



Valorising food waste using black soldier fly larvae: A One Health assessment of mycotoxin bioaccumulation and mycotoxigenic fungi under industrial-scale conditions

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ABSTRACT

Food waste is a major challenge for environmental sustainability and food security. Black soldier fly larvae (BSFL, *Hermetia illucens*) offer a sustainable solution by converting food waste into high-quality protein, aligned with One Health principles. However, concerns over bioaccumulation of contaminants, including fungi and mycotoxins, have limited the use of food waste as insect substrate. This study evaluated whether BSFL bioaccumulate mycotoxins under industrial-scale rearing conditions, using bioaccumulation factors, while also assessing contamination with fungi of mycotoxin-producing potential. To do that, approximately two tonnes of food waste were collected from restaurants in Santarém, Portugal, processed into a test substrate, along with the cereal-based Gainesville diet as a control. Larvae were reared for 14 days, and samples of substrates, larvae, frass, and BSF meal were analysed for fungal contamination and mycotoxin concentration using culture-based methods and liquid chromatography-tandem mass spectrometry, respectively. Mycotoxigenic fungi belonging to *Fusarium*, *Aspergillus*, and *Penicillium* were predominantly below the limit of detection in larvae. Deoxynivalenol (DON), zearalenone (ZEN), and HT-2 toxin were detected in the substrates, but none of these mycotoxins were detected or quantified in larvae, with bioaccumulation factors consistently below one, indicating no significant accumulation. DON and ZEN were also detected in frass, in some cases at higher concentrations than in the corresponding initial substrates. These findings demonstrate that, under the conditions tested, BSFL did not bioaccumulate the mycotoxins detected in the rearing substrates, while their occurrence in frass highlights the importance of considering contaminant distribution across outputs of the bioconversion process. Overall, this study supports the safe use of BSFL for industrial-scale food waste valorisation and highlights their contribution to improving public health.

1. Introduction

More than one billion tonnes of food are wasted globally each year, with significant implications for both environmental sustainability and

food security [1,2]. This volume of wasted food could potentially feed over one million people experiencing hunger [3]. Moreover, food waste undermines the resilience and sustainability of current food systems. Traditional waste management strategies, such as landfilling and

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incineration, contribute to greenhouse gas emissions, generate unpleasant odours, and facilitate the spread of pathogens. At the same time, the global population is projected to reach 9.7 billion people by 2050, further intensifying the demand for alternative and sustainable sources of protein [4].

Addressing food waste and the demand for sustainable protein sources requires an integrated approach that considers the interconnected health of humans, animals, and the environment. The One Health approach highlights this interdependence, recognising that human health is closely linked to animal health and environmental health [5]. Adopting a One Health perspective enables us to develop strategies that can reduce environmental impact, enhance food security, and improve public health. Insect farming aligns with this framework by supporting food security, while contributing to environmental and economic sustainability [6]. Furthermore, the One Health approach is essential in these novel food systems, as it supports the integrated assessment of potential risks and benefits for environmental, animal, and human health [7,8]. Within this context, the use of black soldier fly larvae (BSFL, *Hermetia illucens*) has emerged as a promising strategy to convert food waste into high-quality protein. These larvae can convert various organic substrates, including food waste, into animal feed, fertiliser, biofuel, pharmaceuticals, and cosmetics [9–11]. This process offers a sustainable, health-conscious solution that exemplifies the principles of One Health and the circular economy. However, it is not permitted to use former foodstuffs containing meat or fish as an insect substrate in the European Union (EU) (Regulation (EC) No 1069/2009) [12]. In a scientific opinion by the European Food Safety Authority (EFSA), it was stated that these substrates may be contaminated with various biological and chemical contaminants, including fungi and mycotoxins [13].

Fungi affect foods through the production of mycotoxins, compromising their safety [14]. These naturally occurring toxins produced by fungi can pose significant risks to both human and animal health, ranging from gastrointestinal and kidney disorders to immune deficiency and cancer, with ingestion representing the most common route of exposure [15].

The genera *Aspergillus*, *Fusarium*, *Penicillium*, *Claviceps*, and *Alternaria* are among the most relevant mycotoxin-producing fungi, as they generate mycotoxins of major concern for human and animal health, including Aflatoxins (AFTs), Ochratoxins (OTs), Trichothecenes (TCTs), Fumonisin (FUMs), Zearalenone (ZEN), Patulin (PAT), Citrinin (CT), and Ergot Alkaloids (EAs) [16]. These mycotoxins may induce a wide range of adverse effects, including hepatotoxic, nephrotoxic, immunotoxic, neurotoxic, carcinogenic, impaired growth, and reproductive effects [16–19]. In waste management settings, exposure to several of these mycotoxins has been documented, including deoxynivalenol (DON), ZEN, ochratoxin A (OTA), HT-2 toxin, α -zearalenol (α -ZOL), and β -zearalenol (β -ZOL), highlighting waste as a relevant occupational environment for mycotoxin exposure [20].

Food waste, mouldy bread, cottonseed, spices, tea, cereals, leguminous seeds, oilseeds, industrial by-products, and animal feed may all be contaminated with mycotoxins, posing significant risks to animal health, food safety, and public health [21–23]. Considering the severe health effects of these mycotoxins, already mentioned above, it is essential to determine whether substrates derived from food waste may contain mycotoxins and thus pose a risk within insect-based food systems; however, previous studies have shown that BSFL do not bioaccumulate mycotoxins when reared on contaminated substrates [24–27]. However, most available evidence derives from controlled experiments using artificially contaminated substrates, which are valuable for studying responses to specific mycotoxins but do not fully reproduce the heterogeneous composition and naturally occurring contamination of real food waste. Assessing naturally contaminated food waste under industrial-scale conditions is therefore important to determine whether findings from controlled studies translate to realistic production scenarios.

A second knowledge gap concerns the relationship between fungal contamination and mycotoxin occurrence. Previous assessments of fungal contamination in BSFL have generally relied on total mould and yeast counts, which provide information on overall hygienic quality but do not identify fungi with mycotoxin-producing potential. Moreover, the presence of potentially mycotoxigenic fungi does not necessarily correspond to the occurrence of their associated mycotoxins. Simultaneously characterising mycotoxigenic fungi and mycotoxins therefore provides a more comprehensive assessment of both the biological hazard and the associated chemical hazard within the same bioconversion system.

