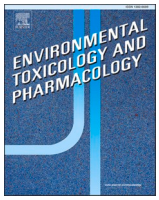
Environmental Toxicology and Pharmacology 117 (2025) 104724

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap



Impact of aflatoxin B_1-exposure on the genotoxic potential of larval extracts of the black soldier fly (*Hermetia illucens*), housefly (*Musca domestica*) and lesser mealworm (*Alphitobius diaperinus*)

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ARTICLE INFO

Edited by Dr. M.D. Coleman

Keywords:
Aflatoxins
Mycotoxin metabolism
Edible insects
Circular food systems
In vitro testing

ABSTRACT

Aflatoxin B_1 (AFB_1)-contaminated crops could serve as insect feed without affecting growth and survival of black soldier fly (BSFL), housefly (HFL), and lesser mealworm (LMW) larvae. However, the genotoxic safety of larvae reared on AFB_1-contaminated substrate remains uncertain. This study assessed the genotoxic effects of larvae reared on 0, 20, and 200 μg/kg AFB_1 spiked substrate through *in vitro* testing. AFB_1 and metabolites were analysed in the feed substrate, larvae and residual material using a LC-MS/MS-based method. Cytotoxic and genotoxic potential of larval extracts were assessed using the Ames MPF™ assay (mini-Ames assay), Ames test, and *in vitro* micronucleus assay.

Results: indicated no effect on survival and biomass for all insects and no bioaccumulation of AFB_1. Species-specific AFB_1 metabolism appeared to occur. HFL exhibited no cytotoxic or genotoxic potential. Further research is required for BSFL and LMW, due to matrix effects and incompatibility with the used *in vitro* tests.

1. Introduction

Aflatoxins, a group of toxic secondary metabolites, are mainly produced by certain species of the *Aspergillus* fungi. These fungi can often be found on food crops such as maize and nuts. All four aflatoxins (aflatoxin B_1, B_2, G_1, G_2) are classified as group 1 carcinogens by IARC, with aflatoxin B_1 (AFB_1) as the most toxic naturally occurring aflatoxin (International Agency for Research on Cancer, 2012; Ostry et al., 2017). In humans and other vertebrates, AFB_1 can be biotransformed by cytochrome P450 (CYP450) enzymes into the reactive AFB_1-*exo*-8,9-epoxide (AFBO) and into other metabolites which are considered less toxic than AFB_1. AFBO is considered the main carcinogenic metabolite of AFB_1 as it can bind with DNA. As such, AFB_1 is considered a genotoxic carcinogen (Schrenk et al., 2020). Other AFB_1 metabolites include aflatoxin M_1 (AFM_1), aflatoxin P_1 (AFP_1) and aflatoxin Q_1 (AFQ_1) (Rushing and Selim, 2019). Additionally, aflatoxicol (AFL) can be formed in the presence of a NADPH reductase, which is not considered less toxic than AFB_1 due to its retaining DNA binding capacity (Berenbaum et al., 2021; Rushing and Selim, 2019). Based on characterised metabolites, CYP450 enzymes are also involved in AFB_1 metabolism in insects (Berenbaum et al., 2021). However, characterisation of metabolites has only been performed on few insect species such as the common fruit fly (*Drosophila melanogaster*) and housefly and the focus was mostly on CYP3 enzymes, as these are thought to be the predominant CYPs involved in insect xenobiotic metabolism (Berenbaum et al., 2021; Feyereisen, 2012).

