limited (Gorman, 2023). For Drosophila melanogaster, it was discussed that non-heme Fe absorption and storage is linked to ferritin and the (acidic) pH in the midgut, which is known as the 'iron region' (Gorman, 2023; Mandilaras et al., 2013). In a recent study on BSFL, Bonelli et al. (2020) noted that parts of the midgut appear to be the main site for Fe absorption and storage as highest level of ferritin RNA transcripts was observed in the anterior and posterior midgut.

The reported Zn content in BSFL was 108 mg/kg DM (Makkar et al., 2014), which is within the range of values observed in our study (96–107 mg/kg DM). The Zn concentration in FD + BCH larvae tended to be higher than those in the FD group (Table 2; $P = 0.087$). Moreover, BAF results showed that Zn accumulated in all substrate groups (BAF >1). However, the Zn BAF was lower in FD + BCH larvae than those in FD + SSP and FD (Fig. 4; $P < 0.05$). Similar to our results, bio-accumulative potential for Cu and Zn in BSFL was also reported (Proc et al., 2020). However, in contrast, Wu et al. (2021) found a Zn BAF for BSFL of 0.80, suggesting it was not accumulated in larvae. This inconsistency for Zn BAF among the studies might be associated with different Zn levels and forms in the feeding substrates (Wu et al., 2021). The Zn yield was not different among

dietary groups (Table 3; $P > 0.10$), and Zn retention was lower in FD + BCH larvae compared with that in FD + SSP and FD larvae (Table 4; $P < 0.05$). Zinc is necessary for the reproduction of insects such as Drosophila melanogaster, however, high Zn concentration can disturb protein function (Cardoso-Jaime et al., 2022).

Larval Cu concentrations observed (10–12 mg/kg DM) were comparable to those previously reported (Ferrari et al., 2022) (13 mg/kg DM) for BSFL fed Gainesville diet. The Cu concentration tended to be higher in FD + BCH larvae compared to those fed on FD (Table 2; $P = 0.052$). Copper BAF was approximately 1 in FD and FD + SSP, whereas it was not accumulated in FD + BCH (BAF <1) (Fig. 4; $P < 0.05$) presumably as a result of the high Cu-level in the feeding substrate (Table S1 in the supplementary data). A comparably low Cu BAF of 0.69 was interpreted as a self-protection mechanism (e.g. Cu excretion) in larvae exposed to a high Cu concentration in the feeding substrate (Wu et al., 2020). The Cu yield tended to be higher in FD + BCH compared to the FD + SSP larvae (Table 3; $P = 0.066$). Moreover, FD + BCH larvae had a lower Cu retention than those larvae fed on FD and FD + SSP substrates (Table 4; $P < 0.05$).

In the present study, the larval concentration of As ranged from 0.05 to 0.12 mg/kg DM (Table 2) which was lower than reported by Ferrari et al. (2022) for BSFL fed Gainesville diet (0.28 mg/kg). Arsenic concentration in FD + SSP larvae was higher than in FD + BCH and FD larvae (Table 2). The As BAF indicates that As was not accumulated in the larval body (BAF <1) of any substrate group (Fig. 5), while larvae fed FD + SSP substrate showed a relatively higher As BAF compared to FD + BCH and FD larvae ($P < 0.05$). Similar to our results, Van der Fels-Klerx et al. (2016) and Van der Fels-Klerx et al. (2018) observed that As was not accumulated in BSFL and

rather the majority of consumed As was not taken up (BAF <1). Moreover, FD + SSP larvae had a higher As yield and retention than those in FD + BCH and FD (Table 3, Table 4 respectively; $P < 0.05$).

The concentration of Cd in BSFL grown on Gainesville diet reported (0.45 mg/kg) (Ferrari et al., 2022) was somewhat lower than observed in the current study (0.63–0.74 mg/kg DM). Cadmium concentration was higher in FD + BCH than in FD larvae (Table 2; $P < 0.05$). Since the maximal Cd concentration in complete feed legally prescribed by the EU Commission is 0.5 mg/kg DM (European Commission, 2002) (Table S2 in the supplementary data), the larval Cd contents observed are above this limit. It is of note however, that the SSR supplementation did not contribute much to the larval accumulation because the Cd content in larvae fed on the control substrate FD was already above the authorized limit suggesting that BSFL are prone to Cd accumulation. This confirms earlier observations of a Cd BAF of 6–8 for control diets and 6 to 10 when the substrates were spiked with extra Cd at 0.5–2 fold the authorized limit for Cd content in total feed (Van der Fels-Klerx et al., 2016). Cadmium had the highest BAF among all measured heavy metals with a tendency for a higher BAF in FD + SSP compared to the FD larvae (Fig. 5; $P = 0.080$). Cadmium yield and retention did not differ among the substrate groups (Table 3, Table 4 respectively; $P > 0.10$). It is known that BSFL accumulate high amounts of Cd (Proc et al., 2020), which is highly toxic and may constitute serious health risks for animal and humans (Truzzi et al., 2019). The Cd toxicity is dependent on oxidation level and methylation grade (Bolan et al., 2014; Egorova and Ananikov, 2017), which was not analysed in our study. In general, little information is available on transport mechanisms of minerals in BSFL. However, it has been suggested that

high BAF for Cd in BSFL could be attributed to the large number of Ca2+ channels in their gut, which facilitate the Cd transport by means of heat shock proteins, resulting in high Cd accumulation compared to other heavy metals (Bessa et al., 2021; Van der Fels-Klerx et al., 2016).

Ferrari et al. (2022) reported that Pb concentration in BSFL fed Gainesville diet was 0.33 mg/kg which is similar to the Pb content of FD larvae in the present study. The FD + BCH larvae had the highest Pb concentration among the substrate groups, followed by larvae fed FD + SSP (Table 2; $P < 0.05$). Moreover, BAF showed that Pb was only accumulated in FD + SSP larvae (Fig. 5; BAF >1), while Pb was not stored in larvae of the FD + BCH and FD groups. Nevertheless, the yield for Pb in FD + BCH larvae was almost 5 times higher than that of FD larvae, whereas the Pb yield of the FD + SSP group was twice of that found in FD (Table 3; $P < 0.05$). The Pb retention resulted in the highest and lowest values in the FD + SSP and FD + BCH groups, respectively (Table 4; $P < 0.05$). The lack of Pb accumulation in the FD + BCH larvae despite its high concentration in the FD + BCH substrate (11 times higher in FD + BCH than in FD + SSP) could be explained by physico-chemical properties of BCH which may affect Pb release (Yang et al., 2018). It has been suggested that the exoskeleton of the BSFL is the storage site for Pb (Diener et al., 2015; Van der Fels-Klerx et al., 2016). Therefore, during processing of larvae, the Pb content in the final larval meal product could be reduced.

The concentration of Hg in BSFL was below the limit of quantification, which is in line with a low Hg content in BSFL fed Gainesville diet (0.007 mg/kg) as reported (Ferrari et al., 2022). In addition, the BAF result for Hg showed no accumulation (Fig. 5; $P > 0.10$).

Recently, the use of processed animal protein derived from insects to partly replace soybean meal and fishmeal to feed aquaculture animals, pigs, and poultry has been authorized by the European Commission (2021). However, nutrient recycling by using organic residues, which are currently defined as waste, still poses health risk that need to be averted for the future (Salemdeeb et al., 2017). Our data show that Fe, Zn, Cu, As, Pb, and Hg contents in BSFL did not exceed the currently EU authorized maximum concentration in complete feed (Table S2 in the supplementary data). However, the Mn and Cd contents of BSFL were above the authorized maximum concentration (Table S2 in the supplementary data).

There are potential limitations to our study. Since BSFL guts were not fully emptied by starvation at harvest, the results obtained on mineral and heavy metal concentrations in BSFL bodies might have been affected to a certain degree by gut filling. However, gut filling does not necessarily imply an elevated concentration of minerals in BSFL. As BAF indicated clear accumulation of certain minerals in BSFL, potential substrate residues in the gut content might have even diluted the true accumulation levels at least for elements shown to have high recovery (e.g. Ca, Mn and Cd). Nevertheless, since all BSFL groups were treated in the same way, potential gut filling effects may be considered negligible.

3.4. Nutrients, minerals and heavy metal composition of frass

Crude fibre, aNDFom, and ADFom contents in frass of FD + SSP were lower than those in the FD group (Table S3 in the supplementary data; $P < 0.05$), which might suggest an increased fibre degradation by FD + SSP larvae or microbiota and fungi in the substrate. Although in the current study the fibre degrading characteristics were not determined in the BSFL, fibre degrading

enzymes such as ligninases and cellulases are known to be present in the larval gut but their pattern depends on the microbiota in the substrate and the substrate composition (Müller et al., 2017). It has been suggested that cellulase activity produced by the fungi Rhizopus oryzae isolated from soil was inhibited by heavy metals such as Cu, Zn, Co or Pb (Murashima et al., 2002), but it remains to be determined if the heavy metal concentration pattern in the BSFL feeding substrate affects the activity of fibre degrading enzymes in bacteria and fungi. Frass of both FD + SSP and FD + BCH groups had a higher Ca, P and Na concentration than FD frass (Table S3 in the supplementary data; $P < 0.05$) and among the recyclate supplemented groups, FD + SSP had the highest Ca, P and Na concentration ($P < 0.05$). Moreover, FD + BCH had the highest Mn, Fe, Zn, Cu, As, Cd, and Pb content in frass among the groups ($P < 0.05$). However, heavy metal content in frass of all groups was below the currently EU authorized maximum concentration for organic fertilizers (Table S4 in the supplementary data) (European Commission, 2019). Frass of BSFL has been suggested as a sustainable and environmentally safe fertilizer (Beesigamukama et al., 2020). In summary, according to our results, frass of FD + SSP contains higher amounts of CP, ash and macro-minerals while heavy metal content was of the same magnitude as in FD frass which suggest that FD + SSP frass might be a better fertilizer.

5. Conclusions

This study provides information on the impact of using sewage sludge recyclates as a mineral-rich supplement to BSFL substrates with the aim of testing the reintegration of minerals from currently legally prohibited wastes and residues into the nutrient cycle. Dietary inclusion of SSP recyclate negatively affected the growth performance of BSFL. The concentrations of the minerals

found in the larvae confirm that the micronutrient profile of BSFL depends on the initial mineral concentrations in the substrate. Both BCH and SSP supplements increased Ca content in BSFL, whereas only SSP increased P content. Inclusion of SSP was associated with lower heavy metal content in larvae and frass as compared to BCH which reflects the lower concentrations of heavy metals in SSP due to the technological reduction process used. Nevertheless, BSFL enriched with minerals from both SSR containing substrates (i.e. SSP, BCH) do not exceed the current EU authorized maximum concentration in complete feed for Fe, Zn, Cu, As, and Hg. However, the EU thresholds for Mn and Cd concentrations are exceeded even for BSFL reared on the control feeding substrate FD without SSR supplementation. Because of the toxicity of excessive Mn and Cd in feed or food, limits on the BSFL incorporation level in animal feed need to be established depending on the SSR and BSFL processing technology. Apart from nutrient recycling and legal considerations in agriculture, supplementation of larval substrate with SSP is not economical and should be used directly as fertilizer. However, lower quality and less expensive SSR processed in an agricultural context could be further considered for a potential re-integration in agri-food nutrient cycles, provided that the heavy metal concentration is already reduced during wastewater treatment.

Credit authorstatement

MMS: data collection, formal analysis, writing original draft, writing-review and editing; MM: data collection, methodology, writing-review and editing; KS: formal analysis; SG: formal analysis; PW: supervision, funding acquisition; JT: Resources; LH: formal analysis; SD: writing-review and editing; GD: conceptualization, formal analysis, methodology, writing-review and

editing; CCM: conceptualization, funding acquisition, supervision, methodology, writing-review and editing. All authors read the article and approved the submitted version.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Prof. Dr. Cornelia C. Metges reports financial support was provided by Leibniz ScienceCampus Phosphorus Research Rostock.

Acknowledgements

This work is an outcome of a sub-project of the Leibniz ScienceCampus Phosphorus Research Rostock, and the first author was partly funded by it. Matching institutional funding of FBN is acknowledged. The authors are grateful to K. Pilz, A. Schulz, B. Mielenz, K. Grot, M. Statz, K. Wilke, R. Gaeth and S. Dwars for their assistance with larval and fly handling, laboratory analyses, and sample collection. We thank the FBN for open access funding and Hermetia Baruth GmbH for their cooperation in setting up a black soldier fly colony. The graphical abstract and the illustration of the experimental design (Fig. 1) were created by BioRender.

Data availability

Data will be made available on request.

References

Algren, M., Costello, C., Landis, A.E., 2022. Phosphorus (P) in animal diets as a driver of embodied P in animal products and net anthropogenic P inputs. J. Ind. Ecol. DOI: https://doi.org/10.1111/jiec.13251.

Arabzadeh, G., Delisle-Houde, M., Tweddell, R.J., Deschamps, M.-H., Dorais, M., Lebeuf, Y., Derome, N., Vandenberg, G., 2022. Diet Composition Influences Growth Performance, Bioconversion of Black Soldier Fly Larvae: Agronomic Value and In Vitro Biofungicidal Activity of Derived Frass. Agronomy 12, 1765. DOI: https://doi.org/10.3390/agronomy12081765.

Arnone, S., De Mei, M., Petrazzuolo, F., Musmeci, S., Tonelli, L., Salvicchi, A., Defilippo, F., Curatolo, M., Bonilauri, P., 2022. Black soldier fly (Hermetia illucens L.) as a high-potential agent for bioconversion of municipal primary sewage sludge. Environ. Sci. Pollut. Res., 1-16. DOI: https://doi.org/10.1007/s11356-022-20250-w.

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

Beesigamukama, D., Mochoge, B., Korir, N.K., Fiaboe, K.K., Nakimbugwe, D., Khamis, F.M., Subramanian, S., Dubois, T., Musyoka, M.W., Ekesi, S., 2020. Exploring black soldier fly frass as novel fertilizer for improved growth, yield, and nitrogen use efficiency of maize under field conditions. Front. Plant Sci. 11, 574592. DOI: https://doi.org/10.3389/fpls.2020.574592.

Ben-Shahar, Y., 2018. The impact of environmental Mn exposure on insect biology. Front. Genet. 9, 70. DOI: https://doi.org/10.3389/fgene.2018.00070.

Bessa, L.W., Pieterse, E., Marais, J., Dhanani, K., Hoffman, L.C., 2021. Food Safety of Consuming Black Soldier Fly (Hermetia illucens) Larvae: Microbial, Heavy Metal and Cross-Reactive Allergen Risks. Foods 10, 1934. DOI: https://doi.org/10.3390/foods10081934.

Bolan, N., Kunhikrishnan, A., Thangarajan, R., Kumpiene, J., Park, J., Makino, T., Kirkham, M.B., Scheckel, K., 2014. Remediation of heavy metal (loid) s contaminated soils–to mobilize or to immobilize? J. Hazard. Mater. 266, 141-166. DOI: https://doi.org/10.1016/j.jhazmat.2013.12.018.

Bonelli, M., Bruno, D., Brilli, M., Gianfranceschi, N., Tian, L., Tettamanti, G., Caccia, S., Casartelli, M., 2020. Black soldier fly larvae adapt to different food substrates through morphological and functional responses of the midgut. Int. J. Mol. Sci. 21, 4955. DOI: https://doi.org/10.3390/ijms21144955.

Broeckx, L., Frooninckx, L., Slegers, L., Berrens, S., Noyens, I., Goossens, S., Verheyen, G., Wuyts, A., Van Miert, S., 2021. Growth of Black Soldier Fly Larvae Reared on Organic Side-Streams. Sustainability 13, 12953. DOI: https://doi.org/10.3390/su132312953.

Broom, L.J., Monteiro, A., Piñon, A., 2021. Recent advances in understanding the influence of zinc, copper, and manganese on the gastrointestinal environment of pigs and poultry. Animals 11, 1276. DOI: https://doi.org/10.3390/ani11051276.

Browne, A., O'Donnell, M.J., 2016. Segment-specific Ca2+ transport by isolated Malpighian tubules of Drosophila melanogaster: A comparison of larval and adult stages. J. Insect Physiol. 87, 1-11. DOI: https://doi.org/10.1016/j.jinsphys.2016.01.005.

Buss, W., Wurzer, C., Manning, D.A., Rohling, E.J., Borevitz, J., Mašek, O., 2022. Mineral-enriched biochar delivers enhanced nutrient recovery and carbon dioxide removal. Commun. Earth Environ. 3, 1-11. DOI: https://doi.org/10.1038/s43247-022-00394-w.

Cardoso-Jaime, V., Broderick, N.A., Maya-Maldonado, K., 2022. Metal ions in insect reproduction: a crosstalk between reproductive physiology and immunity. Curr. Opin. Insect. Sci., 100924. DOI: https://doi.org/10.1016/j.cois.2022.100924.

Chia, S.Y., Tanga, C.M., Khamis, F.M., Mohamed, S.A., Salifu, D., Sevgan, S., Fiaboe, K.K., Niassy, S., van Loon, J.J., Dicke, M., 2018. Threshold temperatures and thermal requirements of black soldier fly Hermetia illucens: Implications for mass production. PloS one 13, e0206097. DOI: https://doi.org/10.1371/journal.pone.0206097.

Chia, S.Y., Tanga, C.M., Osuga, I.M., Cheseto, X., Ekesi, S., Dicke, M., van Loon, J.J., 2020. Nutritional composition of black soldier fly larvae feeding on agro-industrial by-products. Entomologia Experimentalis et Applicata 168, 472-481. DOI: https://doi.org/10.1111/eea.12940.

Cohen, Y., Enfält, P., Kabbe, C., 2019. Production of clean phosphorus products from sewage sludge ash using the Ash2Phos process, Proceedings of the International Fertiliser Society. International Fertiliser Society, Brussels, Belgium, pp. 1-19.

Daş, G., Seyedalmoosavi, M.M., Schleifer, K., Mielenz, M., Metges, C.C., 2023. The validity of the bioaccumulation index versus the bioaccumulation factor for assessment of element accumulation in black soldier fly larvae. J. Insects Food Feed 0, 1 – 16. DOI: https://doi.org/10.3920/JIFF2023.0021.

Dawar, K., Fahad, S., Jahangir, M. M. R., Munir, I., Alam, S. S., Khan, S. A., Mian, I. A. Datta, R., Saud, S., Banout, J., Adnan, M., Ahmad, M. N., Khan, A., Dewil, R., Habib-ur-Rahman, M., Ansari, M. J., Danish, S., 2021. Biochar and urease inhibitor mitigate NH3 and N2O emissions and improve wheat yield in a urea fertilized alkaline soil. Sci. Rep. 11, 17413. DOI: https://doi.org/10.1038/s41598-021-96771-0.

DGE, 2000. Deutsche Gesellschaft für Ernährung e. V.

Diener, S., Zurbrügg, C., Tockner, K., 2015. Bioaccumulation of heavy metals in the black soldier fly, Hermetia illucens and effects on its life cycle. J. Insects Food Feed 1, 261-270. DOI: https://doi.org/10.3920/JIFF2015.0030.

Ding, Y., Liu, Y., Liu, S., Li, Z., Tan, X., Huang, X., Zeng, G., Zhou, L., Zheng, B., 2016. Biochar to improve soil fertility. A review. Agron Sustain Dev 36, 1-18. DOI: https://doi.org/10.1007/s13593-016-0372-z.

Dortmans, B., Diener, S., Bart, V., Zurbrügg, C., 2017. Black soldier fly biowaste processing: a step-by-step guide. eawag.Egle, L., Rechberger, H., Krampe, J., Zessner, M., 2016. Phosphorus recovery from municipal wastewater: An integrated comparative technological, environmental and economic assessment of P recovery technologies. Sci. Total Environ. 571, 522-542. DOI: https://doi.org/10.1016/j.scitotenv.2016.07.019.