Addressing these complementary gaps, this study evaluated mycotoxin occurrence and bioaccumulation in BSFL reared under industrial-scale conditions using heterogeneous, naturally contaminated food waste without artificial mycotoxin spiking, while simultaneously identifying and enumerating fungi with mycotoxin-producing potential. This integrated approach provides a more realistic and risk-oriented assessment of mycotoxin safety in BSFL-based food waste valorisation within a One Health framework.

2. Materials and methods

2.1. Experimental design

An industrial-scale bioconversion assay was conducted at EntoGreen R&D unit's facilities (Santarém, Portugal). Two substrates were considered: a test substrate composed of food waste, and a control substrate consisting of the Gainesville diet. The Gainesville diet is a standardised, nutritionally balanced rearing substrate commonly used for BSFL, typically consisting of approximately 67% water, 17% wheat bran, 6.6% maize flour, and 9.9% alfalfa, ensuring consistent larval growth and performance [28]. For the test substrate, 1847.5 kg of pre- and post-consumer food waste were collected from nine restaurants in Santarém. The post-consumer fraction consisted of a heterogeneous mixture of food leftovers, including fish, meat, cereals, vegetables, eggs, fruits, bones, fish spines, and dessert products. The pre-consumer fraction comprised vegetable peels and trimmings, raw meat and fish, eggshells, and uncooked eggs. The collection was carried out with the support of the Municipality of Santarém, which provided dedicated waste containers to enable selective collection. The residues were transported under refrigerated conditions to the rearing facilities, where they were subsequently used for substrate formulation. The material was ground and mixed with wheat bran to achieve a final moisture content of 65–67%. Only organic residues (e.g., meat, fish, bones, vegetables, and fruits) were included, and physical contaminants such as cutlery or beverage cans were removed.

Juvenile larvae were inoculated onto each substrate and reared for 14 days under controlled industrial conditions at EntoGreen R&D unit's facilities. A total of 114 experimental units were prepared for the test substrate and 144 for the control substrate, each in PVC containers (60 × 40 × 11 cm; EntoGreen, Santarém, Portugal), with a higher number of control units included to increase the statistical robustness of the baseline condition. All procedures followed EntoGreen's standard industrial workflow to ensure realistic production conditions and are described in Ref. [29].

For both substrates, samples of the initial material were collected on day 1, before larval inoculation. On day 14, samples of fresh larvae were collected after separation from the frass by sieving; larvae were not fasted prior to sampling. Meal samples were obtained after drying and milling. The basic nutritional composition of both the food waste test substrate and the Gainesville control diet, together with the corresponding analytical methods, is provided in [Supplementary Table S1](#).

Samples were randomly collected from multiple rearing containers within each treatment and pooled into three independent sample bags, corresponding to three biological replicates. Microbiological analyses were performed immediately after sampling. For mycotoxin analysis, all

samples (substrate, larvae, frass, and meal) were stored at -20°C until processing. Each analyte was determined in each biological replicate using four analytical replicates ($n = 4$).

2.2. Viable mycobiota characterisation

Fungal contamination was assessed in both substrates (test and control) and larvae samples. All samples were collected aseptically in sterile bags and transported to the laboratory refrigerated for immediate processing. Ten grams of each sample were weighed and diluted with 90 mL of Tryptone Soya Broth (dilution 10^{-1}). The suspensions were agitated at $1 \times g$ for 30 min. Serial tenfold dilutions were prepared up until 10^{-3} .

For fungal contamination, 150 μL of each dilution were spread onto Malt Extract Agar (MEA) and Dichloran Glycerol Agar (DG18). Plates were incubated at 27°C for six days. To assess fungi with pathogenic potential, 150 μL were inoculated onto DG18 and incubated at 37°C . All plates were incubated during five to seven days.

Fungal isolates were identified based on macroscopic and microscopic morphological characteristics, including colony morphology and microscopic reproductive and vegetative structures, according to the taxonomic criteria described in the *Atlas of Clinical Fungi* by De Hoog et al. (2000), with identifications reported at genus, section, or species, as applicable [30]. Colony counts were then recorded, and results expressed as $\log_{10}$ CFU/g. The limit of detection (LOD) for fungal enumeration was considered $1.8 \log_{10}$ CFU/g. Molecular methods were not performed for taxonomic confirmation.

2.3. Mycotoxin analysis

2.3.1. Reagents and samples

Samples of substrate, larvae, frass and meal were lyophilised and ground prior to analysis. Mycotoxin determination was performed by liquid chromatography–tandem mass spectrometry (LC-MS), following the procedures defined in Regulation (EU) 2023/2782, which sets out the analytical methods for the control of mycotoxins in food. For each matrix, four independent portions of the same sample were collected and processed as technical replicates (A–D), and each replicate was analysed in duplicate.

Methanol (LC-MS grade) and acetonitrile (gradient grade) were purchased from Merck (Darmstadt, Germany). Acetic acid and ammonium acetate, both of LC-MS grade, were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was produced using an Elix 3 and Simplicity UV purification systems (Millipore, Molsheim, France).

All analytical standards, including both native (unlabelled) and ^{13}C -labelled mycotoxins, were supplied by Romer Labs (Tulln, Austria). Individual stock solutions of the unlabelled and ^{13}C -labelled compounds were combined separately to prepare two working standard mixtures. The first containing the native mycotoxins at the following concentrations: 15-ADON (2303 ng/mL), 3-ADON (2472 ng/mL), AFB₁ (628 ng/mL), AFB₂ (157 ng/mL), AFG₁ (634 ng/mL), AFG₂ (158 ng/mL), α -ZAL (134 ng/mL), α -ZOL (137 ng/mL), β -ZAL (132 ng/mL), β -ZOL (133 ng/mL), DOM-1 (2307 ng/mL), DON (7400 ng/mL), DON-3-glucoside (2076 ng/mL), DAS (1676 ng/mL), FB₁ (2500 ng/mL), FB₂ (2510 ng/mL), FB₃ (1253 ng/mL), FUS-X (1656 ng/mL), HT-2 toxin (4954 ng/mL), MAS (1352 ng/mL), NEO (1333 ng/mL), NIV (4944 ng/mL), OTA (661 ng/mL), T-2 toxin (1651 ng/mL), and ZEN (2469 ng/mL). The second mixture containing the corresponding ^{13}C -labelled analogues: [$^{13}\text{C}_{17}$]-AFB₁ (10 ng/mL), [$^{13}\text{C}_{15}$]-DON (500 ng/mL), [$^{13}\text{C}_{21}$]-DON-3-Glucosid (250 ng/mL), [$^{13}\text{C}_{34}$]-FB₁ (500 ng/mL), [$^{13}\text{C}_{34}$]-FB₂ (250 ng/mL), [$^{13}\text{C}_{22}$]-HT-2 toxin (250 ng/mL), [$^{13}\text{C}_{20}$]-OTA (50 ng/mL), [$^{13}\text{C}_{24}$]-T-2 toxin (10 ng/mL), and [$^{13}\text{C}_{18}$]-ZEN (50 ng/mL). All stock solutions and prepared working mixtures were stored at -20°C . Before use, working solutions were allowed to equilibrate to room temperature.

