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RESEARCH ARTICLE

Can black soldier fly larvae (*Hermetia illucens*) be reared on waste streams for food and feed? – A safety perspective

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Abstract

The use of insects as feed and food can be part of the solution towards a circular economy, in case the safety of insect products is assured. Black soldier fly larvae (BSFL, *Hermetia illucens*) can be reared on different waste streams. However, before BSFL can be legally reared on these streams, the safety of BSFL for feed and food should be assessed thoroughly. This study aimed to investigate several food safety aspects of BSFL grown on waste streams. Therefore, BSFL were reared for 7 days on substrate mixtures of waste streams with similar protein and moisture content. These waste streams included fast food waste (FF), mushroom stem (MS), pig manure solids (PS), poultry meal (PM) and slaughter waste (SW). The substrates, BSFL and the frass were analysed for the presence of metals and veterinary drugs. The substrates and BSFL were also analysed for presence of DNA of ruminant, pig and chicken. Some of the metals accumulated in BSFL, although the concentrations in BSFL (as these would be manufactured as feed) were below maximum limits for feed. Only traces of some of the analysed veterinary drugs were found in the BFSL and no accumulation thereof was observed. DNA of ruminant and pig was traced back in BSFL samples, however, chicken was not. A good understanding of the presence of food safety hazards and possible variance thereof in potential substrates, such as waste streams, and their possible residues in insects is necessary for implementation of this circular way of insect feeding in the food chain.

Keywords

insect – veterinary drugs – metals – DNA – feed

1 Introduction

The acknowledgment of the need for a circular food system has been growing the last decade. The world population is still growing, while the amount of arable land will remain the same or might even shrink. Feed and food resources will become relatively more scarce in the future (OECD *et al.*, 2022; van Huis *et al.*, 2013). During the production of feed and food, materials are left over; the so-called waste streams. To move towards a circular food system, a valuable purpose for these waste streams should be sought. By using these waste

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streams, not only is the amount of waste minimised, but it could also help the feed and food industry in reattributing their resources. This gives the movement towards a circular food system also an economic incentive for the feed and food industry (Maroušek *et al.*, 2023) or can strengthen the feed and food industry in low-income countries (Chaalala *et al.*, 2018). Insects and in particular black soldier fly larvae (BSFL, *Hermetia illucens* (L.), Diptera: Stratiomyidae) can be reared on low value waste streams and can be used as a high-value protein source (Magee *et al.*, 2021; Maroušek *et al.*, 2023; Spranghers *et al.*, 2017). This implies that BSFL have a high bioconversion rate and have the capability to valorise these waste streams (Cadinu *et al.*, 2020). Moreover, rearing of BSFL has capability of up-scaling and industrialising and can therefore also serve an economic advantage compared to conventional feed materials (Cadinu *et al.*, 2020; Maroušek *et al.*, 2023). However, before BSFL can be legally reared on such streams, the safety of the final product should be assessed thoroughly, amongst others. Therefore, on the one hand, there is the challenge of feed and food security, on the other hand there is the opportunity to use waste streams for circular feed and food production through insect rearing (Ojha *et al.*, 2020).

Some food safety hazards can be present in waste streams and may end up in the BSFL. Meyer *et al.* (2021) presented an overview of a variety of chemical hazards that can be present in substrates and can accumulate in insects. In general, the presence of food safety hazards in substrates is very much dependent on the origin of the particular substrate.

Certain heavy metals and metalloids are known to accumulate in BSFL. Especially cadmium has been shown to accumulate in BSFL (Biancarosa *et al.*, 2018; Diener *et al.*, 2015; van der Fels-Klerx *et al.*, 2016; van der Fels-Klerx *et al.*, 2020). Veterinary drugs are another type of chemical hazards that can be present in waste streams from animal materials and in animal manure (Berendsen *et al.*, 2015; Hoek-van den Hil *et al.*, 2022; Massé *et al.*, 2014). In the European Union (EU), several waste streams cannot be used as feed for farmed animals, including reared insects, but certain exemptions have been implemented in recent years. From 2017, farmed insects are allowed to be used as feed for aquaculture and as of 2021 as feed for pigs and poultry. According to EU legislation (Regulation (EC) No 999/2001, Art. 7), it is neither allowed to feed ruminants with animal protein including insects, nor are ruminant processed animal proteins (PAPs) allowed to be fed to non-ruminants. PAPs from pigs may also be fed to poultry, and vice versa, but such materials may not be used as substrates for reared insects due to concerns over carry-over of animal proteins from substrate via insects to target animals. Legislation in Australia has a similar approach towards the use of animal by-products. In Australia, it is allowed to use insects as feed for aquatic animals as well as feed for pigs and poultry (in Queensland and South-Australia), provided that these are not reared on animal by-products (DiGiacomo, 2023). Also in China and Japan, it is not allowed to feed ruminants with animal proteins, because of the risk of transferrable spongiform encephalopathy (Renna *et al.*, 2023). In Canada and the United States, specific legislation for the use of insects in feed is lacking (Larouche *et al.*, 2023; Renna *et al.*, 2023).