Egorova, K.S., Ananikov, V.P., 2017. Toxicity of metal compounds: knowledge and myths. Organometallics 36, 4071-4090. DOI: https://doi.org/10.1021/acs.organomet.7b00605.
Emre, I., Kayis, T., Coskun, M., Dursun, O., Cogun, H.Y., 2013. Changes in antioxidative enzyme activity, glycogen, lipid, protein, and malondialdehyde content in cadmium-treated Galleria mellonella larvae. Annals of the Entomological Society of America 106, 371-377.
European Commission, 2002. Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. Official Journal of the European Communities 140, 10–22.
European Commission, 2003. Commission regulation (EC) No 1334/2003 of 25 July 2003 amending the conditions for authorisation of a number of additives in feedingstuffs belonging to the group of trace elements. Official Journal of the European Union 187, 11.
European Commission, 2006. Commission regulation (EC) No 479/2006 of 23 March 2006 as regards the authorisation of certain additives belonging to the group compounds of trace elements. Official Journal of the European Union 86, 4.
European Commission, 2009. Regulation (EC) No 767/2009 of the European Parliament and of the Council of 13 July 2009 on the placing on the market and use of feed, amending European Parliament and Council Regulation (EC) No 1831/2003 and repealing Council Directive 79/373/EEC, commission directive 80/511/EEC, council directives 82/471/EEC, 93/74/EEC, 93/113/ EC and 96/25/EC and commission decision. Official Journal of the European Union 229, 1-28.
European Commission, 2019. Regulation (EU) 2019/1009 of the European parliament and of the council of 5 June 2019 laying down rules on the making available on the market of EU fertilising products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003. Official Journal of the European Union, 25.6.2019.
European Commission, 2021. Commission-regulation (EU) 2021/1372 of 17 August 2021 amending Annex IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards the prohibition to feed non-ruminant farmed animals, other than fur animals, with protein derived from animals. Official Journal of the European Union 295, 1–17.
Ferrari, L., Sele, V., Silva, M., Bonilauri, P., De Filippo, F., Selmin, F., Ørnsrud, R., Pinotti, L., Ottoboni, M., 2022. Biofortification of selenium in black soldier fly (Hermetia illucens) prepupae reared on seaweed or selenium enriched substrates. J. Insects Food Feed, 1-14. DOI: https://doi.org/10.3920/JIFF2021.0153.
Fesharaki, M., Rath, W., 2018. Verkohlungsanlage Pyreg. Europäisches Patentamt, Patentnummer: EP 3 358 253 A1.
Finke, M.D., 2007. Estimate of chitin in raw whole insects. Zoo biology: published in affiliation with the American zoo and aquarium association 26, 105-115. DOI: https://doi.org/10.1002/zoo.20123.
Finke, M.D., 2013. Complete nutrient content of four species of feeder insects. Zoo Biol. 32, 27-36. DOI: https://doi.org/10.1002/zoo.21012.
Fountain, M.T., Hopkin, S.P., 2001. Continuous monitoring of Folsomia candida (Insecta: Collembola) in a metal exposure test. Ecotoxicology and Environmental Safety 48, 275-286.

Gorman, M.J., 2023. Iron Homeostasis in Insects. Annu. Rev. Entomol. 68, 51-67. DOI: https://doi.org/10.1146/annurev-ento-040622-092836.
Hogsette, J.A., 1992. New diets for production of house flies and stable flies (Diptera: Muscidae) in the laboratory. J. Econ. Entomol. 85, 2291-2294. DOI: https://doi.org/10.1093/jee/85.6.2291.
Hrdina, A., Iatsenko, I., 2022. The roles of metals in insect–microbe interactions and immunity. Curr. Opin. Insect. Sci. 49, 71-77. DOI: https://doi.org/10.1016/j.cois.2021.12.004.
Hunter, M.C., Smith, R.G., Schipanski, M.E., Atwood, L.W., Mortensen, D.A., 2017. Agriculture in 2050: recalibrating targets for sustainable intensification. Bioscience 67, 386-391. DOI: https://doi.org/10.1093/biosci/bix010.
Joseph, S., Cowie, A.L., Van Zwieten, L., Bolan, N., Budai, A., Buss, W., Cayuela, M.L., Graber, E.R., Ippolito, J.A., Kuzyakov, Y., 2021. How biochar works, and when it doesn't: A review of mechanisms controlling soil and plant responses to biochar. GCB Bioenergy 13, 1731-1764. DOI: https://doi.org/10.1111/gcbb.12885.
Lievens, S., Poma, G., De Smet, J., Van Campenhout, L., Covaci, A., Van Der Borght, M., 2021. Chemical safety of black soldier fly larvae (Hermetia illucens), knowledge gaps and recommendations for future research: A critical review. J. Insects Food Feed 7, 383-396. DOI: https://doi.org/10.3920/JIFF2020.0081.
Liland, N.S., Biancarosa, I., Araujo, P., Biemans, D., Bruckner, C.G., Waagbø, R., Torstensen, B.E., Lock, E.-J., 2017. Modulation of nutrient composition of black soldier fly (Hermetia illucens) larvae by feeding seaweed-enriched media. PloS one 12, e0183188. DOI: https://doi.org/10.1371/journal.pone.0183188.
Lugert, V., Thaller, G., Tetens, J., Schulz, C., Krieter, J., 2016. A review on fish growth calculation: multiple functions in fish production and their specific application. Rev. Aquac. 8, 30-42. DOI: https://doi.org/10.1111/raq.12071.
Makkar, H.P., Tran, G., Heuzé, V., Ankers, P., 2014. State-of-the-art on use of insects as animal feed. Anim. Feed Sci. Technol. 197, 1-33. DOI: https://doi.org/10.1016/j.anifeedsci.2014.07.008.
Mandilaras, K., Pathmanathan, T., Missirlis, F., 2013. Iron absorption in Drosophila melanogaster. Nutrients 5, 1622-1647. DOI: https://doi.org/10.3390/nu5051622.
Matsumoto, M., Nishimura, T., 1998. Mersenne twister: a 623-dimensionally equidistributed uniform pseudo-random number generator. ACM Trans. Model. Comput. Simul. 8, 3-30. DOI: https://doi.org/10.1145/272991.272995.
Miranda, C.D., Cammack, J.A., Tomberlin, J.K., 2020. Mass production of the black soldier fly, Hermetia illucens (L.),(Diptera: Stratiomyidae) reared on three manure types. Animals 10, 1243. DOI: https://doi.org/10.3390/ani10071243.
Müller, A., Wolf, D., Gutzeit, H.O., 2017. The black soldier fly, Hermetia illucens–a promising source for sustainable production of proteins, lipids and bioactive substances. Zeitschrift für Naturforschung C 72, 351-363. DOI: https://doi.org/10.1515/znc-2017-0030.
Murashima, K., Nishimura, T., Nakamura, Y., Koga, J., Moriya, T., Sumida, N., Yaguchi, T., Kono, T., 2002. Purification and characterization of new endo-1, 4-β-D-glucanases from

Rhizopus oryzae. Enzyme Microb. Technol. 30, 319-326. DOI: https://doi.org/10.1016/S0141-0229(01)00513-0.
Naumann, C., Bassler, R., Seibold, R., Barth, K., 1997. VDLUFA-Methodenbuch Band III, Die chemische Untersuchung von Futtermitteln. VDLUFA-Verlag, Darmstadt, Germany.
NRC, 1994. National Research Council (NRC). Nutrient Requirements of Poultry: ninth revised edition.
Pliantiangtam, N., Chundang, P., Kovitvadhi, A., 2021. Growth performance, waste reduction efficiency and nutritional composition of black soldier fly (Hermetia illucens) larvae and prepupae reared on coconut endosperm and soybean curd residue with or without supplementation. Insects 12, 682. DOI: https://doi.org/10.3390/insects12080682.
Proc, K., Bulak, P., Wiącek, D., Bieganowski, A., 2020. Hermetia illucens exhibits bioaccumulative potential for 15 different elements–Implications for feed and food production. Sci. Total Environ. 723, 138125. DOI: https://doi.org/10.1016/j.scitotenv.2020.138125.
Raman, S.S., Stringer, L.C., Bruce, N.C., Chong, C.S., 2022. Opportunities, challenges and solutions for black soldier fly larvae-based animal feed production. J. Clean. Prod., 133802. DOI: https://doi.org/10.1016/j.jclepro.2022.133802.
Rose, E., Lee, D., Xiao, E., Zhao, W., Wee, M., Cohen, J., Bergwitz, C., 2019. Endocrine regulation of MFS2 by branchless controls phosphate excretion and stone formation in Drosophila renal tubules. Sci. Rep. 9, 1-12. DOI: https://doi.org/10.1038/s41598-019-45269-x.
Salemdeeb, R., Zu Ermgassen, E.K., Kim, M.H., Balmford, A., Al-Tabbaa, A., 2017. Environmental and health impacts of using food waste as animal feed: a comparative analysis of food waste management options. J. Clean. Prod. 140, 871-880. DOI: https://doi.org/10.1016/j.jclepro.2016.05.049.
Sandrock, C., Leupi, S., Wohlfahrt, J., Kaya, C., Heuel, M., Terranova, M., Blanckenhorn, W.U., Windisch, W., Kreuzer, M., Leiber, F., 2022. Genotype-by-diet interactions for larval performance and body composition traits in the black soldier fly, Hermetia illucens. Insects 13, 424. DOI: https://doi.org/10.3390/insects13050424.
Schmitt, E., Belghit, I., Johansen, J., Leushuis, R., Lock, E.-J., Melsen, D., Kathirampatti Ramasamy Shanmugam, R., Van Loon, J., Paul, A., 2019. Growth and safety assessment of feed streams for black soldier fly larvae: a case study with aquaculture sludge. Animals 9, 189. DOI: https://doi.org/10.3390/ani9040189.
Seyedalmoosavi, M., Dannenberger, D., Pfuhl, R., Görs, S., Mielenz, M., Maak, S., Wolf, P., Daş, G., Metges, C., 2022a. Lipid metabolism, fatty acid composition and meat quality in broilers supplemented with increasing levels of defrosted black soldier fly larvae. J. Insects Food Feed, 1-16. DOI: https://doi.org/10.3920/JIFF2022.0125.
Seyedalmoosavi, M.M., Mielenz, M., Veldkamp, T., Daş, G., Metges, C.C., 2022b. Growth efficiency, intestinal biology, and nutrient utilization and requirements of black soldier fly (Hermetia illucens) larvae compared to monogastric livestock species: a review. J. Anim. Sci. Biotechnol. 13, 1-20. DOI: https://doi.org/10.1186/s40104-022-00682-7.

Shumo, M., Osuga, I.M., Khamis, F.M., Tanga, C.M., Fiaboe, K.K., Subramanian, S., Ekesi, S., van Huis, A., Borgemeister, C., 2019. The nutritive value of black soldier fly larvae reared on common organic waste streams in Kenya. Sci. Rep. 9, 1-13. DOI: https://doi.org/10.1038/s41598-019-46603-z.

Siddique, M.T., Robinson, J.S., 2003. Phosphorus sorption and availability in soils amended with animal manures and sewage sludge. J. Environ. Qual. 32, 1114-1121. DOI: https://doi.org/10.2134/jeq2003.1114.

Soest, P.v., Robertson, J., 1977. What is fibre and fibre in food. Nutr. Rev 35, 12.

Soetemans, L., Uyttebroek, M., Bastiaens, L., 2020. Characteristics of chitin extracted from black soldier fly in different life stages. Int. J. Biol. Macromol. 165, 3206-3214. DOI: https://doi.org/10.1016/j.ijbiomac.2020.11.041.

Søvik, E., LaMora, A., Seehra, G., Barron, A.B., Duncan, J.G., Ben-Shahar, Y., 2017. Drosophila divalent metal ion transporter Malvolio is required in dopaminergic neurons for feeding decisions. Genes Brain Behav. 16, 506-514. DOI: https://doi.org/10.1111/gbb.12375.

Spranghers, T., Ottoboni, M., Klootwijk, C., Ovyn, A., Deboosere, S., De Meulenaer, B., Michiels, J., Eeckhout, M., De Clercq, P., De Smet, S., 2017. Nutritional composition of black soldier fly (Hermetia illucens) prepupae reared on different organic waste substrates. J. Sci. Food Agric. 97, 2594-2600. DOI: https://doi.org/10.1002/jsfa.8081.

Srivastava, N., Majumder, C., 2008. Novel biofiltration methods for the treatment of heavy metals from industrial wastewater. J. Hazard. Mater. 151, 1-8. DOI: https://doi.org/10.1016/j.jhazmat.2007.09.101.

Tomberlin, J.K., Sheppard, D.C., Joyce, J.A., 2002. Selected life-history traits of black soldier flies (Diptera: Stratiomyidae) reared on three artificial diets. Ann. Entomol. Soc. Am. 95, 379-386. DOI: https://doi.org/10.1603/0013-8746(2002)095[0379:SLHTOB]2.0.CO;2.

Truzzi, C., Illuminati, S., Girolametti, F., Antonucci, M., Scarponi, G., Ruschioni, S., Riolo, P., Annibaldi, A., 2019. Influence of feeding substrates on the presence of toxic metals (Cd, pb, ni, as, hg) in larvae of Tenebrio molitor: Risk assessment for human consumption. Int. J. Environ. Res. Public Health 16, 4815. DOI: https://doi.org/10.3390/ijerph16234815.

United-Nations, 2019. World Population Prospects 2019: Highlights.

Van der Fels-Klerx, H., Camenzuli, L., Van Der Lee, M., Oonincx, D., 2016. Uptake of cadmium, lead and arsenic by Tenebrio molitor and Hermetia illucens from contaminated substrates. PLoS One 11, e0166186. DOI: https://doi.org/10.1371/journal.pone.0166186.

Van der Fels-Klerx, H., Camenzuli, L., Belluco, S., Meijer, N., Ricci, A., 2018. Food safety issues related to uses of insects for feeds and foods. Compr. Rev. Food Sci. Food Saf. 17, 1172-1183. DOI: https://doi.org/10.1111/1541-4337.12385.

Van der Kooij, S., van Vliet, B.J., Stomph, T.J., Sutton, N.B., Anten, N.P., Hoffland, E., 2020. Phosphorus recovered from human excreta: A socio-ecological-technical approach to phosphorus recycling. Resour. Conserv. Recycl. 157, 104744. DOI: https://doi.org/10.1016/j.resconrec.2020.104744.

Van Soest, P.v., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583-3597. DOI: https://doi.org/10.3168/jds.S0022-0302(91)78551-2.

Walker, C., 1990. Kinetic models to predict bioaccumulation of pollutants. Funct. Ecol., 295-301. DOI: https://doi.org/10.2307/2389589.

Wu, N., Wang, X., Xu, X., Cai, R., Xie, S., 2020. Effects of heavy metals on the bioaccumulation, excretion and gut microbiome of black soldier fly larvae (Hermetia illucens). Ecotoxicol. Environ. Saf. 192, 110323. DOI: https://doi.org/10.1016/j.ecoenv.2020.110323.

Wu, N., Wang, X., Yan, Z., Xu, X., Xie, S., Liang, J., 2021. Transformation of pig manure by passage through the gut of black soldier fly larvae (Hermetia illucens): Metal speciation, potential pathogens and metal-related functional profiling. Ecotoxicol. Environ. Saf. 211, 111925. DOI: https://doi.org/10.1016/j.ecoenv.2021.111925.

Yang, Y., Meehan, B., Shah, K., Surapaneni, A., Hughes, J., Fouché, L., Paz-Ferreiro, J., 2018. Physicochemical properties of biochars produced from biosolids in Victoria, Australia. Int. J. Environ. Res. Public Health 15, 1459. DOI: https://doi.org/10.3390/ijerph15071459.

Zhao, Y., Zhao, L., Mei, Y., Li, F., Cao, X., 2018. Release of nutrients and heavy metals from biochar-amended soil under environmentally relevant conditions. Environ. Sci. Pollut. Res. 25, 2517-2527. DOI: https://doi.org/10.1007/s11356-017-0668-9.

Zulkifli, N.F.N.M., Seok-Kian, A.Y., Seng, L.L., Mustafa, S., Kim, Y.-S., Shapawi, R., 2022. Nutritional value of black soldier fly (Hermetia illucens) larvae processed by different methods. PloS one 17, e0263924. DOI: https://doi.org/10.1371/journal.pone.0263924.

Table S1. Ingredients and analysed chemical composition of the feeding substrates based on a modified Gainesville fly diet (FD) supplemented with or without sewage sludge recyclates (FD+BCH, FD+SSP) fed to black soldier fly (*Hermetia illucens*) larvae.

Ingredients and composition	**Feeding substrates[1,2]**			**Recyclates**	
	FD	**FD+BCH**	**FD+SSP**	**BCH**	**SSP**
***Modified Gainesville diet*, %**					
FM					
Lucerne	9	9	9	-	-
Sugar beet pulp	7.5	7.5	7.5	-	-
Corn meal	6	5.4	5.4	-	-
Wheat bran[3]	7.5	6.9	6.9	-	-
Biochar (BCH)	0	1.2	0	-	-
Single Superphosphate (SSP)	0	0	1.08	-	-
Water	70	70	70.12		
Chemical composition, g/kg DM					
Dry matter, g/kg FM	854	856	856	-	-
Crude protein	153.7	147.3	147.3	-	-
Crude fat	31.8	29.7	29.7	-	-
Crude fibre	174.6	171.1	171.1	-	-
Starch	201.0	182.0	182.0	-	-
aNDFom	395.0	382.0	382.0	-	-
ADFom	216.0	211.3	211.3	-	-
ME, MJ/kg DM	7.9	7.4	7.4	-	-
GE, MJ/kg DM[4]	10.9	10.2	10.2	-	-

Macrominerals, g/kg DM					
Ca	7.9	9.9	16.9	52.4	208.4
P	4.8	7.0	8.3	65.9	88.8
Mg	2.6	2.8	2.6	7.3	1.8
Na	0.5	0.6	1.1	2.4	14.5
K	14.9	14.6	14.5	4.2	2.1
Heavy metals, mg/kg DM					
Mn	65.8	82.3	65.7	490.0	59.5
Fe	337.0	5,852	351.0	141,064	430
Zn	39.0	101.1	37.9	462.9	8.9
Cu	9.6	27.3	9.6	1,643	23.4
As	0.12	0.23	0.17	3.0	1.3
Cd[5]	0.13	0.14	0.13	<0.02	<0.02
Pb	0.25	3.65	0.33	87.0	1.9
Hg[5]	0.02	0.02	0.02	< 0.02	< 0.02

[1]**FD:** modified Gainesville fly diet**; FD+BCH:** Pyrolysis product of sewage sludge (Biochar; BCH) added to FD (4%); **FD+SSP:** Single Superphosphate (SSP) added to FD (3.6%).

BCH: Biochar was derived from the pyrolysis of sewage sludge produced by the PYREG-process (Fesharaki and Rath, 2018).

SSP: Single Superphosphate originated from recycling of sewage sludge produced by incineration using the Ash2Phos process (Cohen et al., 2019).

[2]The feeding substrates are based on a mixture of 30% ingredients and 70% water. The amount of Single Superphosphate (SSP) was reduced because further grinding and drying had to be conducted, due to clumping of the original substrate. The reduced water content was compensated by reducing the added amount of SSP to 3.6% instead of 4%.

[3]Based on the storage humidity of wheat bran.

[4]ME (Metabolizable energy) was converted to GE (gross energy) by multiplying with the conversion factor of 1.379 (NRC, 1994).

[5]Cd and Hg concentrations in recyclates were lower than the lower limit of quantification.

Abbreviations: FM: Fresh matter; **DM**: Dry matter; a**NDFom**: Neutral detergent fibre after amylase treatment (organic matter); **ADFom**: Acid detergent fibre (organic matter).

Table S2. Heavy metal concentrations of the experimental fly diet (FD) supplemented with two different sewage sludge recyclates (BCH, SSP) and of the black soldier fly (*Hermetia illucens)* larvae fed on these diets compared to the currently authorised maximum concentration for heavy metals in feed for piglets in the European Union.

	Feeding substrates[1]			**Black soldier fly larvae**			**Maximum concentration in feed**
	FD	**FD+BCH**	**FD+SSP**	**FD**	**FD+BCH**	**FD+SSP**	**European Food Safety Authority (EFSA) and European Legislation[2, 3]**
Heavy metals, mg/kg DM							
Mn	65.8	82.3	65.7	285	304	303	150[4, 12, 13]
Fe	337	5,852	351	150	557	339	750[5, 14]
Zn	39.0	101.1	37.9	95.8	107.4	98.5	150[6, 14]
Cu	9.6	27.3	9.6	9.9	12.2	10.1	25[7, 14]
As	0.12	0.23	0.17	0.05	0.07	0.12	2[8, 15]
Cd	0.13	0.14	0.13	0.63	0.77	0.74	0.5[9, 15]
Pb	0.25	3.7	0.33	0.23	1.16	0.54	5[10, 15]
Hg	0.02	0.02	0.02	0.000	0.000	0.001	0.1[11, 15]

[1]**FD:** modified Gainesville fly diet**; FD+BCH:** Pyrolysis product of sewage sludge (Biochar; BCH) added to FD (4%); **FD+SSP:** Single Superphosphate (SSP) added to FD (3.6%).
Total number of observations used for statistical analyses, N = 18 (i.e., n = 6 replicate rearing containers per treatment group within 2 experimental runs).
[2]Currently authorised maximum concentration for heavy metals in complete feed for piglets by EFSA; EFSA, 2010 (4); EFSA, 2016 (5, 7); EFSA, 2014 (6); EFSA, 2009 (8); EFSA, 2004a (9); EFSA, 2004b (10); and EFSA, 2008 (11).
[3]Legally prescribed maximum concentration of heavy metals in complete feed for piglets; European Commission, 2003b (12); European Commission, 2006 (13); European Commission, 2003a (14); European Commission, 2002 (15).

Table S3. Nutrient, macromineral and heavy metal concentrations in frass of black soldier fly (*Hermetia illucens)* larvae fed on a fly diet (FD) supplemented with or without two different sewage sludge recyclates (BCH, SSP).