2.3.2. Sample preparation

Prior to extraction, samples were lyophilised for 48 h at $-46 \pm 2^{\circ}\text{C}$ using a ModulyoD Freeze Dryer (Thermo Scientific) and subsequently ground to obtain a homogeneous material. For extraction, 2.0 g of homogenized sample were accurately weighed into a centrifuge tube and extracted with 8.0 mL of a solvent composed of acetonitrile/water/acetic acid (79:20:1, v/v/v). The mixture was shaken for 60 min at room temperature to promote analyte transfer into the organic phase. After extraction, the samples were centrifuged at $6025 \times g$ for 5 min to separate the solid residue from the liquid extract. An aliquot of 0.4 mL of the supernatant was transferred to a microcentrifuge tube and diluted with 0.4 mL of methanol/water (2:8, v/v). The diluted extract was further clarified by centrifugation at $25857 \times g$ for 30 min. Finally, 80 μL of the clear extract was mixed with 20 μL of the internal standard solution. After vortex mixing to ensure complete homogenization, the samples were analysed by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

2.3.3. HPLC-MS/MS analysis

Chromatographic separation was performed using a Nexera high-performance liquid chromatograph (Shimadzu, Tokyo, Japan). Analytes were separated at 40°C on a Waters AQUITY Premier HSS T3 analytical column (2.1×100 mm, $1.8 \mu\text{m}$; Waters, Milford, MA, USA). The flow rate was set to 0.25 mL/min and the injection volume was 4 μL . Both mobile phases contained 5 mmol/L ammonium acetate. Mobile phase A consisted of methanol/water/acetic acid (10:89:1, v/v/v), and mobile phase B consisted of methanol/water/acetic acid (97:2:1, v/v/v). The gradient program was as follows: a linear increase from 0% to 97% over 0.0–12.0 min B; isocratic elution at 97% B from 12.0 to 14.0 min; followed by column re-equilibration at 0% B for 6.0 min.

Detection was carried out using a 5500 QTrap mass spectrometer (Sciex, Foster City, CA, USA). Data were acquired in a single chromatographic run operating in both positive and negative electrospray ionization modes, using scheduled multiple reaction monitoring (sMRM). The ion source parameters were: curtain gas 30 psi; collision gas medium; ion spray voltage -4500 V (negative mode) and 5500 V (positive mode); source temperature, 500°C ; ion source gas 1 at 60 psi; and ion source gas 2 at 60 psi. Compound-specific MS parameters are summarised in Table S3. Representative MRM chromatograms are provided for a spiked substrate matrix (Fig. 1), a naturally contaminated substrate sample (Fig. 2), and a larval sample (Fig. 3). The retention times and characteristic ion transitions of the target compounds are provided in Table S3.

2.3.4. Method validation

Method performance was evaluated in terms of apparent recovery (Ra), extraction recovery (Re), and matrix effects (ME), as presented in Table S3. In addition, the working range, limits of detection (LOD), limits of quantification (LOQ), and repeatability were determined for four representative matrices (frass, BSF flour, substrate, and dried larvae), with the corresponding values also reported in Table S4. Validation was carried out in accordance with the criteria established in Commission Decision 2002/657/EC.

Recovery experiments were performed by fortifying blank matrix samples (2.00 g) at six concentration levels, each prepared in triplicate. After spiking, samples were left uncovered at room temperature for 4 h to allow solvent evaporation and equilibration of analytes with the matrix. The samples were then briefly shaken to ensure homogenous distribution before extraction and analysis according to the procedure described above.

Matrix effects were assessed using matrix-matched calibration. Blank matrix extracts were prepared, and six calibration levels were generated in triplicate.

LOD and LOQ values were estimated based on signal-to-noise (S/N) ratios obtained from matrix extracts using the less intense mass transition and Analyst 1.6.2 software (Sciex, Framingham, USA). The LOD and