Human exposure to AFB_1 via food consumption is a major concern, especially since climate change and the resulting increasing warm and humid climates encourages *Aspergillus* spp. growth and AFB_1 contamination of food crops (Focker et al., 2023). Besides liver cancer being the most frequently occurring adverse health effect upon human exposure to AFB_1, other toxic effects are known such as immunotoxicity, malnutrition and growth and developmental disorders (Benkerroum, 2020). Moreover, food security is affected as contaminated crops cannot be consumed and need to be downgraded or even burned (Cucina and Tacconi, 2022). Valorisation of AFB_1-contaminated crops can possibly be accomplished by its use as insect feed when the insects can be grown on contaminated produce. Insects are already consumed as regular part of the human diet in Africa and Asia and it is an upcoming alternative

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https://doi.org/10.1016/j.etap.2025.104724
Received 6 February 2025; Received in revised form 21 May 2025; Accepted 21 May 2025
Available online 22 May 2025

protein source for feed and food in Europe (Raheem et al., 2019). In order to make this a viable solution for upgrading contaminated crops, safety testing of the reared larvae is needed. When deemed safe, the insects can subsequently serve as a sustainable protein source (Meyer et al., 2021).

Previous studies on the rearing of edible insects on AFB_1-contaminated substrates, showed that tested insects could be reared on AFB_1-contaminated substrates without adverse effects on survival and growth of the larvae. These studies focused mainly on black soldier fly larvae (BSFL, *Hermetia illucens* (L.); Diptera: Stratiomyidae) (Bosch et al., 2017; Camenzuli et al., 2018; Meijer et al., 2019), yellow mealworm larvae (YMW, *Tenebrio molitor* (L.); Coleoptera: Tenebrionidae) (Bosch et al., 2017; Daniso et al., 2024; Gützkow et al., 2021), lesser mealworm larvae (LMW, *Alphitobius diaperinus* (Panzer); Coleoptera: Tenebrionidae) (Camenzuli et al., 2018; Meijer et al., 2022) and housefly larvae (HFL, *Musca domestica* (L.); Diptera: Muscidae) (Niermans et al., 2023). The studies showed that these insects could be reared on the studied AFB_1-contaminated substrates, with exposure concentrations ranging from 10 to 600 μg/kg dry feed, without adverse effects on survival and yield of the larvae.

In some of these studies, fate and metabolism of AFB_1 in BSFL, HFL, LMW was studied as well, showing that the insect larvae could be reared on AFB_1-contaminated substrate without bioaccumulation of the mycotoxin in their body after starvation (to empty their guts) (Bosch et al., 2017; Camenzuli et al., 2018; Meijer et al., 2022, 2019). The degree of AFB_1-metabolization by the insects and the incomplete recovery of certain known AFB_1 metabolites compared to the spiked concentrations in feed varied between the insect species, suggesting species-specific differences in AFB_1 metabolic pathways. As such, it remained unclear whether other additional or so far unknown metabolites were formed and whether unidentified metabolites were also considered less toxic than the parent compound (Niermans, 2024).

Based on the current literature, no final conclusion could be made whether or not insects convert AFB_1 into less or more toxic metabolites, and thus on the safety of edible insects reared on AFB_1-contaminated substrates. This study aimed to investigate the cytotoxicity and genotoxicity of BSFL, HFL and LMW larvae reared on AFB_1-spiked substrate using different *in vitro* state-of-the-art genotoxicity tests (Ames test and micronucleus assay).

2. Materials and methods

2.1. Insect experiments

BSFL and HFL reared on AFB_1-spiked substrate from previously published studies were used to generate larval extracts for the *in vitro* toxicity tests in this study, and are shortly described below. For the LMW, an insect experiment was conducted on AFB_1-spiked substrate and control substrate to likewise generate larval extracts to perform the *in vitro* toxicity tests.

2.1.1. Black soldier fly larvae

BSFL used in this study were obtained from the study of Shah et al. (2024); these BSFL originated from the BSF colony of the Laboratory of Entomology (Wageningen University, The Netherlands). BSF eggs, laid within a period of six hours, were harvested from the cardboard strips (egg-laying tool) and reared on chickenfeed (Kuikenopfokmeel, Kasper Faunafood, Woerden, the Netherlands) until they were seven-days-old after which they were used in the current experiment.