In the absence of carry-over of animal proteins from substrate to insects, permission of this practice could be considered. This could provide a major contribution to the sustainability potential of reared insects in a circular feed and food system. In order to know if BSFL reared on these waste streams can be used as feed, carry-over of animal DNA was investigated in this study.

The objective of this study was to investigate the food safety with a focus on metals, veterinary drugs and intra-species transfer, of BSFL reared on different types of waste streams for BSFL rearing. This study contributes to the collection of data on the safety of BSFL reared on waste streams for the application as feed.

2 Materials and methods

Insect rearing experiment

In short, five days old and commercially reared BSFL were reared for seven days on substrates prepared from several waste streams (Table 1), which were airtight packed and stored at 4 °C. Before the start of the insect rearing experiment, crates with the sizes 75 × 47 × 15 cm were filled with 10 kg substrate and were positioned one day prior to the rearing experiment in the rearing environment to acclimatise. Six different treatments were used, and 3 crates were used for each treatment. Mixtures of waste streams were used to obtain comparable nutritional compositions in terms of amount of protein and moisture content. The exact diet nutrient composition and larval performance, yield and composition are presented by Naser El Deen et al. (under preparation). The rearing was executed in a dark room with controlled temperature and relative humidity, set at 30 °C and 60%, respectively. Substrate (n = 3), and after seven days of rearing, larvae (n = 3) and frass (n = 3)

TABLE 1 Overview of content of the substrates, which were similar in crude protein (~20%) and moisture content (~70%), used in this study to rear BSFL (in percentages)

Substrate code	Substrate
FF	Fast food[1] (56%), cellulose (2%), water (42%)
FF+PS	Fast food (28%), pig manure solids[2] (53%), water (19%)
FF+MS+PM	Fast food (28%), mushroom stem[3] (63%), poultry meal[4] (3%), cellulose (5%)
FF+MS+SW	Fast food (29%), mushroom stem (33%), slaughter waste[5] (34%), cellulose[6] (4%)
PS+MS+PM	Pig manure solids (54%), mushroom stem (37%), poultry meal (4%), cellulose (6%)
PS+MS+SW	Pig manure solids (54%), mushroom stem (6%), slaughter waste (36%), cellulose (4%)

[1]Fast food included leftovers bread, fries, meat products and vegetables. Source: McDonald's restaurants (The Netherlands); [2]Source: Van Beek SPF Varkens B.V. (Lelystad, The Netherlands); [3]Source: CNC Grondstoffen BV (Milsbeek, The Netherlands); [4]Source: Esbro (Doetinchem, The Netherlands); [5]Source: Sonac Burgum B.V. (Burgum, The Netherlands); [6]Source: VWR International (Amsterdam, The Netherlands).

samples were collected from each crate (random spot in the crate) and weighed. Hereafter, the mass balance and dry matter was determined, which is described in more detail by Naser El Deen *et al.* (under preparation). The larvae were washed with tap water, gently dried with a paper tissue and immediately after harvesting frozen at –20 °C (Naser El Deen *et al.*, under preparation). Homogenisation of samples before undergoing all the analyses mentioned in the following sections consisted of cryogenic grinding. The focus of this article is on feed and food safety, therefore all the results of the safety analyses will be discussed in this paper.

Heavy metals and trace elements analyses

Elemental analysis was performed by ICP-MS using an ISO17025 accredited procedure. This method is equivalent to EN 17053:2018, but was expanded with nickel. Samples were pre-treated using acid digestion with a microwave oven (MARS express, CEM Corporation, Matthews, NC, USA). For the microwave digestion 10 mL of concentrated nitric acid (67-70% RS-Superpure nitric acid, Carlo Erba, Val de Reuil, France) was added to 0.8 g of homogenised sample in Teflon digestion vessels. The samples were digested in the microwave oven at a temperature of 210 °C. The digests were quantitatively transferred to 50 ml polypropylene (PP) centrifuge tubes (Greiner Bio-One, Frickenhausen, Germany) and diluted with de-ionised water to a final volume of 50 ml. The digests were diluted 5-fold and 200-fold prior to analysis with a final acid concentration of 2.8% v/v. Concentrations of 14 elements were determined using ICP-QQQ-MS (iCAP TQ, Thermo Scientific, Waltham, MA, USA). Samples were introduced into the ICP-MS using an ASX-520 autosampler (Teledyne CETAC, Omaha, NE, USA). The ICP-MS was equipped with a PFA-ST3 nebuliser, a quartz cyclonic spray chamber and a quartz torch and injector. Nickel cones were used with a high-matrix insert. Eight elements were analysed in single quadrupole KED (kinetic energy discrimination) mode using helium as a collision gas at a flow rate of 4.675 mL/min. These elements were analysed using the following isotopes: ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{66}Zn, ^{98}Mo, ^{112}Cd. Germanium (m/z 74) was used as internal standard. Arsenic and selenium were analysed in triple quadrupole mass-shift mode using oxygen as a reaction gas with a flow rate of 0.5 mL/min. For arsenic Q1 was set to m/z 75 and Q3 to m/z 91, whereas for selenium Q1 was set to m/z 80 and Q3 to m/z 96. Germanium (Q1 and Q3 set to m/z 74) was used as an internal standard. The determination of mercury (m/z 201) and lead (sum of m/z 206, 207 and 208) was performed in single quadrupole standard (no-gas) using bismuth at m/z 209 as the internal standard. Concentrations were determined using external calibration curves with rhodium as internal standard. Certified reference material BCR 482 lichen was used for quality control.