	Feeding substrates[1]				P-values[2], ≤	
	FD	FD+BCH	FD+SSP	SE	T	B
Nutrients, g/kg DM						
Crude protein[3]	160[b]	158[b]	172[a]	2.6	0.003	0.097
Crude fat	12.3	11.5	11.7	0.9	0.825	0.170
Crude fibre	397[a]	400[a]	331[b]	5.1	0.001	0.036
aNDFom	629[a]	602[b]	548[c]	6.2	0.001	0.055
ADFom	433[a]	431[a]	373[b]	4.7	0.001	0.613
Crude ash	140[c]	182[b]	195[a]	1.5	0.001	0.565
Macrominerals, g/kg DM						
Ca	8.9[c]	11.8[b]	24.7[a]	0.34	0.001	0.574
P	8.8[c]	12.4[b]	15.7[a]	0.17	0.001	0.003
Mg	5.2[a]	5.1[a]	4.8[b]	0.06	0.001	0.109
Na	0.9[c]	1.2[b]	2.5[a]	0.04	0.001	0.040
K	28.7[a]	27.0[b†]	27.9[ab†]	0.28	0.002	0.018
Heavy metals, mg/kg DM						
Mn	66.0[b]	96.8[a]	71.3[b]	1.96	0.001	0.679
Fe	507[b]	11,614[a]	631[b]	200.3	0.001	0.030
Zn	58.2[b]	210.6[a]	58.8[b]	2.8	0.001	0.029
Cu	9.8[c]	52.4[a]	13.3[b]	0.88	0.001	0.006
As	0.12[c]	0.46[a]	0.20[b]	0.013	0.001	0.227
Cd	0.08[b]	0.11[a]	0.08[b]	0.004	0.001	0.333
Pb	0.51[b]	7.11[a]	0.63[b]	0.160	0.001	0.018
Hg	0.02	0.02	0.01	0.005	0.896	0.057

a-c: Values in a row that are marked with different letters differ significantly (Tukey, $P < 0.05$). The symbol † in a row indicates a tendency of two treatments to differ (Tukey, $0.05 < P \leq 0.10$).
[1]**FD:** modified Gainesville fly diet**; FD+BCH:** Pyrolysis product of sewage sludge (Biochar; BCH) added to FD (4%); **FD+SSP:** Single Superphosphate (SSP) added to FD (3.6%).
Total number of observations used for statistical analyses, N = 18 (i.e., n=6 replicate rearing containers per treatment group within 2 experimental runs).
[2]**T**: treatment effect; **B** = block effect (experimental run (2 runs) considered as block).
[3]Calculated as N × 6.25.

Abbreviations: a**NDFom**: Neutral detergent fibre after amylase treatment (organic matter); **ADFom**: Acid detergent fibre organic matter.

Table S4. Heavy metal concentrations in frass of black soldier fly (*Hermetia illucens*) larvae fed on the experimental fly diet (FD) supplemented with two different sewage sludge recyclates (BCH, SSP) compared to the currently authorised maximum concentration for heavy metals in organic solid fertilizer in the European Union.

	Frass of black soldier fly larvae[1]			**Maximum concentration in organic solid fertilizer**
	FD	**FD+BCH**	**FD+SSP**	**European Legislation**
Heavy metals, mg/kg DM				
Mn	66.0	96.8	71.3	-
Fe	507	11,614	631	-
Zn	58.2	210.6	58.8	1,500[2]
Cu	9.8	52.4	13.3	600[2]
As	0.12	0.46	0.20	40[2]
Cd	0.08	0.11	0.08	3[2]
Pb	0.51	7.11	0.63	120[2]
Hg	0.02	0.02	0.01	1[2]

[1]**FD:** modified Gainesville fly diet**; FD+BCH:** Pyrolysis product of sewage sludge (Biochar; BCH) added to FD (4%); **FD+SSP:** Single Superphosphate (SSP) added to FD (3.6%).
Total number of observations used for statistical analyses, N = 18 (i.e., n=6 replicate rearing containers per treatment group within 2 experimental runs).
[2]Legally prescribed maximum concentration of heavy metals in organic solid fertilizer; European Commission, 2019.

References:

Cohen, Y., Enfält, P., Kabbe, C., 2019. Production of clean phosphorus products from sewage sludge ash using the Ash2Phos process, Proceedings of the International Fertiliser Society. International Fertiliser Society, Brussels, Belgium, pp. 1-19.

EFSA, 2004a. Opinion of the Scientific Panel on Contaminants in the Food Chain [CONTAM] related to cadmium as undesirable substance in animal feed. EFSA J. 72: 1-24. DOI: https://doi.org/10.2903/j.efsa.2004.72.

EFSA, 2004b. Opinion of the Scientific Panel on Contaminants in the Food Chain [CONTAM] related to lead as undesirable substance in animal feed. EFSA J. 71: 1-20. DOI: https://doi.org/10.2903/j.efsa.2004.71.

EFSA, 2008. Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on mercury as undesirable substance in feed. EFSA J. 654: 1-76. DOI: https://doi.org/10.2903/j.efsa.2008.654.

EFSA, 2009. EFSA Panel on Contaminants in the Food Chain (CONTAM); Scientific Opinion on Arsenic in Food. EFSA J. 7: 1351. DOI: https://doi.org/10.2903/j.efsa.2009.1351.

EFSA, 2010. Selected trace and ultratrace elements: Biological role, content in feed and requirements in animal nutrition–Elements for risk assessment. EFSA J. 7: 68E. DOI: https://doi.org/10.2903/sp.efsa.2010.EN-68.

EFSA, 2014. FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed). Scientific Opinion on the potential reduction of the currently authorised maximum zinc content in complete feed. EFSA J. 12: 3668. DOI:10.2903/j.efsa.2014.3668.

EFSA, 2016. EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed). Scientific opinion on the safety and efficacy of iron compounds (E1) as feed additives for all animal species: ferrous carbonate; ferric chloride, hexahydrate; ferrous fumarate; ferrous sulphate, heptahydrate; ferrous sulphate, monohydrate; ferrous chelate of aminoacids, hydrate; ferrous chelate of glycine, hydrate, based on a dossier submitted by FEFANA asbl. EFSA J. 14: 4396. DOI:10.2903/j.efsa.2016.4396.

EFSA, 2016. EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed). Scientific opinion on the revision of the currently authorised maximum copper content in complete feed. EFSA J. 14: 4563. DOI: https://doi.org/10.2903/j.efsa.2016.4563.

European Commission, 2002. Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed - Council statement. Official Journal of the European Union 140: 10.

European Commission, 2003a. Opinion of the Scientific Committee for Animal Nutrition on the use of zinc in feedingstuffs. Available online: http://ec.europa.eu/food/fs/sc/scan/out120_en.pdf.

European Commission, 2003b. Commission regulation (EC) No 1334/2003 of 25 July 2003 amending the conditions for authorisation of a number of additives in feedingstuffs belonging to the group of trace elements. Official Journal of the European Union 187: 11.

European Commission, 2006. Commission regulation (EC) No 479/2006 of 23 March 2006 as regards the authorisation of certain additives belonging to the group compounds of trace elements. Official Journal of the European Union 86 (2006): 4.

European Commission, 2019. Regulation (EU) 2019/1009 of the European parliament and of the council of 5 June 2019 laying down rules on the making available on the market of EU fertilising products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003. Official Journal of the European Union, 25.6.2019.

Fesharaki, M., Rath, W., 2018. Verkohlungsanlage Pyreg. Europäisches Patentamt, Patentnummer: EP 3 358 253 A1.

NRC, 1994. National Research Council (NRC). Nutrient Requirements of Poultry: ninth revised edition.

Short title: Recycled-mineral enriched black soldier fly larvae in broiler diets

6. Recycled-mineral enriched whole black soldier fly larvae in broiler diets: effects on growth performance, nutrient intakes, blood metabolites and bone characteristics

In preparation

M.M. Seyedalmoosavi[1,2], G. Daş[1], M. Mielenz[1], S. Maak[3], P. Wolf[4], C.C. Metges[1]

[1] Research Institute for Farm Animal Biology (FBN), Institute of Nutritional Physiology, Wilhelm Stahl Allee 2, 18196 Dummerstorf, Germany

[2] Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB), Department Technology Assessment, Max-Eyth-Allee 100, 14469 Potsdam, Germany

[3] Research Institute for Farm Animal Biology (FBN), Institute of Muscle Biology and Growth, Wilhelm Stahl Allee 2, 18196 Dummerstorf, Germany

[4] University of Rostock, Nutrition Physiology and Animal Nutrition, Faculty of Agricultural and Environmental Sciences, Justus-von-Liebig-Weg 6, 18059 Rostock, Germany

*Corresponding author: metges@fbn-dummerstorf.de

Highlights

Addition of whole BSFL had no adverse effects on nutrient intakes and growth of the birds.

Feeding BSFL to broilers increased serum Ca concentration.

Larvae reared on Biochar reduced serum P concentration.

Altered serum Ca and P concentrations did not affect tibia characteristics.

Abstract

Recycling of critical minerals, i.e., phosphorus, appears to be inevitable for a circular economy based agriculture in the future. Black soldier fly larvae (BSFL) can accumulate certain minerals at varying degrees in their body. Recycled minerals, which are due to current legislations cannot be used in nutrition of classical livestock species, may first be fed to BSFL. Thereafter, mineral enriched BSFL can thenbe used as a feedstuff for other livestock. A feeding experiment was conducted to evaluate the effect of supplementing sewage sludge recycled-mineral enriched whole BSFL to broilers on feed, nutrient, mineral and heavy metal intakes, growth performance, blood metabolites and immunoglobulins, bone characteristics and bone mineral status of the birds. Day-old broiler chicks (Ross 308, N = 80) were assigned to one of 4 groups (n = 6 replicate pens; 2-4 birds/pen). All broilers were fed age-specific basal diets and either had no access to BSFL (CON) or received 15% of the feed intake (FI) of CON birds as defrosted BSFL from three different sources. BSFL used in the broiler experiment were grown either on a Gainesville fly diet (FD) (L-FD) or on the FD supplemented with 4% of biochar (L-BCH), or on the FD supplemented with single-superphosphate sewage sludge recyclate (L-SSP). Broilers in the three larvae supply groups consumed all BSFL on average in 15 min with no significant group difference ($P > 0.05$). Inclusion of 15% of mineral-enriched whole BSFL in broiler rations had no adverse effects on growth performance, nutrient intakes, nutrient conversion efficiency, plasma metabolites and immunoglobulins ($P > 0.05$). Birds in the BSFL supply groups had higher serum Ca concentrations than CON birds ($P < 0.05$). L-BCH supplied birds had a lower serum P than CON birds without larvae supply ($P < 0.05$). Tibia characteristics and tibia mineral status of the birds were not affected by larvae supply ($P > 0.05$). Heavy metal intake of the birds was not affected by the BSFL supply ($P > 0.05$). In conclusion, 15% of mineral enriched larvae supplemented with SSP recyclate can be included in broiler diets without adverse effects on nutrient intakes, growth performance and bone condition.

Key words: Chicken, Sewage sludge recycling, Mineral upcycling, Defrosted BSFL.

1. Introduction

In all animal species, phosphorus (P) is essential because of its important roles in cellular metabolism such as a component of the energy reservoir of the cell, in cellular regulatory mechanisms, and in bone development and mineralization (Bolan et al., 2010; Li et al., 2016). The majority of P used in agriculture or in animal diets is derived from phosphate rock which is a finite resource and increasingly expensive (Shastak et al., 2012). Current P use practices are associated with several environmental concerns (Liu et al., 2008). The excessive application of sewage sludge and animal manure leads to P accumulation in the soil (Bergfeldt et al., 2018). Livestock wastewater and sewage sludge contain a considerable amount of P (Kim et al., 2019). Therefore, recycled P from livestock waste and sewage sludge sources could be an alternative source of P for farm animal diets. However, there is the problem of heavy metals contaminating the sludge and possibly the recyclates, as well as the low bioavailability of P in some recyclate products, depending on the recycling technology (Sartorius et al., 2011).

Black soldier fly larvae (BSFL, Hermetia illucens) have been suggested as alternative source of protein to replace fishmeal and soybean meals in chicken feed (Cutrignelli et al., 2018; Schiavone et al., 2017). Calcium (Ca), P, and potassium (K) are the most abundant macrominerals in the BSFL (Chia et al., 2020), which make the BSFL an interesting source of Ca and P for poultry. However, certain heavy metals in feeding substrate can be accumulated by BSFL (Proc et al., 2020), which may then restrict the use of BSFL as a whole or in meal form in broiler diets.

Dabbou et al. (2018) reported that increasing levels of dietary defatted BSFL meal inclusion (10-15% DM) in male broilers increased body weight and daily feed intake during the starter period, but also negatively affected the feed conversion ratio (FCR) of the birds, suggesting that

lower levels might be more suitable. In a previous study, we found that broilers show a strong interest for eating whole BSFL, and an addition of 20% whole BSFL in broiler rations did not compromise growth performance and nutrient conversion efficiency (Seyedalmoosavi et al., 2022a). Moreover, while 20% whole BSFL in the daily rations did not have negative effects on meat quality and fatty acid (FA) composition, the addition of 30% whole BSFL was associated with altered FA composition in plasma, fat and meat of broilers (Seyedalmoosavi et al., 2022b). Ardennaise chickens fed 8% of whole defrosted larvae (corresponding to 2% on a dry matter basis) had higher body weights than control chickens (Moula et al. (2018). Adding live BSFL (10% of the expected daily feed intake in fresh weight) in turkey increased daily feed intake and body weight gain of BSFL groups compared to the control group resulting in a lower feed conversion ratio (Veldkamp and Van Niekerk, 2019).

In a recent study (Seyedalmoosavi et al.,2023), we have shown that larvae incorporate greater amounts of Ca and P in the larval body depending on the source of mineral recyclates fed to BSFL, the, and show altered macronutrients compositions. As a next step, we hypothesized that the provision of such recycled-mineral enriched whole BSFL in addition to nutritionally balanced broiler diets would improve Ca and P homeostasis without having negative effects on growth performance, metabolism, and heavy metal uptake of broilers. Consequently, the objective of this study was therefore to investigate the effects of feeding mineral enriched BSFL grown on diets supplemented with two different sewage sludge recyclates on broiler performance and health. Nutrient intake, growth performance, blood metabolites, immunoglobulin isotypes, bone characteristics and tibia mineral statues of the birds were then investigated in a broiler feeding experiment.

2. Material and Methods

Ethical approval for the chicken experiment was obtained from the state ethics committee for animal experimentation (Mecklenburg-Western Pomerania State Office for Agriculture, Food Safety and Fisheries, Germany; permission no.: AZ.: 7221.3-2-016/20). Animal care and handling, stunning, necropsies and all sampling procedures were performed by trained and authorized staff following animal welfare rules.

2.1. Experimental design, chickens and management

A completely randomized design with 4 treatments was used in this study. A total of 80 newly-hatched broiler chicks (Ross 308) was obtained from a commercial hatchery and reared at the experimental poultry facility at the Research Institute for Farm Animal Biology (FBN). The chicks were weighed and randomly assigned to one of 4 experimental groups (n = 20 / group). Birds of each group were allocated to 6 replicate pens in four adjacent rooms, each containing 6 pens, i.e., n = 6 replicate pens per group equally allocated to 4 rooms. Depending on pen size, each replicate pen contained 2 - 4 birds. Each pen was equipped with a feeder, a line of drinking nipples, and a deep layer of wood shavings as litter material. Pens in each room were separated from each other by solid panels. Throughout the experiment, birds in the 4 different rooms were raised under the same automatically controlled environmental conditions (Aviagen, 2018). The ambient temperature was 33 °C at the beginning of the experiment and was gradually decreased to 21°C in week (wk) 6, while the humidity was gradually increased from 37% to 70% by wk 6.

2.2. Dietary treatments and BSFL provision

All birds received age-specific basal diets in mesh feed form (Scharnebecker Mühle GmbH, Boizenburg, Germany). The basal diet was designed to meet or exceed age-specific nutrient recommendation of broilers (Aviagen, 2019) in three phases, i.e. starter (d 0 - 14), grower (d 15 - 28) and finisher (d 29 - 42) diets (Table 1). Birds in the control group (CON) received the

age-specific basal diet, and had no access to BSFL. Birds in the other 3 feeding groups received 15% of the feed intake (FI) of CON birds as defrosted whole BSFL in addition to the basal diet.

Table 1. Ingredients and analyzed chemical composition of the age-specific basal diets fed to broilers during the experiment.

	Basal diets		
	Starter (d 1-14)	**Grower (d 15-28)**	**Finisher (d 29-42)**
Ingredients, *%*			
Soybean meal 48%	36	34	35
Wheat	31	28	28
Maize	21.5	28	26.5
Barley	5	4	5
Linseed oil	3	3	3
Vit-Min. Premix[1]	2.5	2.5	2.5
Oyster shells	1.0	0.5	0.0
Chemical analysis, *g / kg DM unless otherwise indicated*			
Dry matter	908	905	906
Crude ash	69.4	71.8	90.5
Crude protein	254.4	275.1	241.7
Crude fat	55.1	48.6	43.1
Crude fiber	30.8	25.4	25.4
Starch[2]	383.3	369.1	406.2
aNDFom	11.0	12.2	11.0
ADFom	37.4	38.7	33.1
ME, *MJ / kg DM*	13.0	12.8	12.6
Minerals, *g / kg DM*			
Ca	12.44	12.27	20.42
P	6.61	6.41	6.29
Mg	2.20	2.43	2.21
Na	1.21	1.77	1.99
K	11.45	12.15	10.49
Heavy metals, *mg/kg DM*			
Mn	117.7	144.2	154.4
Fe	287.3	311.7	386.5
Zn	110.6	139.6	150.2
Cu	18.17	22.54	23.40
As	0.160	0.222	0.410
Cd	0.095	0.064	0.080
Pb	0.159	0.126	0.190
Hg	<0.003	<LLOD	<0.003

[1]Amount of vitamin and minerals provided through premix per kg of feed were as follows: Vit. A 10000 IU, Vit. D3 2000 IU, Vit. E 20 mg, Vit. K3 3 mg, Vit. B1 1 mg, Vit. B2 6 mg, Vit. B6 3 mg, Vit. B12 30 mcg, Niacin 30 mg, Pantothenic acid 10.8 mg, Folic acid 0.4 mg, Biotin 24 µg, Choline 300 mg, Fe 55 mg, Cu 18 mg, Zn 80 mg, Mn 93 mg, I 0.66 mg, Se 0.34 mg, Co 0.05 mg, Phytase 250 FTU.

*: Calculated based on Hahn et al., 2018 (i.e. Chitin = ADF - ADL).

Abbreviations: a**NDFom**: Neutral detergent fiber (organic matter); **ADFom**: Acid detergent fiber (organic matter). LLOD: Lower limit of detection

The larvae (L-) supplied to the three groups differed as they were grown on a modified Gainesville fly diet (L-FD), or on FD supplemented either with approximately 4% of biochar (BCH) (L-BCH) or 3.6% Single Superphosphate (SSP) (L-SSP) mineral-rich sewage sludge recyclates at the expense of wheat bran and corn meal as recently published (Seyedalmoosavi et al., 2023). Biochar (BCH) is a recyclate derived from pyrolysis of sewage sludge produced by the PYREG process (PYREG GmbH; Dörth, Germany) (Fesharaki and Rath, 2018), and SSP originated from the recycling of sewage sludge produced by incineration using the Ash2Phos process developed by EasyMining Sweden AB (Cohen et al., 2019). The mineral-enriched BSFL were harvested, and killed using liquid nitrogen and stored frozen at -20°C until fed to the broilers of the current study.

Except for the first day (d1), the daily amount of BSFL to be fed to the broilers in the L-FD, L-BCH and L-SSP groups was calculated based on FI of the CON birds on the previous day. On d1, FI of birds from previous experiments was used as a reference. Twelve hours before broilers were offered, the frozen larvae were defrosted in a refrigerator ($4^{\circ C}$). Broilers received defrosted BSFL at room temperature and at the same time each day (by 07:30 h). Defrosted larvae provided per pen were weighed and put on a feeding plate on the ground of the pen; the start time of larva eating by the birds was recorded. The pens were observed frequently, and the time at which larvae were no longer on the plate was recorded with a precision of 1 min. The time spent eating BSFL (TSL, min / pen) was then calculated (i.e., end time – start time) for each day.

2.3. Chemical analysis of diets and BSFL

During the experiment, feed and larvae samples were collected regularly and stored at −20°C for chemical analyses. At the end of the experiment, all sub-samples were pooled by larvae (i.e. L-FD, L-BCH and L-SSP) and feed type (i.e. starter, grower, and finisher), and representative samples were analyzed for their nutrient contents. Feed (Table 1) and larvae (Supplementary Table 1) samples were analysed for DM, crude ash, CP, crude fat, starch, crude fibre (CF), neutral detergent fiber (aNDFom), acid detergent fiber (ADFom), and macro- and trace minerals contents by the accredited feed laboratory of Landwirtschaftliche Untersuchungs-und Forschungsanstalt (LUFA), LMS Agrarberatung GmbH (Rostock, Germany) using standard methods (Naumann et al., 1997). The metabolizable energy (ME) of the diets was calculated from CP, CL, starch and sucrose according to the recommendations of the German Society of Nutritional Physiology (GfE, 1999). Because BSFL do not contain starch and sucrose and information on the glycogen content was not available, their ME content is probably underestimated. The mineral and heavy metal contents including Ca, P, magnesium (Mg), sodium (Na), K, manganese (Mn), iron (Fe), zinc (Zn), cupper (Cu), arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg) of the feed (Table 1) and larvae (Supplementary Table 1) were analysed by LUFA Rostock, Germany. The crude fat content was lower while the crude ash content of the L-SSP was higher than L-FD and L-BCH (Supplementary Table 1). The ME content of L-SSP larvae was lower than that of L-BCH and L-FD larvae. In addition, L-SSP larvae had higher Ca, P, Mg, Na and K concentrations than the other larval groups. In contrast, larvae in the L-BCH group had higher Fe, Cd and Pb levels than the other groups (Supplementary Table 1).