These seven-days-old BSFL were subjected to either a control diet or a diet spiked with 20 μg/kg AFB_1, which is the maximum limit of AFB_1 allowed in feed materials at the moment of the experiment was conducted as set in Directive 2002/32/EC (EC, 2002). The control substrate was prepared by mixing 333 g chickenfeed, 618 mL water, and 1.9 mL MeOH without AFB_1 (moisture content 65 %; 0.2 % MeOH). The spiked diet was prepared in the same way, with addition of 1.9 mL of the AFB_1 solution (suspended in MeOH) to the chickenfeed/water mixture (moisture content 65 %; 0.2 % MeOH). 150 BSFL, were placed in a 480 mL rearing container (BugDorm insect pots, MegaView Science Co., Ltd., Taichung, Taiwan) together with 100 g of wet control feed or wet feed spiked with AFB_1. The two treatments were performed in quadruplicate. The containers were placed in a climate chamber at 27 °C, relative humidity of 65 % and a day/night rhythm of 12/12 h for seven days. On the seventh day, all BSFL were separated from the residual material (mixture of left over substrate and frass), washed with room temperature tap water, dried, weighed and transferred onto control feed for one day to remove contaminated substrate from the larvae's digestive system (with the purpose of gut cleaning). On day eight, this procedure was repeated after which the larvae were frozen and stored at −20 °C for further analyses.

2.1.2. Housefly larvae

The HFL eggs used in this study were taken from the HFL colony of the Laboratory of Entomology (Wageningen University, The Netherlands). The colony originated from the Faculty of Science and Engineering (University of Groningen, The Netherlands). The HFL rearing process was identical as described in Niermans et al. (2023).

Like the BSFL, the HFL eggs were subjected to either a control diet or a diet spiked with 20 μg/kg AFB_1. The substrates used for HFL rearing were prepared as described in Niermans et al. (2024). In short, the dry substrate for HFL consisted of a mixture of 37 % wheat bran, 56 % wheat flour, 4 % full fat milk powder (28.2 g fat/100 g) and 2 % dry instant baker's yeast. A total of 600 mL wet control substrate was prepared by manually mixing the 240 g of the dry feed mixture, 144 mL water, 216 mL Nipagin solution in water (final concentration: 0.9 mg/L; Merck, Darmstadt, Germany) and 1.2 mL MeOH without AFB_1 for 15 min (moisture content 65 %; 0.2 % MeOH). The AFB_1-spiked substrate was prepared in the same manner with addition of 1.2 mL of the AFB_1 solution (dissolved in MeOH) to the dry food/water mixture (moisture content 65 %; 0.2 % MeOH). 200 HFL eggs (corresponding to 0.012 g) were added to the same rearing containers as used for BSFL with 60 g of either wet control- or wet spiked substrate, with four replicates per treatment. The containers were placed in a climate chamber at 25 °C, relative humidity of 65 % and a day/night cycle of 12/12 h for five days. At day five, the same harvesting procedures were applied as with BSFL. However, the larvae were put on control feed for ± 5.5 h to empty the contaminated substrate from the larvae's gut. The HFL larvae and all residual material were frozen and stored at −20 °C for further analyses.

2.1.3. Lesser mealworm larvae

Neonate LMW larvae, which had hatched within 24 h before the start of the experiment were obtained from Ynsect NL Nutrition & Health B.V, The Netherlands.

As was done for the other two insect species, the LMW were subjected to either a control diet or a diet spiked with 20 μg/kg AFB1. Additionally, the LMW received a diet spiked at 200 μg/kg (10-times the maximum limit of AFB_1 in feed at the moment of the experiment was conducted (EC, 2002)), as a case to assess the genotoxic potential of the larvae when reared on excessive concentrations of AFB_1. As LMW require feed with lower water content than BSFL and HFL, the substrate was prepared following a different protocol. The control substrate was prepared creating a slurry of 1 kg dry feed (wheat bran-based) and approximately 2300 mL MeOH, which was mixed using a hand mixer. The spiked diet was prepared in the same way, with spiking of two 1 kg batches of dry feed with AFB_1 at intended concentrations of 20 μg/kg and 200 μg/kg using a 250 μg/mL stock solution of AFB_1 (dissolved in MeOH). The slurries were placed in aluminium containers and left in a fume hood for one day to allow the MeOH to evaporate. After one day, the spiked and control feed were weighed to measure the moisture loss. Finally, the feed was transferred into closed containers and stored in a dry and cool place until further usage. At the start of the experiment, for each replicate, 20 g of dry feed mixed with water (ratio 1:1 on weight