The zinc levels in some of the sample digests were reanalysed using an ISO17025 accredited procedure. The determination of zinc was performed using a flame atomic absorption spectrophotometer (FAAS, AAnalyst 800, Perkin Elmer, Waltham, MA, USA) with an air-acetylene flame. Zinc was measured at a wavelength of 213.9 nm and background correction was applied using a deuterium lamp. Concentrations were determined using external calibration curves and certified reference material BCR 482 lichen was used for quality control. An overview of all tested heavy metals and trace elements and the limits of reporting for these are provided in Supplementary Table S1.

Veterinary drugs analyses

Residues of antibiotics, antiparasitic compounds and coccidiostats were analysed. Antibiotic residues were analysed with an LC-MS/MS method. Sample analyses were performed according to Hoek-van den Hil *et al.* (2022) with some slight modifications. The gradient was slightly adapted for this study compared to the previous study by (Hoek-van den Hil *et al.*, 2022). Operating at a flow rate of 0.3 mL/min, the used gradient was: 0-0.5 min, 1% B, 0.5-2.5 min, a linear increase to 25% B, 2.5-5.4 min a linear increase to 70% B, 5.4-5.5 min a linear increase to 100% B with a final hold of 1.0 min and an equilibration time of 1 min.

The antiparasitic compounds were also analysed with an LC-MS/MS method according to the method used in the study by Hoek-van den Hil *et al.* (2022).

For the analyses of coccidiostats, a similar procedure as for the antiparasitic compounds as described by Hoek-van den Hil *et al.* (2022) was carried out. Only the flow rate and gradient were slightly adapted. These analyses were operating at a flow of 0.4 mL/min, the used gradient was: 0-1.0 min, 0% B, 1.0-2.5 min, a linear increase to 45% B, 2.5-8.5 min a linear increase to 100% B with a final hold of 3.0 min and an equilibration time of 2.5 min. The first screening analyses on veterinary drugs included 91 compounds in total. An overview of limits of quantification of all tested veterinary drugs is provided in Supplementary Table S1.

DNA analyses

In order to see if proteins of other animals are present in the reared insects, DNA analyses for animal DNA of ruminant, pig and chicken were performed. For the isolation of DNA from insects and their feed, the procedure for isolating DNA from ruminant Processed Animal Protein (PAPs) as described by the 'EURL-AP SOP DNA extraction' was followed (http://www.eurl.craw.eu/wp-content/uploads/2021/01/EURL-AP-SOP-DNA-extraction-V1.1.pdf). This method entails the DNA isolation of 100 mg material in duplicate from ground material according to an adaptation of the DNA extraction using the "Wizard® Magnetic DNA purification system for Food" kit.

The DNA detection methods that were applied are ruminant (EURL-AP SOP Ruminant PCR: https://www.eurl.craw.eu/wp-content/uploads/2021/05/EURL-AP-SOP-Ruminant-PCR-V1.3.pdf) and pig (EURL-AP SOP pig PCR: https://www.eurl.craw.eu/wp-content/uploads/2021/09/EURL-AP-SOP-Pig-PCR-V1.0.pdf). Since the EURL-AP detection method for chicken/turkey was not officially released at the time of the experiments, a detection method for chicken, targeting the mitochondrial D-loop (Pegels *et al.*, 2012) was applied.