2.4. Feed intake and growth performance of broilers

Pen based daily FI was measured in the morning, and the average daily or weekly total FI per bird was then calculated. The weekly total fresh matter intake (FMI; the sum of feed and larval

intake) and the resulting dry matter intake (DMI) per average bird of each pen were calculated. Based on the amounts of feed and BSFL intakes, and the nutrient, ME, mineral and heavy metal contents of feed and BSFL, pen based nutrients, ME, and mineral and heavy metal intakes were calculated for an average bird. The growth performance of the broilers was determined throughout the experimental period. The pen based BW, FI, FMI, DMI, feed conversion ratio (FCR) with consideration of either FMI (i.e. FCR-1: g FMI per g BW gain) or DMI (i.e. FCR-2: g DMI per g BW gain), protein conversion ratio (PCR: g protein intake to gain 100 g BW) and energy conversion ratio (ECR: MJ ME intake to gain 100 g BW) per average bird was calculated on a weekly basis. To assess the homogeneity in the growth of birds in a pen, a weekly coefficient of variation (CV) of BW was calculated for each pen.

2.5. Blood metabolites and immunoglobulin isotypes

In the end of fattening period on d 42, the birds were weighed, stunned and slaughtered for collection of blood samples (N = 80; 20 birds / group). From each bird 2 tubes (each 5 mL) of slaughter blood were collected for harvesting serum and plasma, put on ice and transported to the laboratory. For serum collection blood samples were kept at room temperature for approximately 1 h to allow for clotting. Blood for plasma was collected in K3-EDTA-coated, evacuated tubes (Sarstedt AG & Co., Nümbrecht, Germany) and kept on ice. Tubes were centrifuged (2,500 × g for 20 min at 4°C) and serum and plasma were isolated and stored in Eppendorf vials (Sarstedt AG & Co., Nümbrecht, Germany) at −20°C before analyses on an automatic enzymatic analyzer (ABX Pentra 400, Horiba Medical, Montpellier, France). Serum was used to analyze alkaline phosphatase (ALP) activity, Ca, P, and Mg concentration with a commercial kit (ALP Kit No. A11A01626). Plasma albumin, cholesterol, glucose, non-esterified fatty acids (NEFA), triglycerides and uric acid (UA) concentrations were analyzed using commercial kits [albumin: Kit No. A11A01664; cholesterol: Kit No. A11A01634;

Glucose: Kit No. A11A01667; triglyceride: Kit No. A11A01640 (Horiba ABX); NEFA: Kit No. 434-91795 (Wako Chemicals GmbH, Neuss, Germany); uric acid: Kit No. LT-UR0010 (MTI diagnostics, Idstein, Germany)]. Plasma immunglobulin (IgY, IgM, IgA) concentration was determined by ELISA kits (IgY: Kit No. E30-104; IgM: Kit No. E30-103; IgA: Kit No. E30-102; Bethyl Laboratories, Inc, Montgomery, TX, USA). The inter-assay CV of plasma albumin, cholesterol, glucose, NEFA, triglycerides, UA and serum ALP, Ca, P, and Mg analysis were 0.70%, 1.08%, 2.51%, 8.56%, 4.29%, 3.86%, 22.89%, 9.83%, 9.09%, and 8.85%, respectively. Moreover, the intra-assay CV of plasma albumin, cholesterol, glucose, NEFA, triglycerides, UA and serum ALP, Ca, P, and Mg analysis were 2.75%, 2.92%, 2.97%, 0.96%, 1.03%, 1.85%, 2.34%, 2.79%, 3.19%, 6.44%, respectively.

2.6. Bone measurements

After slaughtering of broilers, the right tibia was separated from individual birds (n = 12 birds / group) and after carefully removing the cartilage, fat, muscle tissue and other tissues on the bone, the bone fresh weight was measured. Bone samples were stored at –20°C for further analyses. Bone dimensions and bone-breaking strength were assessed. Twenty-four h before measurements, bone samples were kept at 4 °C for defrosting. Using a caliper tibia diameter and length were measured. Bone breaking strength was measured using a 3-point bending Texture Analyzer (model TAXT, Stable Micro Systems, Vienna Court, UK).

The tibia bone samples were lyophilized and placed into a modified soxhlet apparatus (LG-6920-100; Wilmad-Labglass, Vineland, NJ). Following extraction using petroleum ether, they were ashed overnight at 550°C in a muffle furnace (Type 30,400 Furnace; Thermolyne). The P-content was analysed colorimetrically by the ammonium vanadate-molybdate method (Gericke and Kurmies (1952) using a Thermo Spectronic Genesys 5 (336001) UV-visible spectrophotometer (Thermo Fisher Scientific, Waltham, USA) at a wavelength of 365 nm.

Calcium content was determined by flame atomic absorption spectrometry (Solaar AA Spectrometer iCE 3500, Thermo Fisher Scientific, USA) with prior microwave assisted acid digestion using 25 µl of sample solution mixed with 2475 µL lanthanum solution (water 84.71%, hydrochloric acid 9.92%, lanthanum oxide 5.36%).

2.7. Statistical analysis

The pen was the experimental unit for all daily or weekly taken pen-based measurements, e.g. FI, larvae and nutrient intakes and BW. Weekly measured pen based average BW, nutrient and ME intake, and the corresponding weekly nutrient and energy conversion indices calculated (FCR, PCR and ECR) were analyzed using a linear mixed model (PROC MIXED) in SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). The model included fixed effects of the treatment group (1 4), week (1 – 6) and treatment group by week interaction. The blocking effect of rooms (1 – 4) was also included in the model. Pen (n = 24) was considered as repeatedly measured subject over time, and was implemented in the statistical model. For the time spent eating larvae, the abovementioned model was used with day (1 – 42) instead of week effects. For the single-point measurements (e.g. bone and blood metabolites and IgY isotypes) the experimental unit was a bird sampled at slaughter (N = 48). Thus, parameters measured on individual birds were analyzed with analysis of variance using the general linear model procedure of SAS (PROC GLM). The statistical model included the fixed effect of treatment group (1 – 4) and the blocking effects of rooms and pens. Group differences were separated by Tukey-Kramer test. The SLICE statement of PROC MIXED of SAS was used to conduct partitioned analyses of the LSM for interactions between treatments by week. The significance level was preset at $P < 0.05$, and a tendency was declared at $0.05 < P \leq 0.10$. Values are presented as LSM with their SE.

3. Results and discussion

3.1. Feed, nutrient, mineral and heavy metal intakes

With the exception of the first 4 d, the daily BSFL offered to the chickens were completely consumed within a few min in all 3 BSFL groups (Supplementary Figure 1), with no significant group difference for TSL ($P > 0.05$). Consistent with these results, we found in a previous experiment that broilers strongly prefer defrosted whole BSFL to regular feed as all larvae provided up to 30% feed intake of broilers were eaten within a few minutes (Seyedalmoosavi et al., 2022a). Broilers in all larvae supply groups had similar feed intake and FMI as CON group (Table 2; $P > 0.05$). In addition, DMI did not differ among the groups ($P > 0.05$) which resulted in similar nutrient intakes including CP, CL, crude ash, Ca, P and Mg intakes (Table 3; $P > 0.05$). However, birds in L-FD, L-BCH and L-SSP groups had a lower CP : ME intake than those in CON (Figure 1; $P < 0.05$). Although there was no significant difference among the groups for CP intake, the numerically higher CP intake in CON birds might be the reason for the higher CP : ME intake in the CON birds. Despite the differences in heavy metal contents of the different BSFL supplied to the birds, we could not detect differences in heavy metal intakes of broilers.

Table 1. Ingredients and analyzed chemical composition of the age-specific basal diets fed to broilers during the experiment.

	Basal diets		
	Starter (d 1-14)	**Grower (d 15-28)**	**Finisher (d 29-42)**
Ingredients, *%*			
Soybean meal 48%	36	34	35
Wheat	31	28	28
Maize	21.5	28	26.5
Barley	5	4	5
Linseed oil	3	3	3
Vit-Min. Premix[1]	2.5	2.5	2.5
Oyster shells	1.0	0.5	0.0
Chemical analysis, *g / kg DM*			
Dry matter, *g / kg*	908	905	906
Crude ash	69.4	71.8	90.5
Crude protein	254.4	275.1	241.7
Crude fat	55.1	48.6	43.1
Crude fiber	30.8	25.4	25.4
Starch[2]	383.3	369.1	406.2
aNDFom	11.0	12.2	11.0
ADFom	37.4	38.7	33.1
ME, *MJ / kg DM*	13.0	12.8	12.6
Minerals, *g / kg DM*			
Ca	12.44	12.27	20.42
P	6.61	6.41	6.29
Mg	2.20	2.43	2.21
Na	1.21	1.77	1.99
K	11.45	12.15	10.49
Heavy metals, *mg/kg DM*			
Mn	117.7	144.2	154.4
Fe	287.3	311.7	386.5
Zn	110.6	139.6	150.2
Cu	18.17	22.54	23.40
As	0.160	0.222	0.410
Cd	0.095	0.064	0.080
Pb	0.159	0.126	0.190
Hg	<0.003	<LLD	<0.003

[1]Amount of vitamin and minerals provided through premix per kg of feed were as follows: Vit. A 10000 IU, Vit. D3 2000 IU, Vit. E 20 mg, Vit. K3 3 mg, Vit. B1 1 mg, Vit. B2 6 mg, Vit. B6 3 mg, Vit. B12 30 mcg, Niacin 30 mg, Pantothenic acid 10.8 mg, Folic acid 0.4 mg, Biotin 24 μg, Choline 300 mg, Fe 55 mg, Cu 18 mg, Zn 80 mg, Mn 93 mg, I 0.66 mg, Se 0.34 mg, Co 0.05 mg, Phytase 250 FTU.

Abbreviations: a**NDFom**: Neutral detergent fiber (organic matter); **ADFom**: Acid detergent fiber (organic matter). LLD: Lower limit of detection.

Table 2. Effect of inclusion of recycled mineral enriched whole black soldier fly larvae (BSFL) in broiler rations on feed intake, growth performance and nutrient and energy conversion ratios of broilers.

	Dietary treatment groups[1]					P-values[2], ≤		
	CON	**L-FD**	**L-BCH**	**L-SSP**	**SE**	**G**	**W**	**G×W**
Intakes, *g /wk*								
Feed	629	472	498	469	73.7	0.392	0.001	0.396
Larvae	-	91.9	91.8	91.8	-	-	-	-
Fresh matter	629	564	589	561	73.7	0.905	0.001	0.847
Dry matter	570	454	478	451	66.8	0.563	0.001	0.589
Growth performance								
Initial BW, *g*	48.75	49.00	47.88	48.92	0.026	0.750	-	-
Average BW, *g*	1011	779	809	780	176	0.750	0.001	0.621
CV of BW, *%*	30.07	44.75	34.51	46.78	8.92	0.496	0.001	0.301
Nutrient and energy conversion ratios								
FCR-1, *FMI / BWG*	1.87	2.26	2.15	2.47	0.320	0.618	0.201	0.668
FCR-2, *DMI / BWG*	1.67	1.79	1.74	1.92	0.220	0.884	0.070	0.599
PCR, *g CP / 100 g BWG*	47.5	53.0	51.3	56.7	6.77	0.812	0.064	0.676
ECR, *MJ ME / 100 g BWG*	2.17	2.78	2.62	2.95	0.40	0.561	0.168	0.689

[1] Dietary treatments: ad-libitum feed without access to BSFL (CON), or with BSFL amounting to 15% of feed intake of CON birds as defrosted L-FD (BSFL reared on fly diet (FD) without adding recyclate), L-BCH (BSFL reared on fly diet with 4% Biochar recyclate) or L-SSP (BSFL reared on fly diet with 4% SSP recyclate) whole BSFL in addition to the basal diet (referred to as groups L-FD, L-BCH, and L-SSP, respectively).
Total number of observations used for statistical analyses, N = 144 (4 treatments each with 6 replicate pens repeatedly measured over 6 weeks). Number of birds, n = 20 per treatment group.

[2] **G**: group effect, **W** = time effect (weeks), **G×W** = treatment by week interaction.
Abbreviations: **BW**: body weight; **BWG**: body weight gain; **ECR**: energy conversion ratio (i.e., MJ metabolizable energy needed to gain 100 g BW); **FCR-1**: feed conversion ratio based on FMI (i.e., g FM intake per g BW gain); **FCR-2**: feed conversion ratio corrected for DM intake (i.e., g DM intake per g BW gain); **PCR**: protein conversion ratio (i.e., g crude protein needed to gain 100 g BW); **ECR**: energy conversion ratio (i.e., MJ energy needed to gain 100 g BW).

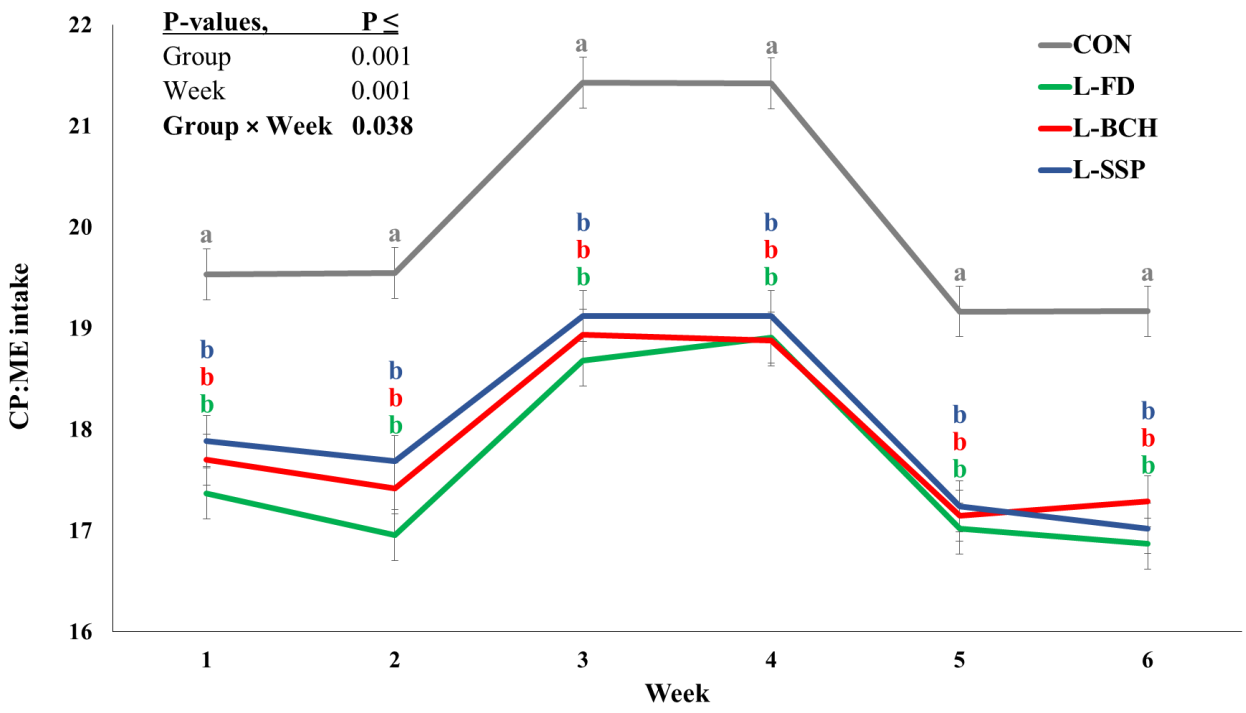


Figure 1. Inclusion of recycled mineral enriched whole black soldier fly larvae in broiler rations on crude protein intake (CPI) to metabolizable energy intake (MEI) ratio of broilers, i.e. CP:ME ratio. Values are LSM with their SE. Dietary treatments: ad-libitum feed without access to BSFL (CON), or with BSFL amounting to 15% of the feed intake of CON birds as defrozen L-FD (BSFL reared on fly diet without adding recyclate), L-BCH (BSFL reared on fly diet with 4% Biochar recyclate) or L-SSP (BSFL reared on fly diet with 3.6 % SSP recyclate) whole BSFL. a-b: Groups denoted with different letters differ significantly (Tukey, $P<0.05$).

Although there were differences in the nutrient, mineral and heavy metal content of the larvae in different groups (Supplementary Table 1), we did not observe differences in feed, nutrient, mineral and heavy metal intakes in broilers. Consistent with the present results, in a previous experiment we found that up to 20% whole BSFL in broiler ration had no effect on feed and nutrient intake, while a higher inclusion rate (30%) reduced dry matter, starch, aNDFom and ME intake of the

birds (Seyedalmoosavi et al., 2022a). Therefore, lack of differences in feed, nutrient, mineral and heavy metal intakes could be attributed to the amount of BSFL (15%) offered to the birds. The inclusion level that we used in this experiment did not compromise the intake of regular feed in larval intake groups. In addition, it should be noted that the nutrient composition of the BSFL used in our previous study was different from that used in the current study. In BSFL of all 3 groups had a lower fat content than that reported in our previous experiment which subsequently was associated with lower ME intake (Seyedalmoosavi et al., 2022a). This can explain why we could not find differences between the broiler groups for crude fat intake, while in our previous experiment, inclusion of increasing levels of BSFL in broiler diets caused a linear increase in fat intake.

3.2. Growth performance and nutrient and energy efficiencies

The body weight of the chickens did not differ among the treatment groups (Table 2; $P > 0.05$). This might be partly due to a relatively large within-group variability (Figure 2) reflected by a coefficient of variation for chicken BW of 30 – 47% ($P > 0.05$). We made similar observations for feed, protein and energy conversions ratios (FCR-1, FCR-2, PCR and ECR) irrespective of dietary treatment (Table 2; $P > 0.05$). As was clearly reflected in the CV of BW, there was generally a large variation within group for most variables, which could explain the lack of significant differences in feed and nutrient intake of the birds.

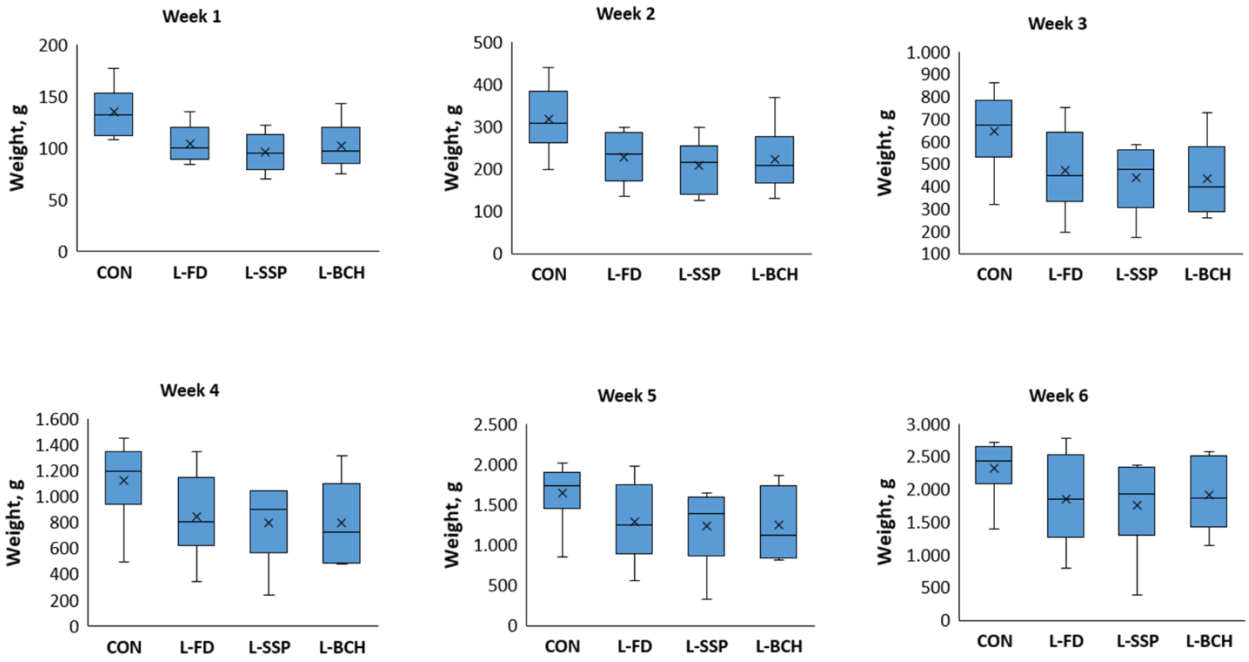


Figure 2. Inclusion of recycled mineral enriched whole black soldier fly larvae in broiler rations on body weight of the chickens during the experimental weeks. Dietary treatments: ad-libitum feed without access to BSFL (CON), or with BSFL amounting to 15% of the feed intake of CON birds as defrozen L-FD (BSFL reared on fly diet without adding recyclate), L-BCH (BSFL reared on fly diet with 4% Biochar recyclate) or L-SSP (BSFL reared on fly diet with 3.6 % SSP recyclate) whole BSFL.