basis) and 300 mg of one-day old LMW were placed in an open container of approximately 20 × 20 cm. The three treatments were performed in triplicate. The rearing containers were placed in a climate chamber at 30 °C, relative humidity of 65 % and a day/night rhythm of 12/12 h for 25 days. Throughout the rearing period, larvae were fed daily, a total of 828 g of prepared feed and 1144 mL of water was ultimately added to the replicate containers. The feed to water ratio was 1:1 from day one and gradually changed to 1:1.25 towards the end. On day 25, 50 % of the larvae subjected to spiked substrate were sieved from the remaining feed as described in Meijer et al. (2022) and moved to a separate rearing container with 11.2 g of control feed for 24 h. After the additional 24-hour period, the larvae in all replicate containers were separated from the residual material, washed, dried and weighed. Per treatment, the larvae and all residual material were placed in clean containers, frozen and stored at −20 °C until further analysis.

2.2. Chemical analyses

In order to quantify the concentrations of AFB_1, AFM_1, AFP_1, AFQ_1 and AFL in the harvested insect larvae, substrates and residual material from the insect rearing period, the following procedures were followed.

2.2.1. Chemicals

Chemical standards for AFB_1, AFL and AFM_1 were purchased from Romer Labs (Getzersdorf, Austria). A standard for AFL was purchased from Enzo Life Sciences (Brussels, Belgium). Standards for AFP_1 and AFQ_1 were purchased from TRC (Toronto, ON, Canada). Moreover, acetic acid 99 – 100 % (100063 Merck KgaA, Darmstadt, Germany), acetonitrile (01203502 HPLC Supra Gradient, Biosolve Chimie, Dieuze, France), MilliQ and DMSO ≥ 99.9 % (472301 Sigma Aldrich Chemie, Schnelldorf, Germany) were used for the sample extractions.

2.2.2. Extraction

Extracts were obtained from the substrates, residual material and insect larvae, namely BSFL, HFL, LMW with starvation, LMW without starvation and their controls. Before extraction, the insect larvae were grinded cryogenically (with liquid nitrogen) into a powder and subsequently stored at −20 °C until further use.

The extraction process of all samples followed the procedure as described by Camenzuli et al. (2018) with adjustments. In short, 2.50 ± 0.01 g of feed/residual material was weighed into 50 mL Greiner centrifuge tubes and mixed with 7.5 mL water. 10 mL extraction solvent was added, consisting of acetonitrile (ACN)/acetic acid 99:1 (*v/v*). After vigorous manual shaking, the tubes were shaken for 30 min in a head-over-head shaker (Reax 2, Heidolph Instruments GmbH & Co, Schwabach, Germany). After addition of 4.0 ± 0.1 g grams of magnesium sulphate ($MgSO_4$, 291184 P, VWR Chemicals, Radnor, USA), the tubes were shaken vigorously by hand and with a vortex shaker (Vortex-genie, scientific industries). Subsequently, the tubes were centrifuged for 10 min at 3500 rpm (Sorvall RC3BP plus low-speed centrifuge, Thermo Scientific, Waltham, USA).

Standard additions were used to quantify AFB_1 and metabolite concentrations in the larvae, substrates and residual material samples. The extracts were prepared with and without the addition of a standard mix containing AFB_1 and four aflatoxin metabolites (AFM_1, AFP_1, AFQ_1 and AFL). Per sample, 200 µl extract was mixed with either 190 µl water and 10 µl of the standard mix or only 200 µl water in a syringeless PTFE filter vial (Mini-UniPrep, Whatman, Marlborough, MA). The vials were capped, vortexed and refrigerated for 30 min after which the vials were closed and stored at 4 °C until liquid chromatography with tandem mass spectrometry (LC-MS/MS) analyses.