In short, a qPCR was performed on the two DNA isolations (on undiluted and 10x diluted sample) including appropriate controls. The sample is considered positive when the signal appears before the determined cut-off as established according to the respective EURL-AP SOPs. The methods are fully validated and performed under accreditation. WFSR participates in the yearly organised Proficiency Tests organised by the EURL-AP, and the cut-off is established yearly as specified by the EURL-AP (https://www.eurl.craw.eu/legal-sources-and-sops/method-of-reference-and-sops/) using the calibrants provided by the EURL-AP. The qPCR for chicken was performed as the EURL methods, but the samples were considered positive when the Cq value was earlier as the established cut-off. The cut-off values for these methods are provided in Supplementary Table S1.

The protocols describe the real-time PCR procedure for the detection of target DNA in a feed sample. Here, the ruminant, pig or chicken is present in a feed as PAP. qPCRs in this work were performed on untreated insects including their gut contents.

Calculation of bioaccumulation factor

The bioaccumulation factor (BAF) was calculated as the concentration of the contaminant corrected for the moisture content in larvae divided by concentration of the contaminant corrected for moisture content in substrate.

3 Results

Heavy metals and trace elements

Concentrations of arsenic, cadmium, lead and manganese in substrate, BSFL and frass are depicted in Figure 1. Arsenic and lead were detected in both BSFL and frass as well in some of the substrates, but concentrations in BSFL were relatively low. However, as can be seen from Figure 1b, BSFL contained higher concentrations of cadmium as compared to the substrates and frass for all treatments. This suggests accumulation of cadmium in BSFL, which is also indicated by the BAF values between 2.9 and 3.4 for cadmium (Table 2).

Arsenic, cadmium, lead, manganese, iron, zinc, copper and selenium accumulated in BSFL (BAF > 1) for one or more treatments. The concentrations of these heavy metals and trace elements are shown in Table 2. Manganese levels in BSFL were also higher than in the respective substrate, showing accumulation of man-