Several studies have been conducted to investigate the effect of partial replacement of soybean meal with BSFL meal or whole BSFL on broiler growth. Inclusion of up to 15% BSFL meal as a substitute for soybean meal in broiler diets did not affect the growth performance and FCR of Cobb broiler chicks (Uushona, 2015). In contrast, 5% of dietary DM as BSFL meal in broiler diets improved growth performance of the birds (Lee et al., 2018). Using up to 10% of defatted BSFL meal as a replacement for soybean meal as dietary protein source positively influenced the growth

performance of broilers but did not affect FCR, while the replacement of soybean meal by 15% defatted BSFL meal reduced broiler growth, which was attributed to the chitin content of BSFL that could reduce the protein digestibility (Dabbou et al., 2018).

Similar to the present study, others also reported the use of whole live or defrosted BSFL as a supplement in broiler rations. In a previous study, we observed that up to 30% of whole BSFL in broiler rations did not affect the growth of the birds, while FCR, PCR and ECR were not affected when up to 20% whole BSFL was included (Seyedalmoosavi et al., 2022a). However, Ipema et al. (2020b) found that provisioning of live BSFL amounting to 10% of dietary DM in broiler diets reduced the growth performance during age d 13 – 27. In contrast, 8% whole defrosted larvae in addition to the regular feed resulted in higher body weights than in the control group (Moula et al., 2018). However, these authors could not find a difference in FCR between the groups. In a study on turkey poults, 10% of the daily feed intake as live BSFL increased daily feed intake and body weight gain and lowered FCR (Veldkamp and Van Niekerk, 2019). Taken together, low inclusion level of BSFL (up to 20% whole and up to 10% meal form) in broiler diet has no negative effects on the growth the birds. However, high inclusion levels (> 30 %) of whole BSFL might be associated with compromised feed conversion and nutrient utilization efficiency.

3.3. Blood metabolites, minerals and immunoglobulins

Plasma albumin, cholesterol, glucose, NEFA and UA were not affected by the treatment groups (Table 4; $P > 0.05$). Plasma triglyceride concentration in birds fed L-SSP tended to be lower than in birds supplied with L-FD ($P = 0.057$), which might be due to the lower fat content of L-SSP larvae than L-FD (Supplementary Table 1). In a previous study, we found that plasma cholesterol and glucose of the birds were not affected by inclusion of different levels of BSFL (up to 30%) in

broiler diets (Seyedalmoosavi et al., 2022b). It should be noted that in the previous study (Seyedalmoosavi et al., 2022b), all birds received BSFL with the same nutrient composition, while nutrient composition of BSFL in the current study differed among the groups. However, in that experiment, plasma NEFA and triglyceride concentrations were higher in birds offered 30% BSFL compared to those given 10% BSFL and the CON groups. This was attributed to the lower ME intake and the higher fat intake in the 30% BSFL group and presumably associated to an increased lipolysis rate. However, in the current study, we could not find any difference between the groups for the fat intake of the birds.

Table 4. Effect of inclusion of recycled mineral enriched whole black soldier fly larvae (BSFL) in broiler rations on selected blood metabolites and immunoglobulin concentrations of broilers.

	Dietary treatment groups[1]					P-value, ≤
	CON	**L-FD**	**L-BCH**	**L-SSP**	**SE**	**Group**
Metabolites						
Albumin, *g / L*	12.41	12.37	12.20	13.07	0.347	0.270
Cholesterol, *mmol / L*	3.00	3.33	3.26	3.29	0.177	0.492
Glucose, *mmol / L*	13.92	14.27	14.40	14.13	0.271	0.596
NEFA, *µmol / L*	207.2	276.7	292.6	283.0	28.68	0.117
Triglyceride, *mmol / L*	0.826	1.16†	1.00	0.803†	0.108	0.057
Uric acid, *µmol / L*	353.1	448.2	415.6	390.7	54.22	0.610
ALP[2], *U / L*	1749	2943	3088	2839	550	0.261
P, *mmol / L*	1.94^{a}	1.56^{ab}	1.42^{b}	1.63^{ab}	0.123	0.022
Ca, *mmol / L*	2.74^{b}	3.21^{a}	3.22^{a}	3.08^{ab}	0.129	0.026
Mg, *mmol / L*	0.936	0.979	0.989	0.992	0.031	0.508
Immunoglobulins						
IgG, *mg / mL*	1.79	1.30	1.33	1.42	0.163	0.107
IgM, *mg / mL*	0.201	0.200	0.202	0.196	0.017	0.994
IgA, *mg / mL*	0.224	0.220	0.209	0.253	0.021	0.464

a-b: Groups denoted with different letters differ significantly (Tukey, $P<0.05$). The signs (†) indicate tendency of two diets to differ (Tukey, $0.05 < P \leq 0.10$).

[1] Dietary treatments: ad-libitum feed without access to BSFL (CON), or with BSFL amounting to 15% of the feed intake of CON birds as defrosted L-FD (BSFL reared on fly diet (FD) without adding recyclate), L-BCH (BSFL reared on fly diet with 4% Biochar recyclate) or L-SSP (BSFL reared on fly diet with 4% SSP recyclate) whole BSFL in addition to the basal diet (referred to as groups L-FD, L-BCH, and L-SSP, respectively).
Total number of observations used for statistical analyses, N = 80 (i.e., 2-4 birds sampled from each of 6 pens allocated to each of 4 treatments at week 6).
Abbreviation: **ALP**: Alkaline phosphatase ([2]measured in serum).

Chickens in L-BCH group had a lower serum P concentration than in the CON group (Table 4; $P < 0.05$). The serum Ca concentration was higher in both L-BCH and L-FD birds than in CON animals ($P < 0.05$). However, serum Ca and P concentrations of the birds in L-SSP were not different to CON ($P > 0.05$). Likewise, serum ALP and Mg concentrations did not differ among the groups (Table 4; $P > 0.05$). We could not find any difference between the four groups for mineral intakes including Ca and P, likely due to the large variability within groups (Table 3). However, the lower serum P in L-BCH broilers could be associated with the lower P content in L-BCH larvae. In contrast to the serum P concentration, the serum Ca concentrations of L-BCH and L-FD broilers were higher than in CON birds. The higher serum Ca in L-BCH and L-FD fed birds could be due to a high Ca content in BSFL (Supplementary Table 1). The differences in serum Ca and P concentrations were not associated with the growth performance of the birds. There is lack of information on the effect of whole BSFL feeding to broilers on serum Ca and P. Dabbou et al. (2018) found a linear and quadratic effect for serum P in broilers fed different levels of defatted BSFL meal. They observed an increased serum P concentration in response to the increasing level of BSFL meal in broiler diet. Plasma immunoglobulins (IgG, IgM and IgA) did not differ among the groups (Table 4; $P > 0.05$). In line with our results, in our previous study, increasing levels of whole BSFL had no effect on the plasma concentration of immunoglobulins in chickens (Seyedalmoosavi et al., 2022 a).

Table 3. Effect of inclusion of recycled mineral enriched whole black soldier fly larvae (BSFL) in broiler rations on nutrient, energy, mineral and heavy metal intakes of broilers.

	Dietary treatment groups[1]					P-values[2], ≤		
	CON	**L-FD**	**L-BCH**	**L-SSP**	**SE**	**G**	**W**	**G×W**
Nutrient and energy intakes								
Crude Protein, *g / wk*	145	121	127	119	16.12	0.669	0.001	0.643
Crude Fat, *g / wk*	26.43	25.80	26.59	24.03	26.43	0.916	0.001	0.547
Crude ash, *g / wk*	46.59	38.94	41.60	39.85	6.01	0.806	0.001	0.796
ME, *MJ / wk*	7.24	6.85	7.08	6.61	0.84	0.954	0.001	0.827
CP:ME ratio	20.04[a]	17.63[b]	17.89[b]	18.01[b]	0.22	0.001	0.001	0.038
Mineral intake, *g / wk*								
Ca	9.55	8.42	9.10	8.85	1.33	0.941	0.001	0.815
P	3.63	2.90	3.06	2.98	0.420	0.611	0.001	0.656
Mg	1.29	1.06	1.12	1.07	0.147	0.640	0.001	0.659
Na	1.04	0.81	0.85	0.81	0.132	0.579	0.001	0.531
K	6.36	5.04	5.30	5.10	0.698	0.521	0.001	0.543
Heavy metal intake, *mg / wk*								
Mn	83.50	62.94	66.35	62.47	10.31	0.448	0.001	0.422
Fe	199	150	159	149	25.69	0.489	0.001	0.421
Zn	80.81	60.95	64.25	60.48	10.03	0.453	0.001	0.421

Cu	12.81	9.65	10.17	9.57	1.56	0.436	0.001	0.417
As	0.180	0.137	0.146	0.137	0.026	0.621	0.001	0.285
Cd	0.049	0.038	0.039	0.037	0.006	0.428	0.001	0.445
Pb	0.092	0.069	0.073	0.069	0.012	0.496	0.001	0.306

a-b: Groups denoted with different letters differ significantly (Tukey, $P<0.05$).

[1] Dietary treatments: ad-libitum feed without access to BSFL (CON), or with BSFL amounting to 15% of the feed intake of CON birds as defrosted L-FD (BSFL reared on fly diet (FD) without adding recyclate), L-BCH (BSFL reared on fly diet with 4% Biochar recyclate) or L-SSP (BSFL reared on fly diet with 4% SSP recyclate) whole BSFL in addition to the basal diet (referred to as groups L-FD, L-BCH, and L-SSP, respectively).

Total number of observations used for statistical analyses, N = 144 (4 treatments each with 6 replicate pens repeatedly measured over 6 weeks). Number of birds, n = 20 per treatment group.

[2] **G**: group effect, **W** = time effect (weeks), **G×W** = treatment by week interaction.

3.4. Tibia characteristics and mineral statues

Tibia characteristics including diameter, length, weight and breaking strength were not affected by the dietary treatments (Table 5; $P > 0.05$). No differences among the groups for tibia ash, tibia ash P and Ca contents could be found (Table 5; $P > 0.05$). Information about the effect of whole BSFL in broiler rations on bone health and mineral statues is limited. Similar to the present results, tibia ash content of local poultry breed fed 8% of defrozen whole BSFL reared on horse manure was not affected (Moula et al., 2018). Ipema et al. (2020a) found that tibia measurements (tibia length, length fluctuating asymmetry, width fluctuating asymmetry and breaking strength) of broilers fed rations in which 5 or 10% of the total dietary DM was replaced with live BSFL were unaffected, except that control birds had a wider tibia than those supplemented with 10% BSFL.

Table 5. Effects of increasing levels of whole black soldier fly larvae in broiler rations on tibia characteristics and mineral statues of broilers.

	Dietary treatment groups[1]					P-value, ≤
	CON	**L-FD**	**L-BCH**	**L-SSP**	**SE**	**Group**
Diameter, cm	0.907	0.792	0.887	0.883	0.055	0.462
Length, cm	9.44	8.44	9.08	9.25	0.380	0.282
Weight, g	11.33	9.14	10.19	11.26	1.18	0.511
Strength, N	281.5	226.0	258.1	267.3	32.19	0.654
Ash (%DM)	52.49	49.02	50.64	47.94	1.82	0.324
P (in ash, %DM)	18.59	18.50	18.40	17.83	0.542	0.739
Ca (in ash, %DM)	36.34	36.89	34.01	34.23	2.19	0.728

a-c: Groups denoted with different letters differ significantly (Tukey, $P<0.05$). The signs (†,*) indicate tendency of two diets to differ (Tukey, $0.05 < P \leq 0.10$).
[1] Dietary treatments: ad-libitum feed without access to BSFL (CON), or with BSFL amounting to 15% of the feed intake of CON birds as defrosted L-FD (BSFL reared on fly diet (FD) without adding recyclate), L-BCH (BSFL reared on fly diet with 4% Biochar recyclate) or L-SSP (BSFL reared on fly diet with 4% SSP recyclate) whole BSFL in addition to the basal diet (referred to as groups L-FD, L-BCH, and L-SSP, respectively).
Total number of observations used for statistical analyses, N = 80 (i.e., 2 birds sampled from each of 6 pens allocated to each of 4 treatments at weeks 4 and 6).
Abbreviation: **ALP**: Alkaline phosphatase ([3]measured in serum).

5. Conclusion

The current experiment provides insight on the effect of using mineral enriched whole BSFL in broiler diets with the aim to reintegrate recycled minerals from currently forbidden residuals into the nutrient cycle. Chickens in all larvae intake groups could consume 15% of mineral enriched whole BSFL in a short time without adverse effects on growth performance, nutrient intakes, nutrient conversion efficiency, blood metabolites and immunoglobulins. Despite the large numerical differences, lack of significant effects on intake and growth performance of broilers could be due to the large within-group variations. Feeding BSFL reared on substrates contained Biochar to broilers caused reduced serum P. These differences in serum Ca and P did not affect the bone characteristics and tibia mineral statues of the birds. However, further studies are required to investigate the effects of higher larval inclusion rates in broiler diets and accordingly; adjust the broiler diets for the Ca and P content to maintain the optimum Ca : P ratio for birds.

Data availability

The data used for analyses in this study is stored in a repository (DOI:xxxxxxxxxxxxx to be provided upon acceptance of the manuscript).

Disclosures: All authors declare that they have no competing interests.

Author's contributions

CCM and GD conceived and designed the study. CCM and PW acquired funding. MMS and GD performed the experiment and contributed to the analysis of the samples and collection of data. MM provided the mineral enriched black soldier fly larvae. MMS performed the statistical analysis

of the data and drafted the manuscript. CCM, GD, MMS, and PW reviewed the manuscript. All authors read and approved the submitted version of the manuscript.

Acknowledgement

This work is an outcome of a sub-project of the Leibniz ScienceCampus Phosphorus Research Rostock, and the first author was partly funded by it. The authors are grateful to B. Mielenz, K. Kàrpàti, U. Lüdtke, E. Wünsche, K. Haß, S. Gaeth, S. Dwars, K. Schleifer, and the other helpful, qualified staff at the Research Institute for Farm Animal Biology (FBN), Dummerstorf, Germany, for their assistance with laboratory analyses and sample collection and making this research project possible.

References

Aviagen, 2018. Broiler Management Handbook, Newbridge, UK, pp. 1–147

Aviagen, 2019. Ross Broiler: Nutrition Specifications. Aviagen: Huntsville, AL, USA.

Bergfeldt, B., Tomasi Morgano, M., Leibold, H., Richter, F. and Stapf, D., 2018. Recovery of phosphorus and other nutrients during pyrolysis of chicken manure. Agriculture 8: 187. https://doi.org/10.3390/agriculture8120187

Bolan, N., Szogi, A., Seshadri, B. and Chuasavathi, T., 2010. The management of phosphorus in poultry litter. Nat Res 7.

Chia, S.Y., Tanga, C.M., Osuga, I.M., Cheseto, X., Ekesi, S., Dicke, M. and van Loon, J.J., 2020. Nutritional composition of black soldier fly larvae feeding on agro-industrial by-products. Entomologia Experimentalis et Applicata 168: 472-481. https://doi.org/10.1111/eea.12940

Cohen, Y., Enfält, P. and Kabbe, C., 2019. Production of clean phosphorus products from sewage sludge ash using the Ash2Phos process, Proceedings of the International Fertiliser Society, Brussels, Belgium. International Fertiliser Society, pp. 1-19.

Cutrignelli, M.I., Messina, M., Tulli, F., Randazzo, B., Olivotto, I., Gasco, L., Loponte, R. and Bovera, F., 2018. Evaluation of an insect meal of the Black Soldier Fly (Hermetia illucens) as soybean substitute: Intestinal morphometry, enzymatic and microbial activity in laying hens. Res Vet Sci 117: 209-215. https://doi.org/10.1016/j.rvsc.2017.12.020

Dabbou, S., Gai, F., Biasato, I., Capucchio, M.T., Biasibetti, E., Dezzutto, D., Meneguz, M., Plachà, I., Gasco, L. and Schiavone, A., 2018. Black soldier fly defatted meal as a dietary protein

source for broiler chickens: Effects on growth performance, blood traits, gut morphology and histological features. J. Anim. Sci. Biotechnol. 9:49 https://doi.org/10.1186/s40104-018-0266-9

Fesharaki, M. and Rath, W., 2018. Verkohlungsanlage Pyreg. Europäisches Patentamt, Patentnummer: EP 3 358 253 A1.

GfE, 1999. Ausschuß für Bedarfsnormen der Gesellschaft für Ernährungsphysiologie, 1999: Empfehlungen zur Energie- und Nährstoffversorgung der Legehennen und Broiler, DLG-Verlag, Frankfurt am Main. 145.

Ipema, A.F., Bokkers, E.A., Gerrits, W.J., Kemp, B. and Bolhuis, J.E., 2020a. Long-term access to live black soldier fly larvae (Hermetia illucens) stimulates activity and reduces fearfulness of broilers, without affecting health. Sci Rep 10: 1-13. https://doi.org/10.1038/s41598-020-74514-x

Ipema, A.F., Gerrits, W.J., Bokkers, E.A., Kemp, B. and Bolhuis, J.E., 2020b. Provisioning of live black soldier fly larvae (Hermetia illucens) benefits broiler activity and leg health in a frequency- and dose-dependent manner. Appl. Anim. Behav. Sci. 230: 105082. https://doi.org/10.1016/j.applanim.2020.105082

Kim, M., Shim, S., Reza, A., Kim, S., Won, S., Jung, B., Kim, J. and Ra, C., 2019. Evaluation of struvite recovered from swine wastewater as an alternative phosphorus source in broiler feed. Agriculture 9: 221. https://doi.org/10.3390/agriculture9100221

Lee, J.-A., Kim, Y.-M., Park, Y.-K., Yang, Y.-C., Jung, B.-G. and Lee, B.-J., 2018. Black soldier fly (Hermetia illucens) larvae enhances immune activities and increases survivability of broiler chicks against experimental infection of Salmonella Gallinarum. J. Vet. Med. Sci.: 17-0236. https://doi.org/10.1292/jvms.17-0236

Li, X., Zhang, D., Yang, T.Y. and Bryden, W.L., 2016. Phosphorus bioavailability: a key aspect for conserving this critical animal feed resource with reference to broiler nutrition. Agriculture 6: 25. https://doi.org/10.3390/agriculture6020025

Liu, Y., Villalba, G., Ayres, R.U. and Schroder, H., 2008. Global phosphorus flows and environmental impacts from a consumption perspective. J. Ind. Ecol. 12: 229-247. https://doi.org/10.1111/j.1530-9290.2008.00025.x

Moula, N., Scippo, M.-L., Douny, C., Degand, G., Dawans, E., Cabaraux, J.-F., Hornick, J.-L., Medigo, R.C., Leroy, P. and Francis, F., 2018. Performances of local poultry breed fed black soldier fly larvae reared on horse manure. Anim Nutr 4: 73-78. https://doi.org/10.1016/j.aninu.2017.10.002

Naumann, C., Bassler, R., Seibold, R. and Barth, K., 1997. VDLUFA-Methodenbuch Band III, Die chemische Untersuchung von Futtermitteln. VDLUFA-Verlag, Darmstadt, Germany.

Proc, K., Bulak, P., Wiącek, D. and Bieganowski, A., 2020. Hermetia illucens exhibits bioaccumulative potential for 15 different elements–Implications for feed and food production. Sci. Total Environ. 723, 138125.

Sartorius, C., von Horn, J. and Tettenborn, F., 2011. Phosphorus recovery from wastewater–state-of-the-art and future potential. Proceedings of the Water Environment Federation 2011: 299-316.

Schiavone, A., Cullere, M., De Marco, M., Meneguz, M., Biasato, I., Bergagna, S., Dezzutto, D., Gai, F., Dabbou, S. and Gasco, L., 2017. Partial or total replacement of soybean oil by black soldier fly larvae (Hermetia illucens L.) fat in broiler diets: Effect on growth performances, feed-choice,

blood traits, carcass characteristics and meat quality. Ital J Anim Sci 16: 93-100. https://doi.org/10.1080/1828051X.2016.1249968

Seyedalmoosavi, M., Mielenz, M., Görs, S., Wolf, P., Daş, G. and Metges, C., 2022a. Effects of increasing levels of whole Black Soldier Fly (Hermetia illucens) larvae in broiler rations on acceptance, nutrient and energy intakes and utilization, and growth performance of broilers. Poult. Sci.: 102202. https://doi.org/10.1016/j.psj.2022.102202

Seyedalmoosavi, M., Dannenberger, D., Pfuhl, R., Görs, S., Mielenz, M., Maak, S., Wolf, P., Daş, G. and Metges, C., 2022b. Lipid metabolism, fatty acid composition and meat quality in broilers supplemented with increasing levels of defrosted black soldier fly larvae. J. Insects as Food Feed: 1-16. https://doi.org/10.3920/JIFF2022.0125

Shastak, Y., Witzig, M., Hartung, K. and Rodehutscord, M., 2012. Comparison of retention and prececal digestibility measurements in evaluating mineral phosphorus sources in broilers. Poult. Sci. 91: 2201-2209. https://doi.org/10.3382/ps.2011-02063

Uushona, T., 2015. Black soldier fly (Hermetia illucens) pre-pupae as a protein source for broiler production, Stellenbosch: Stellenbosch University.