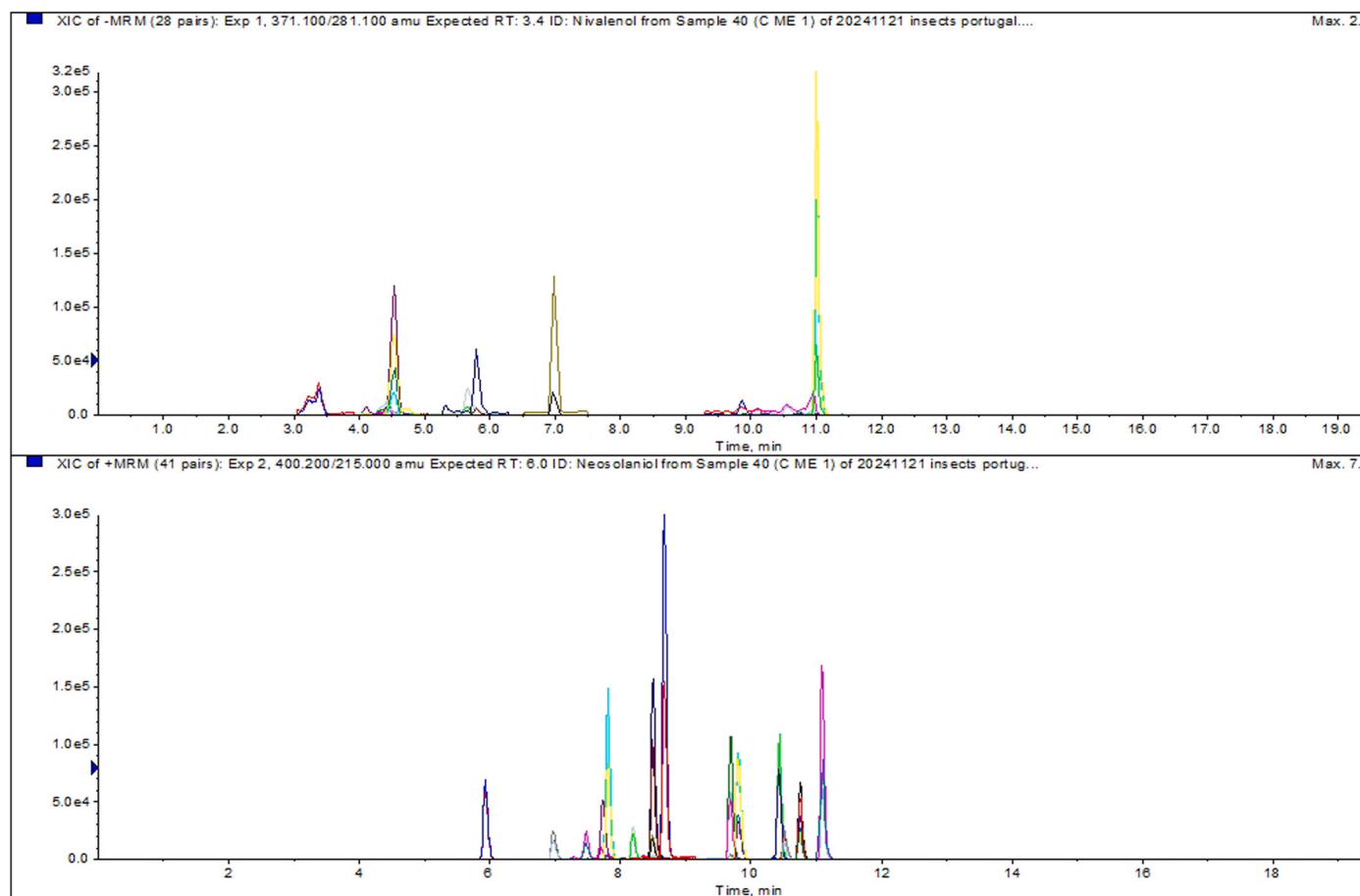


Fig. 1. Representative multiple reaction monitoring (MRM) chromatogram of a substrate matrix spiked with the target mycotoxins. Retention times and characteristic ion transitions of the target compounds are provided in Table S3.

LOQ corresponded to fortification levels yielding S/N ratios of 3 and 10, respectively.

2.4. Bioaccumulation factor

To assess their potential accumulation or reduction by BSFL during the bioconversion process, the bioaccumulation factor (BAF) was calculated based on the quantitative analysis of mycotoxins and serving as an indirect indicator of mycotoxin bioremediation. The BAF was defined as the concentration of each mycotoxin in the larvae, corrected for moisture content, divided by the concentration of the same mycotoxin in the initial substrate, also corrected for moisture content, according to Diener et al. [31]; moisture contents were determined by thermogravimetry and are provided in Supplementary Table S2. For calculation purposes, concentrations below the limit of quantification (LOQ) were assigned a value equal to half the LOQ.

A BAF value lower than 1 indicates a reduction of the mycotoxin during bioconversion, suggesting effective bioremediation, whereas a BAF value greater than 1 indicates bioaccumulation of the mycotoxin in the larvae [32].

2.5. Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics (version 30.0.0.0; IBM Corp., Armonk, NY, USA).

For fungal enumeration and mycotoxin quantification, comparisons between substrates and BSFL were performed separately for control and test groups using the independent samples Mann–Whitney *U* test.

The bioremediation potential of BSFL was assessed by comparing

fungal counts and mycotoxin levels in the substrates prior to bioconversion with those detected in the larvae after feeding.

For statistical purposes, values below the limit of detection (LOD) or the limit of quantification (LOQ) were substituted with LOD/2 or LOQ/2, respectively. Statistical significance was considered at $p < 0.05$.

3. Results

On MEA, fungal contamination was generally low across both control and test groups. In the control group, no statistically significant differences between substrate and larvae were observed for *Fusarium culmorum*, *Fusarium graminearum*, or *Aspergillus* section Nigri (all $p > 0.05$). However, Mucorales was enumerated in both matrices, with significantly higher counts in the substrate ($6.11 \log_{10}$ CFU/g) compared to larvae ($2.43 \log_{10}$ CFU/g) ($p = 0.002$). *Aspergillus fumigatus* remained below the detection limit in both substrate and larvae.

In the test group analysed on MEA, fungal contamination also remained low. No statistically significant differences between matrices were observed for any of the analysed fungi (all $p > 0.05$). *Penicillium* spp. and *Aspergillus* section Flavi remained below the limit of detection in both matrices.

On DG18 at 27 °C, fungal counts in the control group followed a similar pattern. *Fusarium graminearum* was detected in the substrate ($2.6 \pm 0.2 \log_{10}$ CFU/g) but remained below the detection limit in larvae, resulting in a statistically significant difference between matrices ($p = 0.002$). In contrast, *Aspergillus* section Nigri was detected in larvae ($2 \pm 2 \log_{10}$ CFU/g) but not in the substrate. No statistically significant differences were observed for *Aspergillus* sections Flavi and Circumdati, *Penicillium* spp., or the other fungi analysed (all $p > 0.05$).