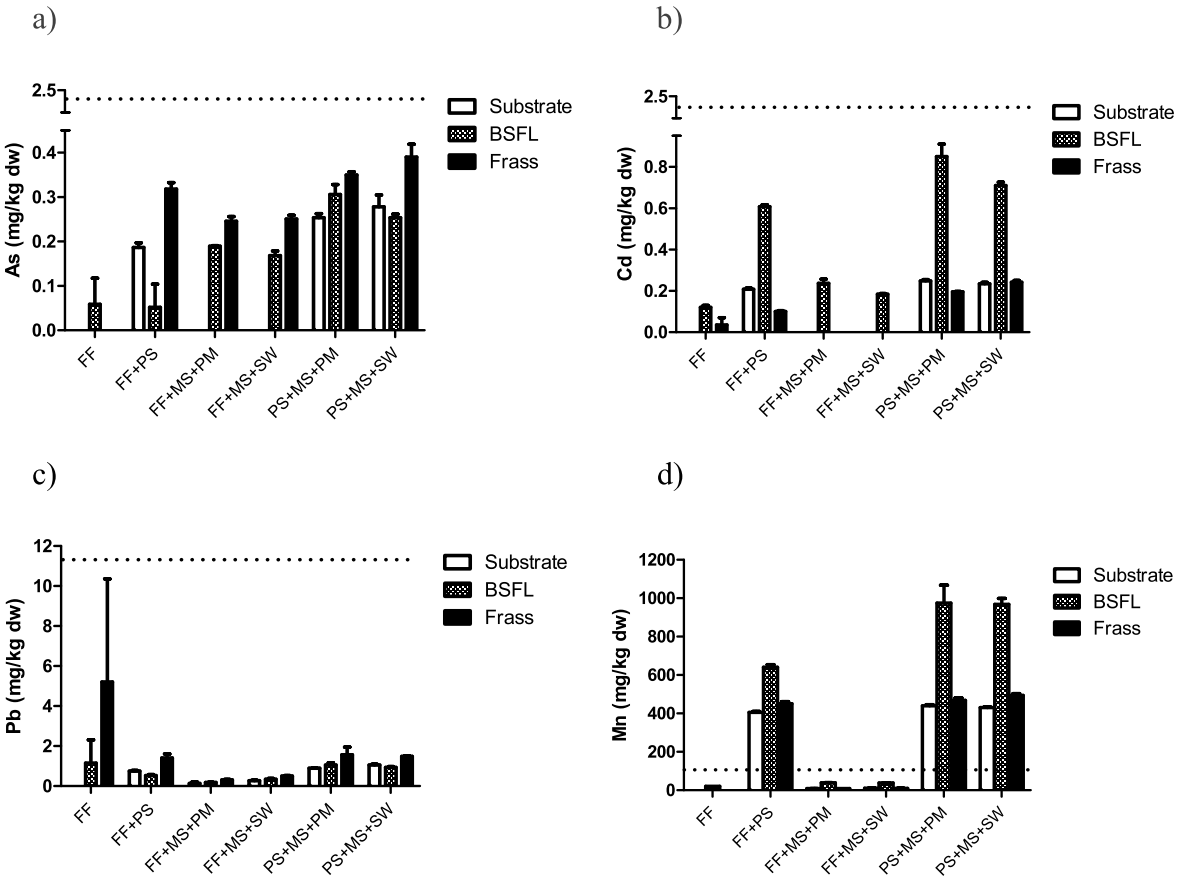

FIGURE 1 Mean concentrations (mg/kg) of a) arsenic, b) cadmium, c) lead and d) manganese in substrate, black soldier fly larvae and frass samples from the different treatments. Data are presented as mean ± SD (n = 3) on dry weight basis and values below limit of reporting are not depicted. The dotted horizontal line represents the EU maximum limit in feed calculated on dry weight basis (the y-axes for a) arsenic and b) cadmium are segmented).

ganese in BSFL reared on several substrates with a BAF varying from 1.6 to 4.7. Furthermore, BSFL reared on solely FF showed a BAF of 5.6 for copper and 2.2 for iron. BSFL reared on FF+PS showed to accumulate selenium. Heavy metals and trace elements that did not accumulate in BSFL (BAF < 1) were mercury, cobalt, nickel, molybdenum, thallium and uranium. Results for these heavy metals and trace elements are presented in Supplementary Table S3.

Veterinary drugs

Samples of both substrate and BSFL reared thereon as well as the frass were analysed to determine the presence of three type of veterinary drugs classes, being antibiotics, antiparasitic substances and coccidiostats. A first, broad screening showed that residues of antibiotics and coccidiostats were present in some of the substrate component, however, no residues of antiparasitic substances were found. Only doxycycline (DOX) and oxytetracycline (OTC) were found in pig manure, slaughter waste and poultry waste as a result of the first, broad screening on the presence of the antibiotic compounds in substrate components. Therefore, DOX and OTC were analysed in the mixed substrate samples, BSFL samples and frass samples with a targeted analysis. Small traces of DOX and OTC were detected in some BSFL samples. However, levels were too low for quantification (LOQ provided in Supplementary Table S1). The same counts for the traces found in some of the substrate samples and frass samples (all below LOQ).

From the 15 coccidiostats that were included in the screening analyses, five different coccidiostats (DNC (marker of nicarbazin), decoquinate, monensin, narasin and salinomycin) were detected in the substrate component slaughter waste and two of these compounds (DNC and salinomycin) also in poultry waste. Subsequently, the substrates, BSFL and frass were subject to targeted analyses on coccidiostats (Table 3). None of the analysed coccidiostats were present in the samples for the treatment FF+PS. Further, other coccidiostats were also not detected in samples for some treatments. The results

TABLE 2 Mean concentrations (mg/kg, n = 3) of heavy metals and trace elements (for bioaccumulation factor > 1) in substrate, black soldier fly larvae and frass samples based on dry weight, followed by its bioaccumulation factor

Metal	Treatment	Substrate (Mean ± SD)	BSFL (Mean ± SD)	Frass (Mean ± SD}	BAF
As	PS+MS+PM	0.25 ± 0.02	0.31 ± 0.04	0.35 ± 0.01	1.2
Cd	FF+PS	0.21 ± 0.01	0.61 ± 0.02	0.10 ± 0.01	2.9
	PS+MS+PM	0.25 ± 0.01	0.85 ± 0.11	0.20 ± 0.00	3.4
	PS+MS+SW	0.23 ± 0.01	0.71 ± 0.03	0.24 ± 0.01	3.0
Pb	FF+MS+PM	0.14 ± 0.12	0.18 ± 0.01	0.32 ± 0.03	1.4
	FF+MS+SW	0.28 ± 0.03	0.35 ± 0.06	0.50 ± 0.04	1.3
	PS+MS+PM	0.89 ± 0.04	1.06 ± 0.18	1.56 ± 0.67	1.2
Mn	FF+PS	405.6 ± 9.2	640.8 ± 22.4	450.5 ± 19.9	1.6
	FF+MS+PM	8.3 ± 0.53	38.9 ± 1.84	8.5 ± 0.38	4.7
	FF+MS+SW	10.6 ± 1.24	37.7 ± 0.74	9.6 ± 1.19	3.6
	PS+MS+PM	439.4 ± 10.2	974.5 ± 160.9	467.9 ± 22.1	2.2
	PS+MS+SW	431 ± 2.91	967.5 ± 53.6	494 ± 14.0	2.2
Fe	FF	28.9 ± 2.57	63.1 ± 2.60	35.9 ± 8.4	2.2
Zn	FF	40.7 ± 0.40	67.0 ± 2.01	59.8 ± 3.95	1.7
	FF+MS+PM	37.6 ± 3.12	74.3 ± 0.26	58.0 ± 1.33	2.0
	FF+MS+SW	55.2 ± 1.08	97.0 ± 3.03	80.3 ± 2.26	1.8
	PS+MS+PM	483.6 ± 12.8	510.4 ± 56.5	636.9 ± 32.0	1.1
Cu	FF	2.06 ± 0.15	11.6 ± 10.2	4.98 ± 4.66	5.6
	FF+MS+PM	6.3 ± 1.02	7.7 ± 0.26	8.3 ± 0.27	1.2
Se	FF+PS	0.53 ± 0.04	0.33 ± 0.06	0.92 ± 0.02	3.3
	FF+MS+PM	0.24 ± 0.21	0.31 ± 0.02	0.57 ± 0.06	1.3
	PS+MS+PM	0.72 ± 0.05	0.80 ± 0.09	0.98 ± 0.06	1.1