2.2.3. Quality control

Quality control procedures included the determination of AFB_1 concentration and homogeneity of freshly spiked substrate. For the BSFL and HFL experiments, eight samples were taken from the spiked substrate and four samples from the spiked residual material for homogeneity determination. Also, from the HFL control feed, four samples were taken to determine the concentration of AFB_1 (to confirm its absence). From the spiked substrates in the LMW experiment, ten samples were taken per treatment to determine homogeneity. Four samples were also taken from the control feed to confirm the absence of AFB_1. The prepared insect diets were considered homogeneous when there was ≤ 20 % difference between the measured concentrations (relative standard deviation) of the samples.

Additional controls were included to confirm extraction efficiency and matrix effect of extracts from all three insect species. This was examined by spiking either 2.5 g of the substrate/residual material with 500 µL of a 0.1 µg/mL AFB_1 stock solution (10 ppb) or 500 µL of a 5 µg/mL stock solution (100 ppb), prior to extraction. Extraction efficiency was evaluated in 0.5 g of grinded insect larvae (BSFL/HFL/LMW starved) fed on the control diet. Further extraction and storage steps were identical as described above.

The limit of quantification (LOQ) was determined per matrix type and per compound by visual inspection of the signal to noise ratio. All values below LOQ were considered zero during data analyses.

2.2.4. LC-MS/MS analyses

The same LC-MS/MS methods were used as described in (Niermans et al., 2023c) to quantify the AFB_1 and metabolites concentrations in the insect larvae extracts, substrates and residual material. For the analysis, the AB Sciex Exion LC (AB Sciex Pte. Ltd., USA) was used together with an AB Sciex QTRAP 6500 triple quad system with an electrospray ionization (ESI) source on positive ionization mode. See Supplementary Table 1 for the instrumental MS/MS parameters. LC separation was performed with an Acquity UPLC HSS T3 1.8 µm 100 × 2.1 mm column (Waters, Milford, MA, USA).

2.2.5. In vitro toxicity testing of BSFL, HFL, LMW extracts

Insect extracts were subjected to several *in vitro* assays to evaluate their mutagenic and genotoxic potential. For this purpose, the Ames MPF™ assay (mini-Ames assay), the Ames test and the *in vitro* micronucleus assay were used with extracts of the insect larvae reared on AFB_1-spiked substrate and their controls.

2.2.6. Optimization of insect extraction

The same extraction method as used for LC-MS/MS analyses was intended to be used to also prepare the insect extracts for the *in vitro* assays. However due to matrix effects of the insect extracts in the Ames MPF™ assay, amendments were made to the extraction process of the three insect larvae species. In summary, 0.50 ± 0.01 g of grinded larvae material of each treatment was thawed at room temperature and put into 50 mL Greiner centrifuge tubes to which 9.5 mL MilliQ water was added. After 15 min, 10 mL of the extraction solvent was added and the extraction procedure was followed as previously described. 10 mL of the extract was filtered using a syringe (Terumo® syringe, latex free, luer locktip) with PTFE filter, 0.45 µm (ACRODISC CR PTFE 0.45 µM 25MM). Moreover, an extra clean-up step was performed using an EMR-Lipid SPE filter (Agilent Captiva EMR-Lipid cartridge). Subsequently, the extracts were evaporated to dryness under a nitrogen flow (35 °C; TurboVap LV, Caliper Life Sciences, Waltham, USA). 455 µl DMSO was added to the extracts before storage at −20 °C until further use. The weight of DMSO was equivalent to the starting weight of 0.50 ± 0.01 g of insect larvae. In this way, the weight:volume ratio of the amount of grams of insects used to obtain the extracts was as comparable as possible, and insect extracts were as representative as possible for the insects themselves when applied in the *in vitro* toxicity assays.