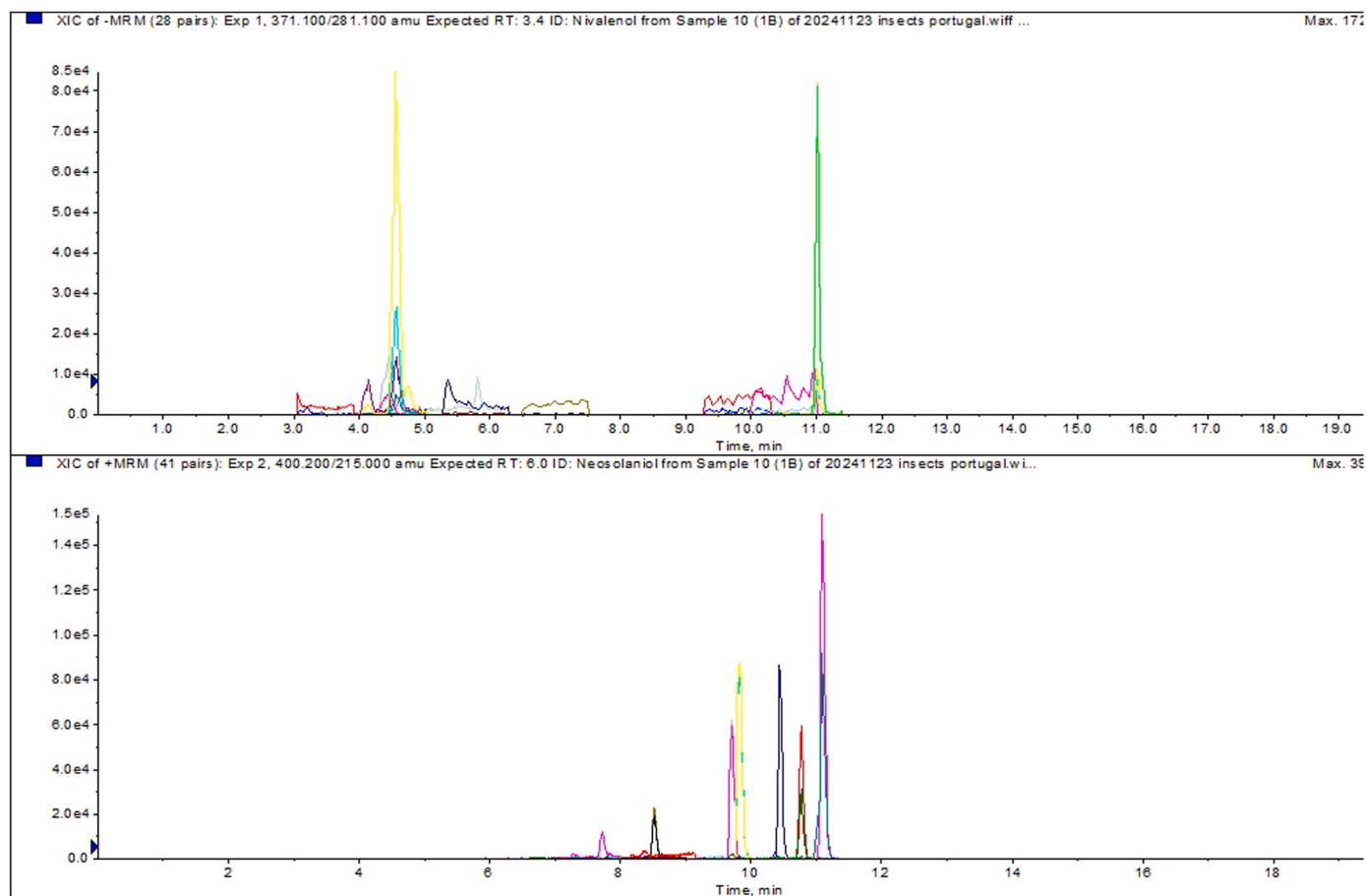


Fig. 2. Representative multiple reaction monitoring (MRM) chromatogram of a naturally contaminated substrate sample. Retention times and characteristic ion transitions of the target compounds are provided in [Table S3](#).

In the test group analysed on DG18 at 27 °C, *Penicillium* spp. were detected in both substrate ($2.6 \pm 0.2 \log_{10}$ CFU/g) and larvae ($2.6 \pm 0.3 \log_{10}$ CFU/g), although no statistically significant differences between matrices were observed ($p = 0.065$). *Aspergillus* sections Nigri, Flavi and Circumdati remained below the detection limit in both matrices.

At DG18 37 °C, fungal growth was limited in most samples. In the control group, *Aspergillus* section Nigri was detected only in larvae ($2.4 \pm 0.0 \log_{10}$ CFU/g) and remained below the detection limit in the substrate, resulting in a statistically significant difference between matrices ($p = 0.002$). *Aspergillus* section Flavi remained below the detection limit in both matrices. In the test group, both *Aspergillus* section Nigri and *Aspergillus* section Flavi remained below the detection limit in substrate and larvae.

All fungi identified throughout the study are described in the text to provide a comprehensive overview of the viable microbiota detected under the different experimental conditions. However, [Table 1](#) specifically highlights the fungi identified in our samples that are recognised as major mycotoxin producers.

A broad spectrum of mycotoxins ($N = 16$) and their metabolites were analysed in the substrate, larvae, meal and frass samples, including Nivalenol, DON, DON-glucoside, Fusarenon-X, deoxydeoxynivalenol, 3-acetyl-DON, 15-acetyl-deoxynivalenol, Neosolaniol, Monoacetoxyscirpenol, Diacetoxyscirpenol, T-2 toxin, HT-2 toxin, Fumonisin B1, B2 and B3, OTA, AFB1, B2, G1 and G2, ZEN, α -zearalenol, and β -zearalenol.

In the control group, only DON, ZEN and HT-2 toxin were detected at concentrations above the limit of detection (LOD) or limit of quantification (LOQ) in at least one of the analysed matrices. In the test group, Zearalenone was the only mycotoxin detected above the LOD/LOQ. All

other mycotoxins and metabolites were below the LOD or LOQ in all matrices analysed, and levels in the meal were below the LOD or LOQ for all mycotoxins in both groups. The LOD and LOQ values for the different mycotoxins in the BSF meal are summarised in [Supplementary Table S4](#).

The concentrations of DON, ZEN and HT-2 toxin detected in the substrate, larvae and frass, along with the bioaccumulation factor (BAF) are summarised in [Table 2](#).

As shown in [Table 2](#), DON was detected in the control group substrate at a mean concentration of $164 \pm 4 \mu\text{g/kg}$ and in frass at $260 \pm 10 \mu\text{g/kg}$, while concentrations in larvae were below the limit of quantification (LOQ = $26.0 \mu\text{g/kg}$). ZEN was quantified in the substrate ($16 \pm 2 \mu\text{g/kg}$) and frass ($32.4 \pm 0.0 \mu\text{g/kg}$) of the control group but remained below the limit of detection (LOD = $0.5 \mu\text{g/kg}$) in larvae. HT-2 toxin was detected in the substrate at $21 \pm 1 \mu\text{g/kg}$, whereas levels in both larvae and frass were below the LOD ($3.8 \mu\text{g/kg}$ and $4.1 \mu\text{g/kg}$, respectively).

In the test group, only ZEN was detected above the LOD, as presented in [Table 2](#). Mean concentrations of ZEN were $3.4 \pm 0.2 \mu\text{g/kg}$ in the substrate and $7.4 \pm 0.7 \mu\text{g/kg}$ in frass, while levels in larvae remained below the LOD ($0.5 \mu\text{g/kg}$).

Bioaccumulation factors, calculated using moisture-corrected concentrations and LOD/2 or LOQ/2 values for non-quantified samples, were all below 1 ([Table 2](#)), indicating the absence of bioaccumulation of detected mycotoxins in BSFL and supporting an overall reduction of contaminants during bioconversion, under the experimental conditions tested.