Veldkamp, T. and Van Niekerk, T., 2019. Live black soldier fly larvae (Hermetia illucens) for turkey poults. J. Insects as Food Feed 5: 301-311. https://doi.org/10.3920/JIFF2018.0031

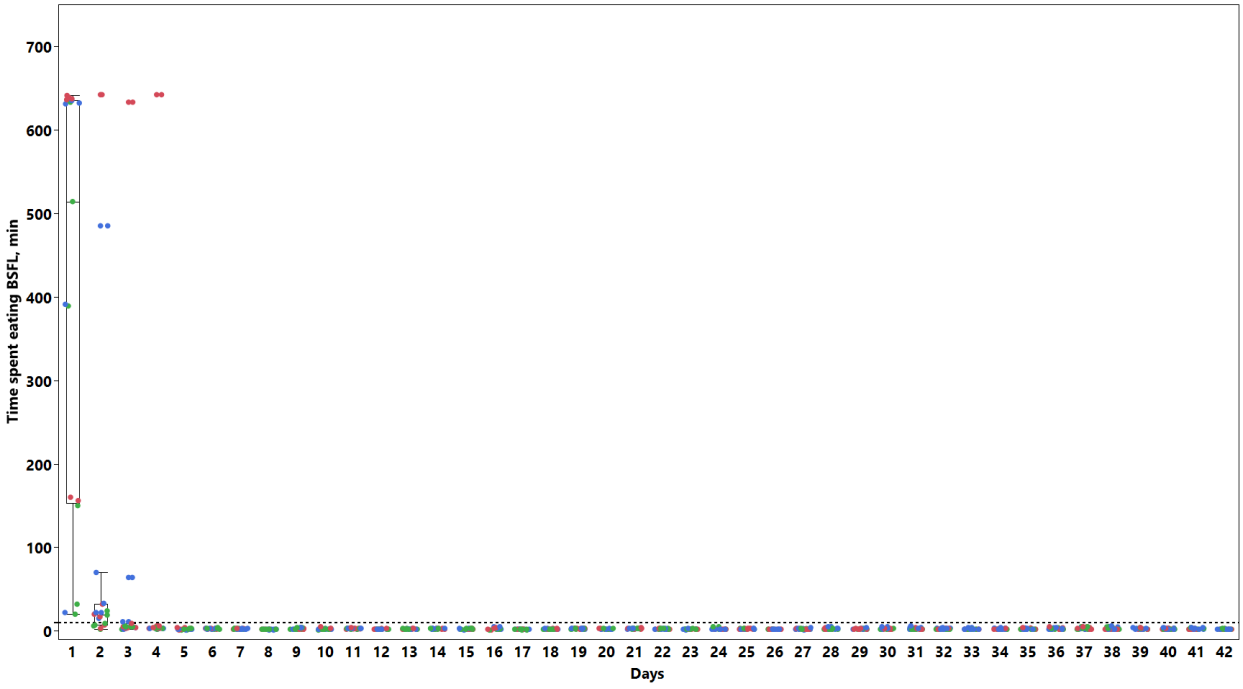


Figure S1. Time spent by broilers for eating defrozen whole black soldier fly larvae during the experimental weeks. Dietary treatments: ad-libitum feed without access to BSFL (CON), or with BSFL amounting to 15% of the feed intake of CON birds as defrozen L-FD (BSFL reared on fly diet without adding recyclate), L-BCH (BSFL reared on fly diet with 4% Biochar recyclate) or L-SSP (BSFL reared on fly diet with 4% SSP recyclate) whole BSFL in addition to the basal diet (referred to as groups L-FD, L-BCH, and L-SSP, respectively). The dashed line on the x-axis indicates an arbitrary threshold of 10 minutes of larva eating time.Total number of observations used for statistical analyses, N = 24 (4 treatments each with 6 replicate pens repeatedly measured over 6 weeks). Number of replicates, n = 6 pens per treatment group.

7. General discussion and conclusions

7.1. Evaluation of methodological, technical and experimental aspects

Phosphorus is one of the essential macro-nutrient in poultry diets which is mainly derived from non-renewable rock phosphate (Li et al., 2016). In addition, low P utilization efficiency in poultry leads to a high P excretion (Nahm, 2007, Rousseau et al., 2012), which increase P emission in the environment. Recently, recycling technologies for P recovery from SS recyclates have received increasing attention in an effort to to limit the environmental impacts of P and minimize the mining of rock phosphate. However, as discussed before, heavy metal content of SS recyclates together with the organic contaminates, feed regulations and hygienic issues are considered as obstacles to extend using recycled P in animal diets.

The first experiment was designed to find the optimum level of whole BSFL in broiler diets. For this, broilers offered whole BSFL at either 10%, 20% or 30% of voluntary FI of CON chickens that received no BSFL. The objective was to assess acceptance, nutrient and energy intakes, growth performance and conversion efficiency, carcass characteristics, alterations in FA compositions in plasma, muscle and abdominal fat tissues, and plasma metabolites (article 1 and 2). We evaluated the hypotheses that using whole BSFL in broiler rations, particularly at high inclusion levels, induces trade-offs in nutrient and energy intake from regular feed and BSFL, ultimately resulting in lower nutrient efficiency and impaired growth performance. Our results confirmed the proposed hypothesis as we observed that high inclusion rates of BSFL (i.e. 30%) negatively affected the feed and energy intake. In the present study, we did not adjust the amount of dietary nutrients, especially energy and protein, in the BSFL fed groups for nutrient content of BSFL. This caused imbalanced nutrient intakes for the chickens. Future research is required to evaluate the effects of

using BSFL in broilers where nutrient content of the diets are adjusted based on the nutrient content of larvae and the larval inclusion rate.

In our experiment, broilers received whole BSFL daily at a certain time in the morning. Therefore, all of the birds had to consume the offered larvae quickly and with no access to whole BSFL for the rest of the day. This may limit positive effects of feeding BSFL to broilers just to the feeding time (i.e. morning). Therefore, instead of feeding all BSFL in one time a day, larvae provision could be prolonged to 3 or 4 times a day to distribute beneficial effects of feeding whole BSFL to broilers during the day. In this regard, Ipema et al. (2020) reported that prolonged access to live BSFL by seven times a day largely increased activity of broilers. In addition, comparison between thawed whole and live BSFL could provide useful information on chicken behavior. Therefore, future experiments are needed to evaluate the provision of live compared to thawed whole BSFL to broilers on behavior of the birds.

In the second experiment, our hypothesis were; 1. BSFL can modify their mineral content in response to the mineral and heavy metal composition in their feeding substrate; and 2. Sewage sludge recyclates (SSR) included in BSFL substrates to produce mineral enriched larvae suitable for inclusion in animal feed. Therefore, our aim was to evaluate the effects of feeding two different mineral-rich SS recyclate in BSFL substrate on LW, development time, and reintegration of SSR minerals in the nutrient cycle by BSFL.

One of the limitations in the second experiment was that we did not count the number of larvae for each box at the beginning of the experiment on d 5. Instead, number of larvae was estimated based on the average individual larval weight. Therefore, we could not calculate the mortality rate of the larvae at the end of the experiment. In previous studies, larvae starvation before killing has

been suggested to reduce the feed and excrement (gut emptying) (Ao at el., 2021). However, in our second experiment, we did not starve the larvae before killing. Therefore, it can be assumed that the mineral and heavy metal analyses in the larvae are not only the representatives of the mineral and heavy metals in the larval body, but also due to the got loading effect.

In the article 4, our hypothesis was that the provision of 15% recycled-mineral enriched whole BSFL in broiler diets has no adverse effects on heavy metal intake, metabolism and growth performance of broilers. Consequently, the objective of this study was to investigate the effects of using BSFL reared on organic materials containing recyclate originated from SS with high P concentration in broiler diets on feed, nutrient and heavy metal intakes, growth performance, blood metabolites, immunoglobulin, tibia traits and tibia mineral statues of the birds. In the third experiment, we used 15% whole BSFL for all BSFL fed groups, however, higher inclusion rates of whole mineral enriched BSFL could be helpful to show more marked effects of using mineral enriched whole BSFL on broiler growth and metabolism.

To evaluate the heavy metal accumulation in broilers fed whole BSFL, the heavy metal content in the body of the chicken is necessary. However, in the third experiment we did not measure the heavy metal content in the birds' body, which remains for the future studies. Moreover, in the third experiment, meat quality of the chickens was not evaluated, therefore, we could not decide if the meat of the chickens was safe and acceptable for human consumption.

7.2. Whole BSFL in broiler rations on broiler growth, physiology metabolism and meat quality

Insects such as BSFL are considered as a natural feed ingredient for free-range poultry species which can improve animal welfare (Spartano and Grasso, 2021, Elahi et al., 2022). In recent years,

there has been a growing number of publications suggesting to include BSFL in broiler diets. However, most of the publications focused on using the meal form of BSFL, which increases the price of the diets and reduces the palatability of BSFL for broilers. In the current thesis, we did not perform cost calculations; however, de Souza Vilela et al. (2021) suggested that using full-fat BSFL in broiler diets reduced the feed cost by 19% when it was replaced up to 15% of the conventional feed ingredients in broiler diets. In a recent study, Sumbule et al. (2021) stated that increasing level of BSFL meal up to 100% reflected the reduced feed cost for broilers, while it did not compromise growth of the birds, which subsequently resulted in increased production profits. In article 1, we observed that up to 20% of whole BSFL did not negatively affect growth performance, nutrient intakes, and utilization efficiency of broilers. These results are encouraging especially, for local farmers, and suggest that using unprocessed BSFL can be included in broiler diets to reduce feed costs without negative effects on the growth performance of the birds.

It should be taken into account that the BSFL's chitin content (9%) may work as a prebiotic in the broilers' intestines, enhancing growth and immune system performance (de Souza Vilela et al., 2021). In addition to chitin, the FA profile of BSFL, which is rich in lauric acid (LA) as well as AMP, which provides an efficient antimicrobial barrier (de Souza Vilela et al., 2021). Our results showed that IgA concentration in broilers that received 30% whole BSFL in their diet tended to be higher than those that received 10% BSFL, which may suggest an activation of the immune system (Seyedalmoosavi et al., 2022a). The AMP derived from BSFL has been suggested as a promising candidate for the replacement of antibiotics in livestock farming, which can improve animal health (Xia et al., 2021).

In conclusion, the results of Article 1 suggested that broiler rations containing up to 20% of the whole BSFL have no detrimental effects on the growth performance and nutrient conversion efficiency of birds. However, higher inclusion rates are associated with lower protein utilization efficiency.

The results of the second experiment suggested that increasing levels of whole BSFL (up to 30%) in broiler diets did not compromise the carcass traits of broilers, which confirmed our hypothesis and was in line with the results of previous studies (Schiavone et al., 2019, Balolong et al., 2020, Lu et al., 2022).

Depending on the larval substrate, the fat content of BSFL (11.8–41.7% of DM) contains high amount of saturated fatty acid (SFA) with up to 76% of total fat and 19% to 40% mono- and poly-unsaturated FA (Seyedalmoosavi et al., 2022b). High proportions of SFA content of BSFL is composed of medium-chain LA (C12:0) and myristic acid (C14:0) (Seyedalmoosavi et al., 2022b). The LA exhibit a wide spectrum of antibacterial activity which has been associated with its ability to disorder lipid membranes of microorganisms making BSFL a functional food to improve immunity of animals (Nekrasov et al., 2022).

In this study, we evaluated selected blood metabolites and FA composition, including 43 individual FA, across plasma, fat, and muscle tissues in 96 birds from two age groups. Our results showed that inclusion of 30% whole BSFL in broiler diets increased SFA levels at the expense of mono-unsaturated fatty acids (MUFA) and poly-unsaturated fatty acids (PUFA) in plasma lipids, breast muscle and abdominal fat tissues. However, broiler diets containing up to 20% whole BSFL did not affect the FA composition in different tissues compared to those in CON group. Lack of effect on FA composition in different tissues of broilers, when their diets included up to 20% whole

BSFL, confirms our hypothesis. However, the alteration of FA composition in different tissues of broilers fed 30% whole BSFL contradicts our hypothesis.

Dietary selected types of LUFA, such as omega-6 and omega-3, play an important role as modulators of cell function and as precursors of lipid mediators (Visioli and Poli, 2020). In contrast, dietary SFA have been associated with higher levels of low-density lipoprotein (LDL) cholesterol and an increased risk of cerebrovascular diseases (Briggs et al., 2017, Visioli and Poli, 2020). Therefore, to produce chicken meat that is healthier for human consumption, high inclusion rates of whole BSFL in broiler diets should be avoided. In the article 2, we suggested that 30% whole BSFL in broiler diets is not recommended, as it might be associated with negative effects on FA composition in plasma, breast muscle and adipose tissues, which in turn reduces the nutritional quality of the meat for human consumption. Our results showed that BSFL contain the C18:2 cis-9, trans-11 FA, an isomer of conjugated linoleic acid (CLA), amounting 0.50% of the total fat. This increased the proportions of CLA in plasma, breast muscle and depot fat tissues of broilers. Hoc et al. (2020) suggested that the occurrence of CLA might be explained by the presence of enzymes such as Δ12 or Δ9 isomerase in BSFL. These enzymes allows the isomeric change from linoleic acid to CLA. Conjugated linoleic acid has been suggested to have positive effects on human health, including prevailing beneficial functions against cancer and arteriosclerosis (Cho et al., 2013). Therefore, the production of CLA-enriched chicken meat for human consumption has become an interesting subject of research.

Our results support inclusion of unprocessed whole BSFL in broiler diets and provide assurance to broiler farmers about the marketability of chicken meat reared on diets that include BSFL. In addition, this issue is important because BSFL have the ability to convert low-grade

organic waste into high-quality biomass. This biomass can then be used as a suitable protein source in animal diets (Seyedalmoosavi et al., 2022b), potentially producing meat of acceptable quality for human consumption. In conclusion, including up to 20% whole BSFL in broiler diets does not adversely affect slaughter weight, meat quality and FA compositions. However, a 30% inclusion of whole BSFL in the diet leads to alterations in FA composition in the plasma, fat and meat of broilers.

7.3. The relationship between larvae feeding and birds' behavior

In modern farming practice, broilers are often housed at high stocking densities and are provided with specially formulated rations, designed to increase growth rates and enhance production efficiency. These conditions impair the welfare, activity, and natural behaviors of broilers, such as foraging, exploration, dustbathing, and perching (De Jong and Gunnink, 2019). Moreover, inability of chickens to express their natural behavior often results in aggressive behavior (Blokhuis and Wiepkema, 1998). Enriched environments enable broilers to perform a wider range of species specific behaviors, leading to improvements in both their biological functioning and welfare aspects (Riber et al., 2018). Generally, insects like BSFL are considered as natural feed ingredients and source of environmental enrichment for free-range poultry species, enhancing animal welfare by promoting their natural behavior (Spartano and Grasso, 2021, Elahi et al., 2022). The inclusion of insect larvae in poultry diets has already approved in European Union (Commission Regulation, 2019); however, there are restrictions, particularly concerning the prohibition of using waste material to feed the larvae.

In this thesis, we did not assess the behavior of the birds, however, there are studies that have evaluated the behavior of birds fed with either live or defrosted whole BSFL. In a previous study,

Ipema et al. (2020) reported that provision of BSFL stimulates natural behaviors and increases the activity of broilers. Recently, Biasato et al. (2022) reported that inclusion of live BSFL in broiler diets stimulated foraging behavior without affecting leg health parameters. Increasing activity of broilers promotes leg bone development and improves leg health (Ipema et al., 2020). Research studies have reported that provision of live BSFL to laying hens has beneficial effects on the behavior of the birds. Star et al. (2020) reported that live larvae provision throughout the day for laying hens improved expression of natural behavior of the birds in searching for feed. In our broiler feeding experiment, all the chickens remained healthy until the end of the experiment and no health issue was observed. Our results indicated that broilers showed a greater interest in consuming whole BSFL even more than regular feed.

7.4. Effect of bio waste feeding for larvae vs. composting

The increasing quantities of bio-waste worldwide, together with inadequate waste management and recycling, can lead to negative environmental effects and pose public health challenges (Salam et al., 2022). Therefore, there is an urgent need for researches to develop new technologies and solutions for waste management. Between 50 – 70 % of solid wastes dumped in landfills are organic, emitting greenhouse gases, and causing unhygienic conditions (Amrul et al., 2022). Composting biowaste is a solution for treating organic waste, which can help reduce the overall volume of waste produced (Amrul et al., 2022). Composting is the aerobic breakdown of biowaste by microorganisms, a natural process that converts organic matter into nutrient-rich soil (Pavlas et al., 2020). Although the composting process is simple, it carries risks such as low quality end products,, excessive odor, and GHG (Reyes-Torres, et al., 2018).

Depending on the raw material, bio-waste may be contaminated with organic pollutants, pathogens, or heavy metals such as Cd or lead (Pb) (Lopes et al., 2011). Consequently, composted organic waste intended for use as fertilizer might contain these undesirable metals. The presence of heavy metals and toxic substances in the final product of conventional composting processes limits the feasibility of using composted organic waste (Mohee and Soobhany, 2014). Due to the diversity of pathogens, composting may not completely eradicate all pathogens, especially those capable of producing spores, despite the fact that high temperatures during the composting process of manure lower the loads of pathogens (Cammack et al., 2021). Therefore, it is important to consider alternative manure management strategies.

In recent years, there has been increasing attention on using BSFL as an effective method for bio-waste conversion (Wu et al., 2020, Salam et al., 2022). Previous reports suggested that composting bio wastes such as manure with BSFL reduces undesirable odor and loads of pathogens (Parodi et al., 2021). Composting manure with insects such as BSFL reduces many types of pathogens, either through direct ingestion by the larvae or through antimicrobial compounds (Cammack et al., 2021). Previous studies on pathogen inoculation support capability of BSFL for hygienic waste management. In a previous study, composting human fecal with BSFL reduced concentration of *Salmonella spp.* and viruses (Lalander et al., 2015). In the current thesis, we did not evaluate the microbial analysis of the gut and frass of BSFL, leaving this aspect for future studies.

Previous studies reported different bioaccumulation patterns of metals and heavy metals in BSFL. These findings suggest that BSFL can selectively regulate the concentration of

micronutrients, including minerals and heavy metals, in their bodies in response to the mineral content of their substrate (Shumo et al., 2019, Proc et al., 2020).

The term “heavy metal” refers to metalloid elements with and atomic number greater than 20 and a specific gravity higher than about 5 g cc^{-1} such as As, Cd, chromium, Pb and Hg (Ali and Khan, 2018). It has been suggested that heavy metals are essential for certain biochemical and physiological functions in trace concentrations (ppb to 10 ppm). However, excessive concentration of heavy metals has been linked to cellular and tissue damage (Tchounwou et al., 2012). In our experiment, the heavy metal content in SSP recyclate was lower than that in BCH recyclate leading to differences in the bioaccumulation of heavy metals in the larval body. According to our result, although the Mn content was higher in BCH recyclate compared to SSP, this did not lead to significant differences in Mn concentration among the groups.

In the article 3, we discussed theadverse effects of excess Mn on larvae, animal and human. According to NRC (1994), Mn recommendation in chicken diet is 20 – 0 mg/kg. Considering the optimum inclusion level of whole BSFL in broiler diets, which is 20% of feed, it seems that using either whole BSFL or the defatted meal does not exceed the Mn recommendation level for broiler diets. However, Grummer et al. (1950) reported that 50 – 100 mg Mn/kg diet might cause some symptoms of toxicity in pigs. Therefore, BSFL should be used with greater caution in pig diets, as they may exceed the recommended Mn levels of 2 – 4 mg/kg (Broom et al., 2021). Also care should be taken for humans in which the Mn recommendation is only 2 – 5 mg/d (DGE, 2000). However, processing technique of BSFL is possibly of importance because Mn might be located mainly in the puparia as discussed by Ushakova et al. (2018).

8- Future perspectives

1- Since we observed that chickens had a high interest for consuming whole BSFL over feed, it could be of interest to provide broilers with ad-libitum amount of whole BSFL to evaluate productive and behavioral traits of birds.

2- High P content of SSR makes it a potential mineral rich supplement in animal nutrition as a substitute for rock P which could improve nutrient recycling. However, depending on the recycling technologies, SSR also contain heavy metals and contaminations that may cause infectious diseases. Currently, according to EU legislation, using SSR in animal feed is not permitted. Therefore, future studies are needed to develop sewage sludge treatment technologies to reduce heavy metal content in the final products (i.e. recyclates), so that it could be directly included in animal diets.

3- Considering that the mineral hemostasis in BSFL are largely unknown, future research should focus on the regulatory pathways of minerals, and specifically Ca and P absorption in BSFL.

4- Since whole BSFL contains high amounts of heavy metal, future researches could adjust the broiler diets for the Ca and P content to maintain the optimum Ca : P ratio for birds.

9. Summary

The expected future depletion of non-renewable rock phosphorus (P) highlights the urgent need for P recycling. Recovered P could be an alternative source of rock P. In order to reintroduce recovered P into the nutrient cycle, the present study examined whether this could be achieved by enriching larvae of the black soldier fly (BSFL). We evaluated the effects of supplementing mineral enriched whole BSFL reared on recycled minerals originated from sewage sludge with high P concentration in broiler diets on feed intake (FI), growth performance, alterations in P-relevant metabolites in blood and bone of broilers. The investigations were carried out separately in three experiments at the Research Institute for Farm Animal Biology (FBN) in Dummerstorf.