2.2.7. Ames MPF™ assay (mini-Ames)

The mutagenic potential of insect extracts of BSFL, HFL and LMW fed on either a control diet or a diet spiked with AFB_1 was determined using the Ames MPF™ 98/100 Microplate Format Mutagenicity Assay of

Xenometrix (Allschwil, Switzerland). The test kit uses the *Salmonella typhimurium* strains TA98 and TA100 to detect base substitution mutations and frameshift mutations, respectively. The insect extracts were subjected to the test conditions with and without a metabolic system via the addition of a 30 % phenobarbital/ß-naphthoflavone-induced rat liver S9 mix (TA98-, TA100-, TA98 + and TA100 +).

The *S. typhimurium* strains (TA98, TA100), ampicillin (50 mg/mL), 2-nitrofluorene (2NF; 50 μg/mL), 4-nitroquinoline N-oxide (4NQO; 2.5 μg/mL), S9 100/1537 booster solution, growth medium, exposure medium and indicator medium (pH indicator medium lacking histidine) were provided as part of the Ames MPF™ 98/100 Mutagenicity Assay kit. The phenobarbital/ß-naphthoflavone-induced rat liver S9, and S9-cofactors (S9-Buffer-Salts, G-6-P, NADP, buffer M) originated from the PCO-0800 S9 Cofactor Kit from Xenometrix AG (Allschwil, Switzerland).

25 μL (TA98, TA100) of freshly thawed frozen strains were inoculated in 10 mL of sterile growth medium together with 10 μL ampicillin (50 mg/mL) in a sterile 50 mL Greiner tube. Simultaneously, the latter was performed in absence of the strains as a negative control. The caps of the Greiner tubes were sealed with parafilm. Inoculated medium was incubated in an orbital shaking incubator, Innova 44 (New Brunswick Scientific, Edison, USA) overnight (14–15 h) at 37 °C, 250 rpm, 1 in. amplitude. OD_{600} (Helios Epsilon, Thermo Scientific, Waltham, USA) of the overnight cultures were determined to confirm sufficient growth. OD_{600} of TA98 and TA100 should be $\geq$ 2.0, whereas the OD_{600} of the negative control (incubated growth medium) needed to be $\leq$ 0.05 to rule out contamination. Each biological replicate of the sample, was tested as a technical triplicate in the Ames MPF™. Therefore, 10 μL of sample (Table 1) was added to three different wells within a 24-well plate following the lay-out shown in Supplementary Figure 1a. This exact procedure was done for four 24-well plates (TA98 +, TA98-, TA100 + and TA100-).

The TA98- and TA100- were prepared by diluting TA98 (10 %) or TA100 (5 %) in exposure medium. For TA98 + and TA100 + this was done by diluting TA98 or TA100 in a mixture of the S9 100/1537 booster solution + exposure medium (ratio 1:667) and 4.50 % S9 mix. This S9 mix was prepared freshly by mixing 54.32 % S9-Buffer-Salts, 2.52 % G-6-P, 3.17 % buffer M, 10 % NADP with 29.99 % phenobarbital/ß-naphthoflavone-induced rat liver S9 and was kept on ice prior to use. 240 μL of the TA98-, TA100-, TA98 + and TA100 + mixtures were transferred to each well of their respective 24-well plate and incubated at 37 °C, 250 rpm, 1 in. amplitude, for 90 min in an orbital shaking incubator (Innova 44, New Brunswick Scientific, Edison, USA). After incubation, 2.8 mL of the indicator medium was added to each well. Final concentration of DMSO in the well was 0.33 %. Then, 50 μL of the sample/indicator medium aliquots were dispersed from a single well of the 24-wells plate to 48 wells of a 384-well plate (Supplementary Figure 1b).

After all samples were transferred, the 384-well plates were incubated at 37 °C for 48 h in an orbital shaking incubator, shaking shut off (Innova 44, New Brunswick Scientific, Edison, USA). During incubation, the cells that have undergone mutagenicity grew into suspension. The increased growth resulting from this reduces the pH of the medium and induces a colour change from purple to yellow. Therefore, after incubation, the OD_{590} value of the 384-well plates was determined in a platereader (Synergy HTX - Multi-Well Plate Reader - BioTek, Agilent Technologies, Santa Clara, USA), via which the number of positive wells (yellow) out of 48 wells per technical replicate was counted. From this a fold induction over baseline was determined, which was done by dividing the mean number of positive wells for all technical and biological replicates per sample by the baseline. The baseline was calculated by adding one standard deviation to the mean number of positive well of the solvent control sample. When the fold induction was < 2, the outcome was considered as negative, whereas a fold induction $\geq$ 2, was considered as a positive result indicating mutagenicity.