Mycotoxin analysis showed significant absence of bioaccumulation of ZEN, DON, and HT-2 toxin by BSFL in the control group ($p < 0.001$), and a significant of ZEN in the test group ($p < 0.001$).

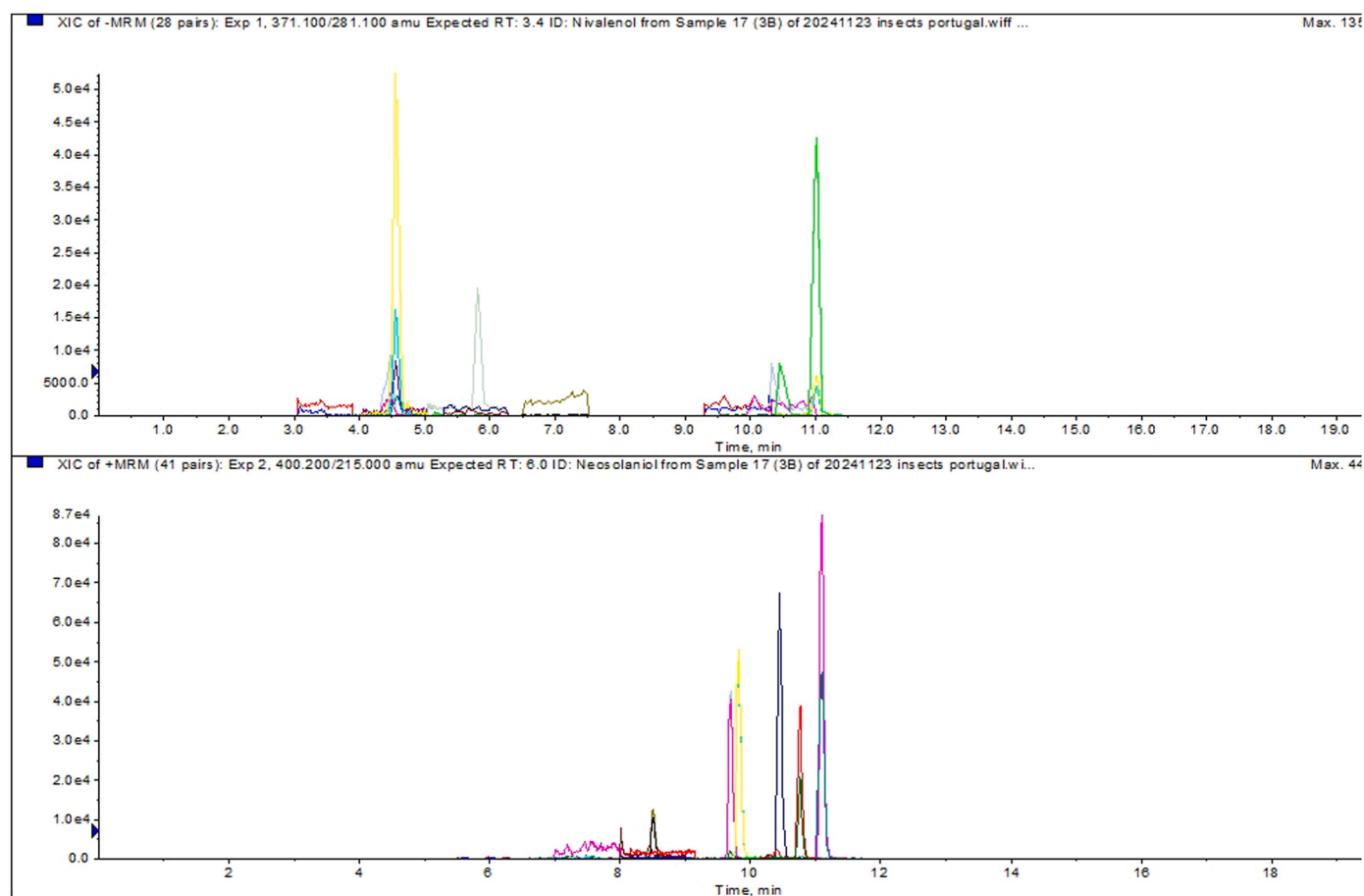


Fig. 3. Representative multiple reaction monitoring (MRM) chromatogram of a larval sample collected at the end of the rearing trial. Retention times and characteristic ion transitions of the target compounds are provided in Table S3.

Table 1

Viable counts of potentially mycotoxigenic fungi in control and test substrates and BSFL under different culture and incubation conditions.

Fungi	Control		Test	
	Substrate	Larvae	Substrate	Larvae
MEA				
<i>F. graminearum</i>	2.0 ± 3.5	<LOD	<LOD	<LOD
<i>F. culmorum</i>	<LOD	<LOD	<LOD	<LOD
<i>A. section Nigri</i>	<LOD	<LOD	<LOD	2.4 ± 0.0
<i>Penicillium</i> spp.	<LOD	<LOD	<LOD	<LOD
<i>A. section Flavi</i>	<LOD	<LOD	<LOD	<LOD
DG18, 27 °C				
<i>A. section Nigri</i>	<LOD	2 ± 2	<LOD	<LOD
<i>A. section Flavi</i>	<LOD	<LOD	<LOD	<LOD
<i>A. section Circumdati</i>	<LOD	<LOD	<LOD	<LOD
<i>Penicillium</i> spp.	<LOD	<LOD	2.9 ± 0.2	2.6 ± 0.3
<i>F. graminearum</i>	2.6 ± 0.2	<LOD	<LOD	<LOD
DG18, 37 °C				
<i>A. section Nigri</i>	<LOD	2.4 ± 0.0	<LOD	<LOD
<i>A. section Flavi</i>	<LOD	<LOD	<LOD	<LOD

Values are expressed as mean ± SD (log₁₀ CFU/g). SD, standard deviation; CFU, colony-forming units; LOD, limit of detection; MEA, Malt Extract Agar; DG18, Dichloran Glycerol Agar. Substrate samples correspond to the initial substrate (day 1, before larval inoculation), whereas larval samples correspond to the final day of the rearing trial (day 14).

4. Discussion

This study evaluated fungal contamination, mycotoxin occurrence and potential bioaccumulation in BSFL reared on food waste under

Table 2

– Mean levels with standard deviation of mycotoxins in substrate, larvae and frass from control and test groups.