In a larvae feeding experiment, we investigated growth, nutrient and mineral composition of 5 day old BSFL (after hatching) reared either on a modified Gainesville fly diet (FD) or on FD supplemented with either 4% of biochar (BCH) or single-superphosphate (SSP) recyclate. Larvae were harvested between 13 to 15 days after hatching. The SSP recyclate compromised growth performance of BSFL. The concentrations of the minerals found in the larvae confirm that the micronutrient profile of BSFL depend on the initial concentrations in the substrate. Both BCH and SSP supplements increased Ca content in BSFL, whereas only SSP increased P content. Except for cadmium and manganese, heavy metal concentration in BSFL were below the current EU limitations.

The first broiler feeding experiment was conducted to investigate the inclusion of whole BSFL in broiler diets on growth performance of the birds, carcass characteristics, and fatty acid (FA) compositions in plasma, muscle and abdominal fat tissues. Newly hatched Ross-308 chicks received whole BSFL at levels of 10% (L10), 20% (L20), or 30% (L30) of voluntary FI of control

(CON) chickens that received no BSFL but only age-specific diets for 42 days. The results showed a strong preference of broilers for consumption of BSFL over regular feed. Inclusion of up to 20% whole BSFL in broiler diets did not adversely affect the growth performance and nutrient conversion efficiency. Dietary inclusion of up to 30% whole BSFL did not negatively affect meat quality and carcass characteristics, however, 30% whole BSFL caused increased saturated fatty acids content at the expense of monounsaturated fatty acids and polyunsaturated fatty acids in plasma, breast and abdominal fat, which might reduce meat quality for human consumption.

A second broiler feeding experiment was performed using day-old Ross 308 broiler chicks. Broilers in the CON group had no access to BSFL but received age specific diets. Birds in the three other groups received 15% of the FI of CON birds as defrosted whole BSFL which were produced in the insect feeding experiment. Inclusion of mineral enriched whole BSFL in broiler diets did not compromise nutrient intakes and growth performance, immunoglobulins, tibia statues and tibia mineral content of the birds. In conclusion, the results of the present dissertation suggests that recycled-mineral enriched whole BSFL with high P concentration could be included in broiler diets without negative effects on growth performance of the birds.

10. Zusammenfassung

Die zukünftig zu erwartende Erschöpfung der nicht erneuerbaren Quellen von Phosphor (P) aus Gestein macht deutlich, wie dringend notwendig das P-Recycling ist. Um zurückgewonnenes P wieder in den Nährstoffkreislauf einzubringen, wurde in der vorliegenden Arbeit geprüft, ob dies über eine Anreicherung von Larven der Schwarzen Soldatenfliege realisierbar ist. Dazu haben wir Futter für Masthähnchen mit ganzen Schwarze Soldatenfliegenlarve (SSFL), deren Substrat mit recycelten Mineralien aus Klärschlamm mit hoher P-Konzentration supplementiert, und deren Auswirkungen auf die Futteraufnahme, die Wachstumsleistung und die Veränderungen der P-relevanten Metaboliten in Blut und Knochen von Masthähnchen untersucht. Die Untersuchungen wurden in drei Versuchen am Forschungsinstitut für Nutztierbiologie (FBN) in Dummerstorf durchgeführt.

In einem Larvenfütterungsexperiment untersuchten wir das Wachstum, die Nährstoff- und Mineralzusammensetzung von 5 Tage alten SSFL (nach dem Schlüpfen), die entweder mit einer modifizierten Gainesville-Fliegendiät (FD) oder mit FD, ergänzt mit entweder 4 % Biokohle (BCH) oder Superphosphat (SSP)-Rezyklat aufgezogen wurden. Die Larven wurden 13 bis 15 Tage nach dem Schlüpfen geerntet. Das SSP-Rezyklat beeinträchtigte die Wachstumsleistung von BSFL. Die Mineralstoffkonzentrationen in den Larven bestätigte, dass das Mikronährstoffprofil von BSFL von den Ausgangskonzentrationen im Substrat abhängt. Sowohl die BCH- als auch die SSP-Ergänzungen erhöhten den Ca-Gehalt in BSFL, wohingegen nur SSP den P-Gehalt erhöhte. Mit Ausnahme von Cadmium und Mangan lagen die Schwermetallkonzentrationen in BSFL unter den geltenden EU-Grenzwerten.

Im ersten Experiment zur Broilerfütterung wurde die Supplementierung ganzer BSFL zum Futter auf Wachstumsleistung, Schlachtkörpereigenschaften sowie der Fettsäurezusammensetzung in Plasma, Muskeln und Bauchfettgewebe der Broiler untersucht. Frisch geschlüpfte Ross-308-Küken erhielten über 42 Tage ganze BSFL in Mengen von 10 % (L10), 20 % (L20) oder 30 % (L30) der Futteraufnahme einer Kontrollgruppe (CON), die keine SSFL, sondern nur altersspezifische Diäten erhielt. Die Ergebnisse zeigten, dass Broiler den Verzehr von BSFL gegenüber normalem Futter stark bevorzugen. Die Supplementierung des Futters mit 20 % BSFL hatte keinen negativen Einfluss auf die Wachstumsleistung und die Effizienz der Nährstoffumwandlung. Auch ein Anteil von bis zu 30 % BSFL im Futter hatte keinen Einfluss auf Fleisch- und Schlachtkörperqualität. Jedoch waren in dieser Gruppe die Konzentrationen an gesättigten Fettsäuren auf Kosten von einfach ungesättigter und polyungesättigter Fettsäuren in Plasma, Brust- und Bauchfett erhöht, was zu einer Verringerung der Fleischqualität für den menschlichen Verzehr führen könnte.

Im zweiten Broilerfütterungsexperiment wurden Eintagsküken des Genotyps Ross 308 4 Fütterungsgruppen zugeteilt, die auf altersspezifischen Diäten basierten. Broiler der CON-Gruppe hatten keinen Zugang zu BSFL, während Vögel in den drei anderen Gruppen 15 % der Futteraufnahme der Broiler der CON-Gruppe als aufgetaute ganze BSFL, die im Insektenfütterungsexperiment produziert wurden. Die Supplementierung der mit Mineralien angereichertem BSFL im Broilerfutter hatte keinen Einfluss auf die Nährstoffaufnahme und Wachstumsleistung, Immunglobuline, Tibia-Status und Tibia-Mineralstoffgehalt der Vögel. Zusammenfassend legen die Ergebnisse der vorliegenden Dissertation nahe, dass mit recycelten Mineralien angereicherte BSFL mit hoher P-Konzentration im Futter von Broilern supplementiert

werden können, ohne dass negative Auswirkungen auf die Wachstumsleistung der Broiler zu erwarten sind.

11. Appendix

11.1. Acknowledgments

It is with pleasure and honor to express my great acknowledge and gratitude to my supervisor Prof. Dr. Cornelia C. Metges for her uncountable help, invaluable expertise in research, patient guidance, support and advice throughout my Ph.D. I would also like to thank my co-supervisor, Dr. Gürbüz Daş for his great support which directed me to successfully complete my dissertation. I would like to express my extreme acknowledge to Dr. Manfred Mielenz for his invaluable advice, continuous support, and help to my project. I would like also to thank Dr. Solvig Görs for her help and support which inspired me to move forward. I want to express my deepest gratitude to F. Feldt and co-workers in the FBN slaughterhouse, as well as B. Mielenz, K. Kàrpàti, U. Lüdtke, E. Wünsche, K. Wilke, R. Gaeth and S. Dwars for their help and support for the animal and various laboratory analyses and with sample collection. I wish to thank Dr. Dirk Dannenberger, and his team particularly Birgit Jentz and Maria Dahm in the Institute of Muscle Biology and Growth who assisted with sample preparation and GC analysis of FA. I express my gratitude to Ralf Pfuhl and his team (FBN, Dummerstorf, Germany) for providing the carcass data.

I specially thank Prof. Jens Tränckner, at University of Rostock, Institute of Water Management, for proving recyclates. I would also like to thank Liane Hüther and Sven Dänicke at Institute of Animal Nutrition, Federal Research Institute for Animal Health, Braunschweig, Germany who provided me with the results of mineral measurements.

Special thanks to the whole my colleagues at FBN, Dr. Quentin L. Sciascia and Dr. Zeyang Li and to all my friends and colleagues for sharing the good time. Lastly, I would like to thank my

family (my mother, my father, and my sisters), whom without them this would have not been possible.

This research project was part of the Leibniz ScienceCampus Phosphorus Research Rostock and was co-funded by the funding line strategic networks of the Leibniz Association.

11.2. List of own publications

Seyedalmoosavi, M. M., Mielenz, M., Veldkamp, T., Daş, G., & Metges, C. C. (2022). Growth efficiency, intestinal biology, and nutrient utilization and requirements of black soldier fly (*Hermetia illucens*) larvae compared to monogastric livestock species: a review. Journal of Animal Science and Biotechnology, 13(1), 1-20. DOI: 10.1186/s40104-022-00682-7

Seyedalmoosavi, M. M., Mielenz, M., Görs, S., Wolf, P., Daş, G., & Metges, C. C. (2022). Effects of increasing levels of whole Black Soldier Fly (*Hermetia illucens*) larvae in broiler rations on acceptance, nutrient and energy intakes and utilization, and growth performance of broilers. Poultry Science, 101 (12) 1-15. DOI: 10.1016/j.psj.2022.102202

Seyedalmoosavi, M. M., Dannenberger, D., Pfuhl, R., Görs, S., Mielenz, M., Maak, S., Wolf, P., Daş, G., & Metges, C. C. (2022). Lipid metabolism, fatty acid composition and meat quality in broilers supplemented with increasing levels of defrosted black soldier fly larvae. Journal of Insects as Food and Feed, 1-16. DOI: 10.3920/JIFF2022.0125

Seyedalmoosavi, M. M., Mielenz, M., Schleifer, K., Görs, S., Wolf, P., Tränckner, J., Hüther, L., Dänicke, S., Daş, G., Metges, C.C. (2023). Upcycling of recycled minerals from sewage sludge through black soldier fly larvae (*Hermetia Illucens*): impact on growth and mineral accumulation. Journal of Environmental Management 344 (2023): 118695. https://doi.org/10.1016/j.jenvman.2023.118695

Seyedalmoosavi, M. M., Daş, G., Mielenz, M., Maak, S., Wolf, P., Metges, C.C. (2023). Recycled-mineral enriched whole black soldier fly larvae in broiler diets: growth performance, nutrient intakes, blood metabolites and bone characteristics. (In preparation).

11.3. List of presentations

Seyedalmoosavi S.M.M., Daş G., Mielenz M., Wolf, P., Metges C.C. (2020). Potentiality of Black Soldier Fly Larvae (BSFL) reared on recycled phosphorus-rich substrates to be included in broiler diets. Beginner level. Day of the Doctoral Student. Leibniz Institute for Farm Animal Biology (Oral presentation).

Seyedalmoosavi S.M.M., Daş G., Metges C.C. (2020). Potentiality of Black Soldier Fly Larvae (BSFL) reared on recycled phosphorus-rich substrates to be included in broiler diets. P-campus symposium (Online presentation).

Seyedalmoosavi S.M.M., Daş G., Mielenz M., Wolf, P., Tränckner, J., Metges C.C. (2021). Black Soldier Fly Larvae reared on recycled phosphorus-rich substrates as a feed component for broilers. Lecture for the online presentation of the P-campus Ringvorlesung.

Seyedalmoosavi S.M.M., Daş G., Metges C.C. (2021). Influence of different amounts of black soldier fly larvae (BSFL) in the ration on nutrient and energy utilization and growth of broilers. 75th Digital Conference of the Society of Nutrition Physiology (Oral presentation).

Seyedalmoosavi S.M.M., Daş G., Mielenz M., Metges C.C. (2021). Black Soldier Fly larvae reared on feed mixed with recycled sewage sludge accumulate Ca and P. 4th Phosphorus in Europe Research Meeting (PERM) (Presented as poster).

Seyedalmoosavi S.M.M., Daş G., Mielenz M., Schleifer, K., Wolf, P., Tränckner, J., Metges C.C. (2021). Growth performance, body composition and mineral bio-accumulation of black soldier fly larvae reared on a fly diet supplemented with sewage sludge recyclates. Day of the Doctoral Student. Leibniz Institute for Farm Animal Biology (Oral presentation).

Seyedalmoosavi S.M.M., Daş G., Maak S., Mielenz M., Metges C.C., Wolf, P. (2022). Effects of different levels of whole black soldier fly larvae in broiler rations on bone characteristics. 76th Conference of the Society of Nutrition Physiology (Oral presentation).

Seyedalmoosavi S.M.M., Daş G., Dannenberger D., Maak S., Mielenz M., Wolf, P., Metges C.C. (2022). Whole black soldier fly larvae in broiler rations: impact on carcass characteristics, blood metabolites and fatty acids profiles in plasma, muscle and fat tissues. 76th Conference of the Society of Nutrition Physiology (Oral presentation).

Seyedalmoosavi S.M.M., Mielenz M., Daş G., Metges C.C. (2022). Broiler eating rate suggests preference for black soldier fly larvae (BSFL) over regular feed. 73rd European Federation of Animal Science (Oral presentation).

11.4. Declaration:

I hereby, declare under oath that I have completed the work submitted here independently and have composed it without outside assistance. Furthermore, I have not used anything other than the resources and sources stated and where I have taken sections from these works in terms of content or text, I have identified this appropriately.

Dummerstorf, 29. 06. 2023

Mohammad M. Seyedalmoosavi

12. References

Ali, H. and E. Khan. 2018. What are heavy metals? Long-standing controversy over the scientific use of the term 'heavy metals'–proposal of a comprehensive definition. Toxicological & Environmental Chemistry 100(1):6-19.

Amann, A., O. Zoboli, J. Krampe, H. Rechberger, M. Zessner, and L. Egle. 2018. Environmental impacts of phosphorus recovery from municipal wastewater. Resources, Conservation and Recycling 130:127-139.

Amrul, N. F., I. Kabir Ahmad, N. E. Ahmad Basri, F. Suja, N. A. Abdul Jalil, and N. A. Azman. 2022. A review of organic waste treatment using black soldier fly (Hermetia illucens). Sustainability 14(8), 4565.

Ao, Y., C. Yang, S. Wang, Q. Hu, L. Yi, J. Zhang,... and C.Yu. 2021. Characteristics and nutrient function of intestinal bacterial communities in black soldier fly (Hermetia illucens L.) larvae in livestock manure conversion. Microbial biotechnology 14(3), 886-896.

Arthurson, V. 2008. Proper sanitization of sewage sludge: a critical issue for a sustainable society. Applied and environmental microbiology 74(17):5267-5275.

Balolong, C. J. L., B. S. Jumawan, and E. C. Taer. 2020. Carcass quality evaluation of broilers fed with black soldier fly (Hermetia Illucens) larvae. J. Environ. Sci. Computer Sci. Eng. Technol. 9:272-280.

Bava, L., C. Jucker, G. Gislon, D. Lupi, S. Savoldelli, M. Zucali, and S. Colombini. 2019. Rearing of Hermetia Illucens on Different Organic By-Products: Influence on Growth, Waste Reduction, and Environmental Impact. Animals 9(6):289.

Bellezza Oddon, S., I. Biasato, A. Imarisio, M. Pipan, D. Dekleva, E. Colombino, M. T. Capucchio, M. Meneguz, S. Bergagna, and R. Barbero. 2021. Black Soldier Fly and Yellow Mealworm live larvae for broiler chickens: Effects on bird performance and health status. Journal of Animal Physiology and Animal Nutrition 105:10-18.

Bergfeldt, B., M. Tomasi Morgano, H. Leibold, F. Richter, and D. Stapf. 2018. Recovery of phosphorus and other nutrients during pyrolysis of chicken manure. Agriculture 8(12):187.

Biasato, I., S. Bellezza Oddon, G. Chemello, M. Gariglio, E. Fiorilla, S. Dabbou, M. Pipan, D. Dekleva, E. Macchi, and L. Gasco. 2022. Welfare implications for broiler chickens reared in an insect larvae-enriched environment: Focus on bird behaviour, plumage status, leg health, and excreta corticosterone. Frontiers in Physiology:1634.

Blokhuis, H. J., and P. R. Wiepkema. 1998. Studies of feather pecking in poultry. Veterinary Quarterly 20(1), 6-9.

Bol, R., G. Gruau, P.-E. Mellander, R. Dupas, M. Bechmann, E. Skarbøvik, M. Bieroza, F. Djodjic, M. Glendell, and P. Jordan. 2018. Challenges of reducing phosphorus based water eutrophication in the agricultural landscapes of Northwest Europe. Frontiers in Marine Science 5:276.

Bolan, N., A. Szogi, B. Seshadri, and T. Chuasavathi. 2010. The management of phosphorus in poultry litter. Nat Res 7.

Briggs, M. A., K. S. Petersen, and P. M. Kris-Etherton. 2017. Saturated fatty acids and cardiovascular disease: replacements for saturated fat to reduce cardiovascular risk. Page 29 in Proc. Healthcare. MDPI.

Broom, L. J., A. Monteiro, and A. Piñon. 2021. Recent advances in understanding the influence of zinc, copper, and manganese on the gastrointestinal environment of pigs and poultry. Animals 11(5):1276 DOI: https://doi.org/10.3390/ani11051276.

Bruno, D., M. Bonelli, F. De Filippis, I. Di Lelio, G. Tettamanti, M. Casartelli, D. Ercolini, and S. Caccia. 2019. The intestinal microbiota of Hermetia illucens larvae is affected by diet and shows a diverse composition in the different midgut regions. Appl. Environ. Microbiol. 85(2):e01864-01818.

Cammack, J. and J. Tomberlin. 2017. The impact of diet protein and carbohydrate on select life-history traits of the black soldier fly Hermetia illucens (L.)(Diptera: Stratiomyidae). Insects 8(2):56.

Cammack, J. A., C. D. Miranda, H. R. Jordan, and J. K. Tomberlin. 2021. Upcycling of manure with insects: current and future prospects. Journal of Insects as Food and Feed 7(5), 605-619.

Casartelli, M., M. Bonelli, D. Bruno, S. Caccia, G. Sgambetterra, S. Cappellozza, and G. Tettamanti. 2019. Structural and functional characterization of Hermetia illucens larval midgut. Frontiers in physiology 10:204.

Chande, S., and C. Bergwitz. 2018. Role of phosphate sensing in bone and mineral metabolism. Nature Reviews Endocrinology, 14(11): 637-655.

Chia, S. Y., C. M. Tanga, I. M. Osuga, X. Cheseto, S. Ekesi, M. Dicke, and J. J. van Loon. 2020. Nutritional composition of black soldier fly larvae feeding on agro-industrial by-products. Entomologia Experimentalis et Applicata 168(6-7):472-481.

Cho, S., C. Ryu, J. Yang, D. T. Mbiriri, C.-W. Choi, J.-I. Chae, Y.-H. Kim, K.-S. Shim, Y. J. Kim, and N.-J. Choi. 2013. Effect of conjugated linoleic acid feeding on the growth performance and meat fatty acid profiles in broiler: meta-analysis. Asian-Australasian journal of animal sciences 26(7):995.

Cieślik, B. and P. Konieczka. 2017. A review of phosphorus recovery methods at various steps of wastewater treatment and sewage sludge management. The concept of "no solid waste generation" and analytical methods. Journal of Cleaner Production 142:1728-1740.

Cohen, Y. 2009. Phosphorus dissolution from ash of incinerated sewage sludge and animal carcasses using sulphuric acid. Environmental Technology 30(11):1215-1226.

Cohen, Y., P. Enfält, and C. Kabbe. 2019. Proceedings of the International Fertiliser Society 832, Brussels, Belgium UK, 13th December, 2013. Pages 1-19 in Proc. Proceedings-International Fertiliser Society. International Fertiliser Society.

Commission Regulation (EU) No 68/2013 of 16 January 2013 on the Catalogue of Feed Materials. Available online: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32013R0068 (accessed on 16 September 2019).

Cross, H., H. Debiec, and M. Peterlik. 1990. Mechanism and regulation of intestinal phosphate absorption. Miner Electrolyte Metab 16(2-3):115-124.

Cutrignelli, M. I., M. Messina, F. Tulli, B. Randazzo, I. Olivotto, L. Gasco, R. Loponte, and F. Bovera. 2018. Evaluation of an insect meal of the Black Soldier Fly (Hermetia illucens) as soybean substitute: Intestinal morphometry, enzymatic and microbial activity in laying hens. Research in veterinary science 117:209-215.