Table 1
Overview of samples tested in the Ames MPF™ 98/100 Microplate Format Mutagenicity Assay.

Abbreviation	Subscription	Final conc. during pre-incubation
Solvent control	DMSO	
chem bl.	Final extraction solvent	
chem bl.+AFB_1	Final extraction solvent spiked with 1.05 μg/mL AFB_1 (final conc. of extract)	AFB_1: 4.18E−02 μg/mL
BSFL, HFL, LMW	BSFL, HFL or LMW fed on clean feed (n = 3)	
BSFL 0.02, HFL 0.02	BSFL or HFL fed on a diet spiked with 0.02 mg/kg AFB_1 (n = 3)	
LMW 0.2	LMW fed on a diet spiked with 0.2 mg/kg AFB_1 (n = 3)	
15x BSFL 0.02, 15x HFL 0.02, 15x LMW 0.2	15x dilution of the BSFL, HFL or LMW fed on the AFB1-spiked substrate (n = 3)	
BSFL + AFB_1, HFL + AFB_1, LMW + AFB_1	Pooled sample (n = 3) of the BSFL, HFL or LMW fed on a clean diet spiked with 1.05 μg/mL AFB_1 (final conc. of extract) prior to extraction	
BSFL + 2NF, HFL + 2NF, LMW + 2NF	Pooled sample (n = 3) of the BSFL, HFL or LMW fed on a clean diet spiked with 50 μg/mL 2NF to the extract	2NF: 2 μg/mL
+	Positive control: 2.38 μg/mL AFB_1 (TA98 + and TA100 +), final conc. 50 μg/mL 2NF (TA98-), and final conc. 2.5 μg/mL 4NQO (TA100-)	AFB_1: 9.52E−02 μg/mL 2NF: 2 μg/mL 4NQO: 0.1 μg/mL

2.2.8. Cytotoxicity check of Ames MPF™ assay strains

In parallel to the Ames MPF™ assay, the optical density (OD600) after 90 min and 48 h of incubation was measured to determine the strains' ability to grow in the presence of insect extracts. This was performed to prevent false negative results due to cytotoxicity of Ames MPF™ assay strains. For this, 10 μL of each of the sample (Table 1) was added to an additional 24-well plate. 240 μL of the TA98 + mixture was added to each well and incubated together with the rest of the 24-wells plates for 90 min (37 °C, 250 rpm). 50 μL from one well of the 24-well plate was then transferred to four wells of a 96-well plate, after which the OD_{600} value was determined in a platereader (Synergy HTX -Multi-Well Plate Reader - BioTek, Agilent Technologies, Santa Clara, USA). Differently from the rest of the plates, this 96-well plate was kept as is and was incubated together with the 384-well plates for 48 h at 37 °C after which the OD_{600} value was determined again. Cytotoxicity was confirmed when the average OD_{600} values of the biological and technical replicates of the sample were $\leq$ baseline OD_{600} minus three times the standard deviation (3 *SD). Baseline was calculated based on the OD_{600} values of the technical replicates of the positive control (AFB_1 dissolved in DMSO).

2.2.9. Cytochrome P450 inhibition assay (CYP3A4, 3A5 and 3A7)

To evaluate whether the insect matrices interfered with cytochrome P450 (CYP3A4, CYP3A5 and CYP3A7) activity, the P450-Glo™ CYP3A4 Assay (Luciferin-PPXE) DMSO Tolerant Assay 10 mL V891 (Promega Benelux, Leiden, the Netherlands) was used. The biological replicates of the larval extracts of BSFL, HFL and LMW were pooled and tested as technical replicates. The pooled samples were subjected to the assay undiluted and diluted (3x, 10x, 30x, 100x and 1000x), with and without metabolic activation (rat liver S9-mix).