Mycotoxin	Group	Substrate (µg/kg)	Larvae (µg/kg)	Frass (µg/kg)	BAF
DON	Control	164 ± 4	<loq	260 ± 10	0.081
	Test	<lod	<loq	<loq	-
ZEN	Control	16 ± 2	<lod	32.4 ± 0.0	0.81
	Test	3.4 ± 0.2	<lod	7.4 ± 0.7	0.047
HT-2 Toxin	Control	21 ± 1	<lod	<lod	0.092
	Test	<lod	<lod	<lod	-

BAF, bioaccumulation factor; LOD, limit of detection; LOQ, limit of quantification. LOD values (µg/kg) for DON were 4.8 in frass, 6.2 in larvae, and 6.7 in substrate; for ZEN, 0.5 in frass, 0.6 in larvae, and 0.9 in substrate; and for HT-2 toxin, 4.1 in frass, 2.9 in larvae, and 3.8 in substrate. LOQ values (µg/kg) for DON were 14 in frass, 19 in larvae, and 20 in substrate; for ZEN, 1.6 in frass, 1.9 in larvae, and 2.8 in substrate; and for HT-2 toxin, 12 in frass, 8.7 in larvae, and 12 in substrate. Substrate samples correspond to the initial substrate (day 1, before larval inoculation), whereas larval samples correspond to the final day of the rearing trial (day 14).

industrial-scale conditions. Overall, no statistically significant differences in fungal contamination were observed between substrates and larvae across most fungal species, culture media and incubation temperatures analysed. In both control and test groups, fungal counts were frequently below the detection limit, particularly in larvae, indicating generally low fungal contamination and a tendency towards lower fungal counts in larvae than in the corresponding substrates. These findings are consistent with a previous observation reported for BSFL

reared on different food waste streams [33].

It is important to consider that the control substrate was cereal-based, whereas the test substrate consisted of a homogeneous mixture of food waste. As these substrates differ in composition and origin, they are expected to harbour distinct fungal profiles. Accordingly, different organic waste substrates can induce significant shifts in both bacterial and fungal communities within the BSFL gut microbiota [34–36].

While the overall pattern was characterised by the absence of significant differences, isolated statistically significant effects were observed for specific fungal contaminations under defined conditions, mainly in the control group. These findings suggest that fungi present in the substrate are not readily transferred to the larvae. Similar observations were reported by Kuznetsova et al. [36], who found that mycelial fungi present in the substrate were eliminated after larval incubation, and by Alagappan et al. [33], who observed lower yeast and mould counts in larvae than in the corresponding substrates. However, the fungal patterns observed in the present study were not uniform across taxa. Mucorales counts were significantly lower in the final larval biomass than in the initial substrate, whereas no significant difference was observed for *Penicillium* spp. This suggests that the transfer or persistence of fungi in larval biomass may be taxon dependent. Such differences may reflect variation among fungal taxa in their ecological characteristics, susceptibility to BSFL-associated antimicrobial factors, or interactions with the larval gut environment [37]. However, these mechanisms were not specifically investigated and should therefore be considered hypotheses requiring further investigation.

Microbial interactions may also contribute to the observed patterns. BSFL bioconversion can substantially alter substrate- and gut-associated microbial communities [37], and competition between bacteria and fungi for nutrients and ecological niches may potentially influence fungal survival and persistence [38–40]. However, as the present study compared the initial substrate with the final larval biomass and did not monitor microbial populations longitudinally, temporal patterns of bacterial proliferation and fungal suppression during bioconversion cannot be inferred.

Unlike a previous study that focused on total mould and yeast counts [33], this study identifies and enumerates fungi with potential mycotoxin-producing capacity under conditions designed to mimic industrial rearing, thereby providing a more risk-oriented and application-relevant perspective. This methodological difference also limits direct comparison with previous studies reporting changes in overall mould and yeast counts, since changes in total fungal load may mask different responses among individual fungal taxa.

The lower fungal counts observed in larvae may be partly explained by the antimicrobial activity of BSFL. Larvae produce antimicrobial peptides and lipid-derived compounds, such as lauric acid and glycerol monolaurate, that can inhibit microbial growth [10,41–43]. In addition, BSFL-associated microorganisms may contribute to antifungal activity, as frass has been shown to inhibit the growth of several fungal plant pathogens [44,45]. Together, these mechanisms may contribute to the reduced fungal contamination observed in larval biomass. The potential antifungal properties associated with BSFL are particularly relevant in the context of emerging challenges related to fungal contamination and mycotoxin occurrence. Climate change is expected to favour the expansion of fungal pathogens and increase the risk of mycotoxin contamination in agrifood systems, particularly in vulnerable regions such as the Mediterranean basin [46–48]. At the same time, the extensive use of azole fungicides has contributed to the emergence of resistant fungal strains, highlighting the need for alternative and sustainable mitigation strategies [48]. In this context, the antifungal properties of BSFL and their associated microbiota may represent a complementary biological approach for reducing fungal contamination. Nevertheless, further research is needed to evaluate the effectiveness of these mechanisms.

A limitation of the present study is that fungal identification relied on macroscopic and microscopic morphological characteristics, without

molecular confirmation. Although established morphological criteria enabled the characterisation of the viable mycobiota and, where sufficiently distinctive characteristics were observed, identification at the species level, closely related fungal taxa may share overlapping phenotypic characteristics, which may introduce uncertainty in species-level assignments. Therefore, the taxonomic identifications reported here should be interpreted considering the morphological nature of the identification approach. Future studies incorporating molecular methods could complement morphological identification and provide additional confirmation of species-level assignments.

Although lower fungal counts were observed in the larvae, several fungi identified in the substrates have the potential to produce mycotoxins, representing an additional chemical hazard that should be considered when assessing the safety of food and feed systems.

The substrates used in this study were naturally contaminated with low concentrations of mycotoxins, with most analysed compounds remaining below detection or quantification limits and all detected levels complying with current EU regulatory limits for food and feed [49–51]. In the control substrate, the detection of *Fusarium graminearum* was consistent with the presence of DON, and ZEN. In contrast, ZEN was detected in the test substrate despite *Fusarium* counts being very low or below detection limits, likely reflecting residual contamination, as mycotoxins may persist after the producing fungi are no longer detectable [52]. Conversely, although *Penicillium* was detected, ochratoxin A remained below the detection limit, suggesting either that toxin concentrations were too low to be detected or that conditions were not favourable for its production. These findings highlight that the occurrence of potentially mycotoxigenic fungi does not necessarily correlate with the detection of their corresponding mycotoxins, as toxin production and persistence are influenced by multiple ecological, environmental and methodological factors [53,54].