TABLE 3 Mean concentrations (μg/kg, n = 3) of coccidiostat residues in substrates, black soldier fly larvae and frass samples from the different treatments based on dry weight

Coccidiostat	Treatment	Substrate (Mean ± SD)	BSFL (Mean ± SD)	Frass (Mean ± SD)
Ponazuril	PS+MS+PM	3.1 ± 0.8	<LOQ	4.1 ± 0.7
	PS+MS+SW	3.5 ± 0.6	<LOQ	4.7 ± 0.7
DNC	FF+MS+SW	4.7 ± 8.1	<LOQ	20.00 ± 26.5
	PS+MS+SW	<LOQ	1.0 ± 0.1	2.8 ± 0.5
Decoquinate	FF+MS+SW	<LOQ	<LOQ	13.7 ± 3.9
	PS+MS+SW	<LOQ	<LOQ	0.9 ± 1.6
Narasin	FF+MS+SW	<LOQ	<LOQ	12.5 ± 7 .3
Salinomycin	FF+MS+SW	<LOQ	<LOQ	3.9 ± 3.3

for the samples for which no residues were detected are not provided in Table 3.

Similar to the antibiotic residues, residues of coccidiostats in the samples of this study were generally low, often only small traces were found that could not be quantified in most cases. The highest levels of coccidiostats were found in the frass samples. The coccidiostats detected in substrate samples were ponazuril and DNC (marker of nicarbazin), however, the concentrations found were just above the LOQ (2.5 μg/kg and 1.0 μg/kg, respectively, also provided in Supplementary Table S1). These trace residues of these coccidiostats were not detected in BSFL reared on the respective substrates. DNC (marker of nicarbazin) was the only coccidiostat that was detected in quantifiable amounts in BSFL samples in this study, which were reared on PS+MS+SW. However, the amounts quantified were around the LOQ (1.0 μg/kg). For some coccidiostat-matrix combinations, it was not possible to determine the presence of the respective coccidiostat. This could be due to the fact

TABLE 4 Overview of qualitative results of DNA analyses of ruminant, pig and poultry on substrates and black soldier fly larvae. + means positive for the respective DNA, – means negative and blank means not analysed

Treatments	Substrates			BSFL		
	Ruminant DNA	Pig DNA	Chicken DNA	Ruminant DNA	Pig DNA	Chicken DNA
FF (n = 3)	+	+	+	+	+	–
PS+MS+PM (n = 3)		+	+		+ (n = 2) – (n = 1)	–
PS+MS+SW (n = 3)		+	+		+	–

that these matrices of biowaste streams as substrates, BSFL and frass are different from commonly tested matrices.

DNA

DNA analyses have been performed on the substrates FF, PS+MS+PM, PS+MS+SW, and BSFL reared on these substrates as showed in Table 4. The analyses of ruminant DNA were only performed on the substrate FF, because cattle is the only ruminant expected to be present in this substrate, and possibly on BSFL reared on this substrate. The results were positive for both FF substrate and BSFL.

Pig DNA was detected in all these substrates and subsequently also in BSFL reared on these substrates, except for one insect sample with the treatment PS+MS+PM. Conversely, all the substrate samples were positive for chicken DNA, but no chicken DNA was detected in BSFL reared thereon.