Dabbou, S., F. Gai, I. Biasato, M. T. Capucchio, E. Biasibetti, D. Dezzutto, M. Meneguz, I. Plachà, L. Gasco, and A. Schiavone. 2018. Black soldier fly defatted meal as a dietary protein

source for broiler chickens: Effects on growth performance, blood traits, gut morphology and histological features. Journal of animal science and biotechnology 9(1):1-10.
Danisi, G. and R. W. Straub. 1980. Unidirectional influx of phosphate across the mucosal membrane of rabbit small intestine. Pflügers Archiv 385(2):117-122.
De Jong, I. C., and H. Gunnink. 2019. Effects of a commercial broiler enrichment programme with or without natural light on behaviour and other welfare indicators. Animal 13(2), 384-391.
De Smet, J., E. Wynants, P. Cos, and L. Van Campenhout. 2018. Microbial community dynamics during rearing of black soldier fly larvae (Hermetia illucens) and impact on exploitation potential. Applied and Environmental Microbiology 84(9):e02722-02717.
de Souza Vilela, J., N. M. Andronicos, M. Kolakshyapati, M. Hilliar, T. Z. Sibanda, N. R. Andrew, R. A. Swick, S. Wilkinson, and I. Ruhnke. 2021. Black Soldier Fly larvae in broiler diets improve broiler performance and modulate the immune system. Animal Nutrition 7(3):695-706.
Devereux, C., M. Smart, F. P. Kalt, and N. Takei. 1994. Animal feeds: Phosphate supplements. In CEH Marketing Research Report; HIS Chemical: Englewood, CO, USA.
DGE. 2000. Deutsche Gesellschaft für Ernährung e. V.
Diener, S., C. Zurbrügg, F. R. Gutiérrez, D. H. Nguyen, A. Morel, T. Koottatep, and K. Tockner. 2011. Black soldier fly larvae for organic waste treatment—prospects and constraints. Proceedings of the WasteSafe 2011:2nd.
Egle, L., H. Rechberger, J. Krampe, and M. Zessner. 2016. Phosphorus recovery from municipal wastewater: An integrated comparative technological, environmental and economic assessment of P recovery technologies. Science of the Total Environment 571:522-542.
Elahi, U., C.-c. Xu, J. Wang, J. Lin, S.-g. Wu, H.-j. Zhang, and G.-h. Qi. 2022. Insect meal as a feed ingredient for poultry. Animal Bioscience 35(2):332.
Ferrer Llagostera, P., Z. Kallas, L. Reig Puig, and D. Amores De Gea. 2019. The use of insect meal as aa sustainable feeding alternative in aquaculture: Current situation, Spanish consumers' perceptions and willingness to pay.
Fesharaki, M. and W. Rath. 2018. Verkohlungsanlage Pyreg. Europäisches Patentamt, Patentnummer: EP 3 358 253 A1.
Foley, J. A., N. Ramankutty, K. A. Brauman, E. S. Cassidy, J. S. Gerber, M. Johnston, N. D. Mueller, C. O'Connell, D. K. Ray, and P. C. West. 2011. Solutions for a cultivated planet. Nature 478(7369):337.
Gariglio, M., S. Dabbou, M. Crispo, I. Biasato, F. Gai, L. Gasco, F. Piacente, P. Odetti, S. Bergagna, and I. Plachà. 2019. Effects of the dietary inclusion of partially defatted black soldier fly (Hermetia illucens) meal on the blood chemistry and tissue (Spleen, Liver, Thymus, and Bursa of Fabricius) histology of muscovy ducks (Cairina moschata domestica). Animals 9(6):307.
Grummer, R., O. Bentley, P. Phillips, and G. Bohstedt. 1950. The role of manganese in growth, reproduction, and lactation of swine. Sci. J. Anim. Sci. 9(2):170-175 DOI: https://doi.org/10.2527/jas1950.92170x.
Havukainen, J., M. T. Nguyen, L. Hermann, M. Horttanainen, M. Mikkilä, I. Deviatkin, and L. Linnanen. 2016. Potential of phosphorus recovery from sewage sludge and manure ash by thermochemical treatment. Waste management 49:221-229.

Herzel, H., O. Krüger, L. Hermann, and C. Adam. 2016. Sewage sludge ash—A promising secondary phosphorus source for fertilizer production. Science of the Total Environment 542:1136-1143.

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

Hudcová, H., J. Vymazal, and M. Rozkošný. 2019. Present restrictions of sewage sludge application in agriculture within the European Union. Soil and Water Research 14(2):104-120.

Ipema, A. F., E. A. Bokkers, W. J. Gerrits, B. Kemp, and J. E. Bolhuis. 2022. Provision of black soldier fly larvae (Hermetia illucens) in different ways benefits broiler welfare and performance, with largest effects of scattering live larvae. Physiology & Behavior:113999.

Ipema, A. F., W. J. Gerrits, E. A. Bokkers, B. Kemp, and J. E. Bolhuis. 2020. Provisioning of live black soldier fly larvae (Hermetia illucens) benefits broiler activity and leg health in a frequency-and dose-dependent manner. Appl. Anim. Behav. Sci. 230:105082 DOI: https://doi.org/10.1016/j.applanim.2020.105082.

Kawasaki, K., Y. Hashimoto, A. Hori, T. Kawasaki, H. Hirayasu, S.-i. Iwase, A. Hashizume, A. Ido, C. Miura, and T. Miura. 2019. Evaluation of black soldier fly (Hermetia illucens) larvae and pre-pupae raised on household organic waste, as potential ingredients for poultry feed. Animals 9(3):98.

Klärschlammverordnung. 2017. Verordnung über die Verwertung von Klärschlamm, Klärschlammgemisch und Klärschlammkompost (Klärschlammverordnung) vom 27. September 2017 (BGBl. I S. 3465), die zuletzt durch Artikel 137 der Verordnung vom 19. Juni 2020 (BGBl. I S. 1328) geändert worden ist.

Lalander, C., Å. Nordberg, and B. Vinnerås. 2018. A comparison in product-value potential in four treatment strategies for food waste and faeces–assessing composting, fly larvae composting and anaerobic digestion. GCB Bioenergy 10(2):84-91 DOI: https://doi.org/10.1111/gcbb.12470.

Lalander, C. H., J. Fidjeland, S. Diener, S. Eriksson, and B. Vinnerås. 2015. High waste-to-biomass conversion and efficient Salmonella spp. reduction using black soldier fly for waste recycling. Agronomy for Sustainable Development 35, 261-271.

Lähteenmäki-Uutela, A., S. B. Marimuthu, and N. Meijer. 2021. Regulations on insects as food and feed: a global comparison. Journal of Insects as Food and Feed 7(5): 849-856.

Li, R., J. Yin, W. Wang, Y. Li, and Z. Zhang. 2014. Transformation of phosphorus during drying and roasting of sewage sludge. Waste management 34(7):1211-1216.

Li, X., D. Zhang, T. Y. Yang, and W. L. Bryden. 2016. Phosphorus bioavailability: a key aspect for conserving this critical animal feed resource with reference to broiler nutrition. Agriculture 6(2):25.

Liland, N. S., I. Biancarosa, P. Araujo, D. Biemans, C. G. Bruckner, R. Waagbø, B. E. Torstensen, and E.-J. Lock. 2017. Modulation of nutrient composition of black soldier fly (Hermetia illucens) larvae by feeding seaweed-enriched media. PloS one 12(8):e0183188.

Lopes, C., M. Herva, A. Franco-Uría, and E. Roca. 2011. Inventory of heavy metal content in organic waste applied as fertilizer in agriculture: evaluating the risk of transfer into the food chain. Environmental Science and Pollution Research 18(6):918-939.

Lu, S., N. Taethaisong, W. Meethip, J. Surakhunthod, B. Sinpru, T. Sroichak, P. Archa, S. Thongpea, S. Paengkoum, and R. A. P. Purba. 2022. Nutritional Composition of Black Soldier Fly Larvae (Hermetia illucens L.) and Its Potential Uses as Alternative Protein Sources in Animal Diets: A Review. Insects 13(9):831.

Macombe, C., S. Le Feon, J. Aubin, and F. Maillard. 2019. Marketing and social effects of industrial scale insect value chains in Europe: case of mealworm for feed in France. Journal of Insects as Food and Feed:1-10.

Maurer, V., M. Holinger, Z. Amsler, B. Früh, J. Wohlfahrt, A. Stamer, and F. Leiber. 2016. Replacement of soybean cake by Hermetia illucens meal in diets for layers. Journal of Insects as Food and Feed 2(2):83-90.

Mohee, R., and N. Soobhany. 2014. Comparison of heavy metals content in compost against vermicompost of organic solid waste: Past and present. Resources, Conservation and Recycling. 92, 206-213.

Moula, N., M.-L. Scippo, C. Douny, G. Degand, E. Dawans, J.-F. Cabaraux, J.-L. Hornick, R. C. Medigo, P. Leroy, and F. Francis. 2018. Performances of local poultry breed fed black soldier fly larvae reared on horse manure. Animal Nutrition 4(1):73-78.

Müller, A., D. Wolf, and H. O. Gutzeit. 2017. The black soldier fly, Hermetia illucens–a promising source for sustainable production of proteins, lipids and bioactive substances. Zeitschrift für Naturforschung C 72(9-10):351-363.

Nahm, K. 2007. Efficient phosphorus utilization in poultry feeding to lessen the environmental impact of excreta. World's Poultry Science Journal 63(4):625-654.

Nekrasov, R. V., G. A. Ivanov, M. G. Chabaev, A. A. Zelenchenkova, N. V. Bogolyubova, D. A. Nikanova, A. A. Sermyagin, S. O. Bibikov, and S. O. Shapovalov. 2022. Effect of Black Soldier Fly (Hermetia illucens L.) Fat on Health and Productivity Performance of Dairy Cows. Animals 12(16):2118.

Nguyen, T. T., J. K. Tomberlin, and S. Vanlaerhoven. 2015. Ability of black soldier fly (Diptera: Stratiomyidae) larvae to recycle food waste. Environmental entomology 44(2):406-410.

NRC. 1994. National Research Council (NRC). Nutrient Requirements of Poultry: ninth revised edition. National Academy Press, Washington, DC, USA.

Onsongo, V., I. Osuga, C. Gachuiri, A. Wachira, D. Miano, C. Tanga, S. Ekesi, D. Nakimbugwe, and K. Fiaboe. 2018. Insects for income generation through animal feed: effect of dietary replacement of soybean and fish meal with black soldier fly meal on broiler growth and economic performance. Journal of economic entomology 111(4):1966-1973.

Oonincx, D. G. and I. J. De Boer. 2012. Environmental impact of the production of mealworms as a protein source for humans–a life cycle assessment. PloS One 7(12):e51145 DOI: https://doi.org/10.1371/journal.pone.0051145.

Pahlow, M., P. Van Oel, M. Mekonnen, and A. Y. Hoekstra. 2015. Increasing pressure on freshwater resources due to terrestrial feed ingredients for aquaculture production. Science of the Total Environment 536:847-857.

Parodi, A., W. J. Gerrits, J. J. Van Loon, I. J. De Boer, A. J. Aarnink, and H. H. Van Zanten. 2021. Black soldier fly reared on pig manure: Bioconversion efficiencies, nutrients in the residual material, greenhouse gas and ammonia emissions. Waste Management 126, 674-683.

Patterson, P. H., P. A. Moore Jr, and R. Angel. 2005. Phosphorus and poultry nutrition. Phosphorus: Agriculture and the environment 46:635-682.

Pavlas, M., J. Dvořáček, T. Pitschke, and R.Peche. 2020. Biowaste Treatment and waste-to-energy—Environmental benefits. Energies. 13(8), 1994.
Penido, M. G. M. and U. S. Alon. 2012. Phosphate homeostasis and its role in bone health. Pediatric nephrology 27(11):2039-2048.
Proc, K., P. Bulak, D. Wiącek, and A. Bieganowski. 2020. Hermetia illucens exhibits bioaccumulative potential for 15 different elements–Implications for feed and food production. Science of The Total Environment 723:138125.
Proszkowiec-Weglarz, M. and R. Angel. 2013. Calcium and phosphorus metabolism in broilers: Effect of homeostatic mechanism on calcium and phosphorus digestibility. Journal of Applied Poultry Research 22(3):609-627.
Reyes-Torres, M., E. R.Oviedo-Ocaña, I. Dominguez, D. Komilis, and A. Sánchez. 2018. A systematic review on the composting of green waste: Feedstock quality and optimization strategies. Waste management. 77, 486-499.
Riber, A. B., H. A. Van De Weerd, I. C. De Jong, and S. Steenfeldt. 2018. Review of environmental enrichment for broiler chickens. Poultry science 97(2), 378-396.
Rousseau, X., M.-P. Létourneau-Montminy, N. Meme, M. Magnin, Y. Nys, and A. Narcy. 2012. Phosphorus utilization in finishing broiler chickens: effects of dietary calcium and microbial phytase. Poultry Science 91(11):2829-2837.
Ruark, M. D., K. A. Kelling, and L. W. Good. 2014. Environmental concerns of phosphorus management in potato production. American Journal of Potato Research 91(2):132-144.
Sabbagh, Y., H. Giral, Y. Caldas, M. Levi, and S. C. Schiavi. 2011. Intestinal phosphate transport. Advances in chronic kidney disease 18(2):85-90.
Salam, M., A. Shahzadi, H. Zheng, F. Alam, G. Nabi, S. Dezhi, W. Ullah, S. Ammara, N. Ali, and M. Bilal. 2022. Effect of different environmental conditions on the growth and development of Black Soldier Fly Larvae and its utilization in solid waste management and pollution mitigation. Environmental Technology & Innovation 28:102649.
Schiavone, A., M. De Marco, S. Martínez, S. Dabbou, M. Renna, J. Madrid, F. Hernandez, L. Rotolo, P. Costa, and F. Gai. 2017. Nutritional value of a partially defatted and a highly defatted black soldier fly larvae (Hermetia illucens L.) meal for broiler chickens: apparent nutrient digestibility, apparent metabolizable energy and apparent ileal amino acid digestibility. Journal of animal science and biotechnology 8(1):1-9.
Schiavone, A., S. Dabbou, M. De Marco, M. Cullere, I. Biasato, E. Biasibetti, M. Capucchio, S. Bergagna, D. Dezzutto, and M. Meneguz. 2018. Black soldier fly larva fat inclusion in finisher broiler chicken diet as an alternative fat source. Animal 12(10):2032-2039.
Schiavone, A., S. Dabbou, M. Petracci, M. Zampiga, F. Sirri, I. Biasato, F. Gai, and L. Gasco. 2019. Black soldier fly defatted meal as a dietary protein source for broiler chickens: Effects on carcass traits, breast meat quality and safety. Animal 13(10):2397-2405 DOI: https://doi.org/10.1017/S1751731119000685.
Schmitt, E., I. Belghit, J. Johansen, R. Leushuis, E.-J. Lock, D. Melsen, R. Kathirampatti Ramasamy Shanmugam, J. Van Loon, and A. Paul. 2019. Growth and safety assessment of feed streams for black soldier fly larvae: a case study with aquaculture sludge. Animals 9(4):189.
Seleiman, M. F., A. Santanen, and P. S. Mäkelä. 2020. Recycling sludge on cropland as fertilizer–Advantages and risks. Resources, Conservation and Recycling 155:104647.

Seyedalmoosavi, M. M., M. Mielenz, T. Veldkamp, G. Daş, and C. C. Metges. 2022b. Growth efficiency, intestinal biology, and nutrient utilization and requirements of black soldier fly (Hermetia illucens) larvae compared to monogastric livestock species: a review. Anim. Sci. Biotechnol. 13(1):1-20 DOI: https://doi.org/10.1186/s40104-022-00682-7.

Seyedalmoosavi, M., M. Mielenz, S. Görs, P. Wolf, G. Daş, and C. Metges. 2022a. Effects of increasing levels of whole Black Soldier Fly (Hermetia illucens) larvae in broiler rations on acceptance, nutrient and energy intakes and utilization, and growth performance of broilers. Poultry Science 101(12):102202.

Sheppard, D. C., G. L. Newton, S. A. Thompson, and S. Savage. 1994. A value added manure management system using the black soldier fly. Bioresource technology 50(3):275-279.

Sheppard, D. C., J. K. Tomberlin, J. A. Joyce, B. C. Kiser, and S. M. Sumner. 2002. Rearing methods for the black soldier fly (Diptera: Stratiomyidae). Journal of Medical Entomology 39(4):695-698.

Shumo, M., I. M. Osuga, F. M. Khamis, C. M. Tanga, K. K. Fiaboe, S. Subramanian, S. Ekesi, A. van Huis, and C. Borgemeister. 2019. The nutritive value of black soldier fly larvae reared on common organic waste streams in Kenya. Scientific reports 9(1):1-13.

Smetana, S., M. Palanisamy, A. Mathys, and V. Heinz. 2016. Sustainability of insect use for feed and food: Life Cycle Assessment perspective. Journal of Cleaner Production 137:741-751.

Spartano, S. and S. Grasso. 2021. Consumers' perspectives on eggs from insect-fed hens: a UK focus group study. Foods 10(2):420.

Spranghers, T., M. Ottoboni, C. Klootwijk, A. Ovyn, S. Deboosere, B. De Meulenaer, J. Michiels, M. Eeckhout, P. De Clercq, and S. De Smet. 2017. Nutritional composition of black soldier fly (Hermetia illucens) prepupae reared on different organic waste substrates. Journal of the Science of Food and Agriculture 97(8):2594-2600.

Star, L., T., Arsiwalla, F., Molist, R., Leushuis, M., Dalim, and A. Paul. 2020. Gradual provision of live black soldier fly (Hermetia illucens) larvae to older laying hens: effect on production performance, egg quality, feather condition and behavior. Animals 10(2), 216.

Sumbule, E. K., M. K. Ambula, I. M. Osuga, J. G. Changeh, D. M. Mwangi, S. Subramanian, D. Salifu, P. A. Alaru, M. Githinji, and J. J. Van Loon. 2021. Cost-effectiveness of black soldier fly larvae meal as substitute of fishmeal in diets for layer chicks and growers. Sustainability 13(11):6074.

Surendra, K., R. Olivier, J. K. Tomberlin, R. Jha, and S. K. Khanal. 2016. Bioconversion of organic wastes into biodiesel and animal feed via insect farming. Renewable energy 98:197-202.

Swiatkiewicz, S., M. Swiatkiewicz, A. Arczewska-Wlosek, and D. Jozefiak. 2015. Chitosan and its oligosaccharide derivatives (chito-oligosaccharides) as feed supplements in poultry and swine nutrition. Journal of animal physiology and animal nutrition 99(1), 1-12.

Tao, X. and X. Huang. 2007. Releasing characteristics of phosphorus and other substances during thermal treatment of excess sludge. Journal of Environmental Sciences 19(10):1153-1158.

Tchounwou, P. B., C. G. Yedjou, A. K. Patlolla, and D. J. Sutton. 2012. Heavy metal toxicity and the environment. Molecular, clinical and environmental toxicology:133-164.

Tschirner, M. and A. Simon. 2015. Influence of different growing substrates and processing on the nutrient composition of black soldier fly larvae destined for animal feed. Journal of Insects as Food and Feed 1(4):249-259.

United Nations. 2015. Transforming our world: The 2030 agenda for sustainable development. New York: United Nations, Department of Economic and Social Affairs.

United-Nations. 2019. Department of Economic and Social Affairs, Population Devision (2019). World Population prospects 2019: Highlights (ST/ESA/SER.A/423).
Ushakova, N., A. Dontsov, N. Sakina, I. Ratnikova, N. Gavrilova, N. Y. Garmash, A. Bastrakov, and A. Kozlova. 2018. Melanin and melanogenesis at different life stages in Hermetia illucens. Biol. Bull. 45(1):47-50 DOI: https://doi.org/10.1134/S1062359018010120.
Van Huis, A. 2013. Potential of insects as food and feed in assuring food security. Annual review of entomology 58:563-583.
Van Huis, A., H. Van Gurp, and M. Dicke. 2014. The insect cookbook: food for a sustainable planet. Columbia University Press.
Van Huis, A., J. Van Itterbeeck, H. Klunder, E. Mertens, A. Halloran, G. Muir, and P. Vantomme. 2013. Edible insects: future prospects for food and feed security. No. 171. Food and Agriculture Organization of the United Nations.
Visioli, F. and A. Poli. 2020. Fatty acids and cardiovascular risk. Evidence, lack of evidence, and diligence. Nutrients 12(12):3782.
Wang, Y.-S. and M. Shelomi. 2017. Review of black soldier fly (Hermetia illucens) as animal feed and human food. Foods 6(10):91.
Wu, N., X. Wang, X. Xu, R. Cai, and S. Xie. 2020. Effects of heavy metals on the bioaccumulation, excretion and gut microbiome of black soldier fly larvae (Hermetia illucens). Ecotoxicology and Environmental Safety 192:110323.
Wuhan, C. P. 2018. The 2nd International Conference 'Insects to Feed the World'(IFW 2018). Journal of Insects as Food and Feed 4:1.
Xia, J., C. Ge, and H. Yao. 2021. Antimicrobial peptides from black soldier fly (Hermetia illucens) as potential antimicrobial factors representing an alternative to antibiotics in livestock farming. Animals 11(7):1937.
Yan, F., R. Angel, and C. Ashwell. 2007. Characterization of the chicken small intestine type IIb sodium phosphate cotransporter. Poultry science 86(1):67-76.
Zharkova, M. S., D. S. Orlov, O. Y. Golubeva, O. B. Chakchir, I. E. Eliseev, T. M. Grinchuk, and O. V. Shamova. 2019. Application of antimicrobial peptides of the innate immune system in combination with conventional antibiotics–a novel way to combat antibiotic resistance? Frontiers in cellular and infection microbiology 9:128.
Zhou, F., J. K. Tomberlin, L. Zheng, Z. Yu, and J. Zhang. 2013. Developmental and waste reduction plasticity of three black soldier fly strains (Diptera: Stratiomyidae) raised on different livestock manures. Journal of medical entomology 50(6):1224-1230.