Across both control and test groups, mycotoxin concentrations in larval biomass remained below detection or quantification limits and all calculated bioaccumulation factors were well below 1, indicating the absence of bioaccumulation in BSFL. These findings demonstrate that, under the conditions tested, BSFL do not concentrate mycotoxins present in the feed substrate. Similar observations have been reported for aflatoxins and *Fusarium* toxins, including DON, FB1, FB2 and ZEN, which were generally absent or present at negligible concentrations in larvae despite being detected in the substrate [24–26,55,56]. *Fusarium* toxins, such as DON, FB1, FB2, and ZEN, were found in the substrates but mostly absent in the larvae, with the compounds being excreted in the frass [24,56]. These findings are consistent with the detection of these mycotoxins in frass in the present study; however, as the larvae were not subjected to a fasting period prior to frass collection, the contribution of residual, non-digested substrate cannot be excluded, and, in the absence of a complete mycotoxin mass balance, the relative distribution of mycotoxins among the different outputs of the bioconversion process cannot be fully determined.

The higher concentrations of DON and ZEN observed in frass than in the initial substrate may also reflect a passive concentration effect associated with substrate consumption and the consequent reduction in residual material during larval bioconversion, rather than an increase in the absolute amount of mycotoxins. A recent study similarly reported low DON concentrations in BSFL accompanied by high concentrations in the resulting frass [57]. Nevertheless, concentration-based comparisons alone cannot distinguish between concentration, excretion, degradation, and redistribution within the bioconversion system.

The absence of detectable parent mycotoxins in larval biomass also does not exclude their biotransformation into metabolites that were not targeted by the analytical method. Evidence for such processes has been reported for AFB1: Niermans et al. [58] demonstrated that both BSFL and substrate-associated microorganisms contributed to its biotransformation, including the formation of aflatoxin P1 and aflatoxicol. In addition, transcriptomic evidence indicates that BSFL possess and regulate genes involved in xenobiotic metabolism following AFB1

exposure [59]. However, these findings cannot be directly extrapolated to DON, ZEN, or HT-2 toxin. As non-targeted metabolite analysis was not performed in the present study, potential transformation of the detected mycotoxins into other compounds remains to be investigated.

From a One Health perspective, the occurrence of residual mycotoxins in frass warrants attention because this material may subsequently be used as an organic fertiliser, thereby providing a potential route for residual contaminants to enter agricultural soils. The environmental fate and potential effects of these residues should therefore be considered alongside the safety of the harvested larval biomass. Consequently, the absence of mycotoxin bioaccumulation in BSFL should not be interpreted as evidence that all outputs of the bioconversion process are free from mycotoxin-related risks. Further studies addressing mycotoxin mass balance, transformation products, and the environmental fate of residual mycotoxins following frass application are needed to provide a more comprehensive assessment of BSFL-based food waste valorisation within a One Health framework.

The absence of bioaccumulation may be explained by both larval detoxification mechanisms and the activity of associated microbial communities. Transcriptomic studies have shown that BSFL upregulate genes involved in xenobiotic metabolism following mycotoxin exposure, indicating an active detoxification response (Shah et al., 2024). In parallel, gut-associated and substrate-associated microorganisms contribute to the transformation of mycotoxins into less toxic metabolites through biodegradation pathways, facilitating their detoxification and elimination [58,60].

In contrast to most previous studies, which relied on artificially contaminated substrates, the present study evaluated mycotoxin behaviour under industrial-scale rearing conditions using heterogeneous food waste streams without artificial contamination, providing a more realistic and industry-relevant assessment of mycotoxin risks associated with BSFL production.

Although BSFL did not bioaccumulate mycotoxins in this study, proactive mitigation strategies remain important to minimise fungal contamination and mycotoxin formation during industrial rearing [61]. These include removing visibly affected substrates, controlling temperature and humidity, and ensuring appropriate storage conditions for both feed materials and derived products, including meal and frass.

5. Conclusions

The present study demonstrates that, under the experimental conditions evaluated, BSFL reared on naturally contaminated food waste under industrial-scale conditions did not bioaccumulate the tested mycotoxins in their biomass. Importantly, the use of heterogeneous, naturally contaminated food waste under industrial-scale rearing conditions provides an industry-relevant assessment that complements previous studies predominantly based on artificially contaminated substrates. The integrated assessment of mycotoxins and potentially mycotoxigenic fungi across the bioconversion system further strengthens the relevance of these findings from a One Health perspective.

Nevertheless, some limitations should be considered when interpreting these results. The study was based on a single food-waste mixture collected within one geographical setting and one rearing cycle, and the relatively low mycotoxin concentrations observed limit extrapolation to substrates with substantially higher contamination levels. Fungal identification relied on morphological methods, and conclusions regarding bioaccumulation are restricted to the mycotoxins investigated under the conditions tested. In addition, the absence of a fasting period prior to larval sampling should be considered when interpreting mycotoxin distribution across bioconversion outputs, as residual non-digested substrate may influence the composition of the recovered frass.

Building on these findings, future studies should evaluate different food-waste streams, contamination levels and rearing cycles, and longitudinal sampling at multiple time points throughout the bioconversion

process, rather than relying solely on measurements of the initial substrate and final larval biomass. Such an approach would allow a more detailed assessment of the dynamics and fate of mycotoxins throughout bioconversion. The analysis of frass following a defined larval fasting period, combined with complete mycotoxin mass-balance approaches, would further help clarify the relative contribution of excretion, degradation, biotransformation and passive concentration during the process. Targeted and non-targeted analysis of mycotoxin metabolites and transformation products, together with molecular confirmation of fungal identification, would further strengthen the mechanistic understanding and safety assessment of BSFL-based food waste valorisation.

CRedit authorship contribution statement

Joana Oliveira: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Renata Cervantes:** Writing – review & editing, Methodology. **Pedro Pena:** Writing – review & editing, Methodology. **Bianca Gomes:** Writing – review & editing, Methodology. **Robert Kosicki:** Writing – review & editing, Methodology, Formal analysis. **Alexandre Trindade:** Writing – review & editing, Supervision. **Daniel Murta:** Writing – review & editing, Supervision, Funding acquisition. **Carla Viegas:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization. **Magdalena Twarużek:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Ricardo Assunção:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Daniel Murta reports financial support was provided by European Commission. Daniel Murta Daniel Murta was employed by the company Ingredient Odissey SA—Entogreen. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jafr.2026.103356>.

Data availability

Data will be made available on request.

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