4 Discussion

This study investigated the safety of BSFL, which were reared on mixtures of different waste streams. Higher levels of some metals were found in BSFL compared to the levels found in the substrates, as can also be seen in the calculated BAF values (>1), indicating that some metals accumulated in BSFL. Accumulation in BSFL was observed for the metals cadmium, manganese, iron, zinc, copper and selenium in BSFL in some of the treatments. The BAF for the metals arsenic and lead were around the value of 1, indicating no accumulation of these heavy metals. In a study on the conversion of municipal primary sewage sludge by BSFL, it was also observed that cadmium, manganese, copper and zinc accumulated in BSFL (Arnone *et al.*, 2022). On the contrary, whereas lead did not accumulate in the samples of this study, accumulation of lead was observed in the study with municipal primary sewage sludge (Arnone *et al.*, 2022). None of the other metals investigated in that study, being arsenic, cobalt, chromium, iron, potassium, molybdenum and nickel accumulated in BSFL (Arnone *et al.*, 2022). The metals cadmium, copper, chromium, lead and zinc were also analysed in substrates and BSFL by Elechi *et al.* (2021). In that study, the accumulation of a metal in BSFL varied per substrate. Nonetheless, accumulation of cadmium and chromium was observed in BSFL reared on the substrates food waste and fruit waste. Accumulation of lead was observed in BSFL reared on chicken mash. A slight accumulation of copper and lead (BAF=1.18 and 1.07, respectively) was observed in BSFL reared on brewery waste (Elechi *et al.*, 2021).

Despite the accumulation of some metals (up to 5 times) in BSFL during this study, the concentrations in BSFL samples were well below the EU maximum limits set for some metals in animal feed calculated on 12% moisture content (calculated, data not given). Maximum limits are laid down in Directive 2002/32/EC on undesirable substances in animal feed and are 2 mg/kg for arsenic and cadmium each and 10 mg/kg for lead for feed materials of animal origin with 12% moisture content. There is also a maximum limit established in EU law for mercury, but no mercury was detected in BSFL samples during this study. Therefore, the concentrations of heavy metals found in BSFL in this study were not of concern for using these BSFL in feed. Although the fact that the studied food safety hazards are initially present in low quantities in the substrates and even with accumulation are traced back in low quantities in BSFL, the waste streams as components to these substrates are prone to high variability. It might be possible, for example, that these waste streams when produced in another season or period contain higher initial concentrations of metals. A limitation of this study is that substrate components originated from one batch, resulting in having analysed only one point in time of supply of these substrate components. Furthermore, the results presented in this paper only represent one sample from each of the three rearing crate. As such, it is important to monitor the presence of especially cadmium in

waste streams used for the substrates for BSFL rearing in order to prevent bioaccumulation of higher concentrations resulting in concentrations in BSFL exceeding the maximum limits. As an example, higher BAF values were seen in study by van der Fels-Klerx *et al.* (2016), which had spiked the substrates. For cadmium, the highest BAF was 9.5 in BSFL reared on substrates spiked with 0.25 mg/kg cadmium, while the highest BAF was 3.4 in BSFL reared on a substrate with 0.25 mg/kg in the current study. Bioaccumulation of cadmium in BSFL was observed in several other studies (Biancarosa *et al.*, 2018; Diener *et al.*, 2015; van der Fels-Klerx *et al.*, 2020). It can be concluded that cadmium accumulate in BSFL, only the height of the BAF can vary between studies, which could be attributed to the different substrates.

Some of the trace elements are used as a feed additive, and can be added up to a maximum level in complete feed. There are maximum levels laid down in EU law to which some trace elements may be added to feed. The most stringent maximum levels laid down in EU law (animal species dependent) in complete feed with 12% moisture content are 1 mg/kg for cobalt (European Commission, 2014), 15 mg/kg for copper (European Commission, 2018), 250 mg/kg for iron (European Commission, 2017b), 100 mg/kg manganese (European Commission, 2017a), 2.5 mg/kg molybdenum (European Commission, 2019b), 0.5 mg/kg selenium (European Commission, 2019a), 120 mg/kg for zinc (European Commission, 2016). However, these maximum levels for feed additives are in respect to the levels in complete feed and not just to the levels in one feed component, for instance insects. At an inclusion level of 15% of BSFL in complete feed the levels of these trace elements were below these limits, except for manganese in BSFL reared on the mixtures PS+MS+PM and PS+MS+SW.

Three classes of veterinary drugs were analysed in this study; antibiotics, antiparasitics and coccidiostats. No antiparisitics were found in the substrate components used in this study. EU legislation lays down a maximum residue limit (MRL) for pharmaceutical active substances in food producing animals in Regulation (EU) No 37/2010. The lowest MRL for the antibiotic DOX is 100 μg/kg for muscle tissue of food producing animals. The lowest MRL for the antibiotic OTC is the same as for DOX. Results of this study taught us that the naturally present levels of these two antibiotics in substrates and consequently in BSFL were well below these MRLs. This is in line with our previous study were oxytetracycline was also found in very low concentration in pig manure and BSFL reared in substrates containing this type of manure (Hoek-van den Hil *et al.*, 2023 (submitted). This is also in line with the study of Liu *et al.* (2021), which investigated the degradation of antibiotics in frass. In that study, it was observed that BSFL were able to degrade OTC and that OTC levels were lower in frass than in the original substrate. A degradation of the antibiotics enrofloxacin and tylosin in a spiked swine manure substrate by BSFL was also observed in the study by (Mei *et al.*, 2022). It is possible that these breakdown products could still be bioactive. Therefore, possible breakdown of veterinary drugs by insect larvae should be further studied.

On the other hand, high DOX concentrations in larvae were observed in a study, which investigated substrates spiked with DOX at levels, which could be reasonably found in manure. The authors of that study described that the levels found in larvae would exceed the maximum limit for DOX in meat products (Hoek-van den Hil *et al.*, 2022).

In case of the coccidiostats, only DNC (marker of nicarbazin) was found in BSFL for one treatment, namely PS+MS+SW. The level detected was well below the most stringent maximum limit described for nicarbazin (mother compound of DNC) in feed by Directive 2002/32/EC, being 1.25 mg/kg based on 12% moisture content. Furthermore, coccidiostats could not be quantified in many samples, due to the low concentrations.

Overall, no concerning levels of veterinary drugs residue were found in BSFL after rearing on the mixtures of waste streams in this study. However, further verification to account for variance in contamination of substrates is needed for definitive conclusions on the safety of tested type of substrates. Monitoring of manure prior to the use can help in selecting manure types, which could possibly be used as substrate for insect rearing, to ensure safety of insect products.

PCR analyses were performed on the substrates and BSFL to determine the presence of DNA from conventional livestock species, i.e. pigs, chickens and ruminants. All substrates contained pig DNA, which was also detected in BSFL grown thereon. Remarkably, pig DNA was also traced in the substrate component poultry meal. A possible explanation for this finding could be cross contamination at processing lines for processing slaughter meal, since these are waste streams and not yet intended to come into a new feed or food system. On the contrary, chicken DNA was found in all substrates except for pig manure, but was not detected in BSFL samples. At this early stage in performing this type of research, we do not have an explanation for this. DNA of ruminant was traced back in substrate samples with fast food and in BSFL samples grown thereon. To the

best of our knowledge, this is the first time that samples from an experiment with BSFL for feed were analysed for the presence of DNA of ruminant, pig and chicken. In this study, the analysed BSFL still contained their gut contents, which could be an explanation for the positive DNA results in BSFL. Therefore, in future research, it would be recommended to study the presence of animal DNA in BSFL that were subjected to a fasting period after rearing on substrates containing animal materials or BSFL could receive a substrate without animal materials during the last phase of rearing. Moreover, BSFL in this study were not subjected to any further processing step besides the homogenisation of samples before analyses. Further processing steps, such as a heat treatment, could alter the DNA in the insects. As a result, the DNA of the animal proteins in the insects could be detectable to a lesser extent or even be undetectable.

Overall, the highest concentrations of food safety hazards in this study were found in frass. This can be related to the reduction of the amount of waste (substrate), due to the use of nutrients by the insects, which makes the concentration of the food safety hazards relatively higher compared to the original concentration in the substrate. This is in line with the findings from Hoek-van den Hil *et al.* (2022) for veterinary drugs and van der Fels-Klerx *et al.* (2016) and van der Fels-Klerx *et al.* (2020) for metals. For this reason, it would be important to consider the type of application of frass, since several food safety hazards will be more concentrated in frass (on dry weight basis). Since 2021, frass is legally defined and allowed to be applied as fertiliser after being heated at 70 °C for 1 hour in Regulation (EU) No 142/2011 as amended by Regulation (EU) 2021/1925. This Regulation does not provide maximum limits for metals in insect frass as fertiliser, but additional national requirements may apply. Although BSFL seems a promising solution towards the bioconversion of waste streams in a circular feed and food system, the use of the investigated waste streams is currently not allowed in many jurisdictions due to the presence of animal proteins and can thus not be applied in practice at the moment.

5 Conclusions

This study investigated the potential of BSFL to be reared on mixtures of several waste streams with special attention to the safety of BSFL to be used as feed and food. BSFL were able to grow on the tested substrates (Naser El Deen *et al.*, under preparation). From all the food safety hazards analysed in this study, only a couple of compounds showed accumulation in BSFL. It was seen that some metals can accumulate in BSFL and attention should be specifically paid to the heavy metal cadmium. Veterinary drugs were only detected in low concentrations in the substrates and even to a lesser extent in BSFL, which were not of concern when using these BSFL for feed. Ruminant and pig DNA was detected in the analysed samples of both substrates and BSFL, but chicken DNA was not detected in BSFL although it was detected in the substrates. To conclude, based on the safety parameters assessed in this study, it is possible to rear BSFL for feed on waste streams provided that the presence of cadmium would be monitored. However, further verification to account for variance in contamination of substrates is needed for definitive conclusions on the safety of the tested substrates. Next to this, further research is needed on the presence of animal proteins in insects that e.g. were subjected to a fasting period or that are further processed after feeding of animal materials.

Supplementary material

Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.24849840

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