P450glo (50 mM), luciferin detection reagent and reconstitution buffer were provided by the P450-Glo™ CYP3A4 Assay (Luciferin-PPXE) DMSO Tolerant Assay 10 mL V891 (Promega Benelux, Leiden, the Netherlands). The phenobarbital/ß-naphthoflavone-induced rat liver S9, and S9-cofactors (S9-Buffer-Salts, G-6-P, NADP) originated from the PCO-0800 S9 Cofactor Kit from Xenometrix AG (Allschwil, Switzerland).

Firstly, a 500 μM P450glo mixture was prepared by diluting P450glo

(50 mM) 100x in Tris-HCl buffer (100 mM; pH 7.5). Then a S9 co-factor mix was made by adding 4.53 % buffer M, 77.59 % S9-Buffer-Salts, 3.60 % 6-G-P and 14.28 % NADP together. Afterwards, a S9 co-factor mix with and without S9 were prepared. The S9 co-factor mix without S9 was prepared by mixing 25 % of the 500 μM P450glo mixture (final concentration 125 μM) and 60 % of a 0.33 M KPO_4 buffer (pH 7.4; final concentration 200 mM) and 15 % of the S9 co-factor mix. Whereas the S9 co-factor mix with S9 contained 23.92 % of the 500 μM P450glo mixture (final concentration 125 μM) and 57.42 % of a 0.33 mM KPO4 buffer (pH 7.4; final concentration 200 mM) and 14.35 % of the S9 co-factor mix and 4.31 % of phenobarbital/ß-naphthoflavone-induced rat liver S9.

6.40 μL of sample extract was added to 153.60 μL of either the mastermix with or without 4.31 % S9 (Table 2) and mixed by pipetting up and down, after which 50 μL was added to each of three wells (technical triplicates) of a 96-well plate. The plates were then pre-incubated for 15 min at room temperature. Afterwards, 50 μL of reconstituted luciferin detection reagent was added to each well and gently mixed by tapping the 96-well plate for 10 s. The plates were then incubated for 30 min at room temperature in order to stabilise the luminescent signal. Luminescence in relative light units (RLU) was recorded using a platereader (CLARIOstar microplate reader, BMG Labtech, Ortenberg, Germany).

Table 2
Overview of samples tested in the P450-Glo™ CYP3A4 Assay (Luciferin-PPXE) DMSO Tolerant Assay.

Sample	S9 co-factor mix: with (w) or without (w/o) S9	(also see description Ames MPF™)
Minus-P450	w/o	CYP-independent background signal
Control inhibitor	w	Itraconazole (final concentration in well 120 μM; CAS: 648625–61–6)
Untreated	w	Total CYP activity, no inhibitor or test compound present
BSFL-, HFL-, LMW-	w/o	Pooled sample (n = 3) of the BSFL, HFL or LMW fed on clean feed
BSFL+ , HFL+ , LMW+ (undiluted, 3x, 10x, 30x, 100x, 1000x)	w	Pooled sample (n = 3) of the BSFL, HFL or LMW fed on clean feed, either undiluted, or diluted (3x, 10x, 30x, 100x or 1000x)
BSFL0.02 + , HFL0.02 + (undiluted, 3x, 10x, 30x, 100x, 1000x)	w	Pooled sample (n = 3) of the BSFL, HFL fed on a diet spiked with 0.02 mg/kg AFB_1, either undiluted, or diluted (3x, 10x, 30x, 100x or 1000x)
LMW0.2 + (undiluted, 3x, 10x, 30x, 100x, 1000x)	w	Pooled sample (n = 3) of the LMW fed on a diet spiked with 0.2 mg/kg AFB_1, either undiluted, or diluted (3x, 10x, 30x, 100x or 1000x)
Solvent control	w	DMSO
chem bl.	w	Extraction solvent
chem bl.+AFB_1	w	Extraction solvent spiked with AFB_1 prior to extraction
BSFL + AFB_1, HFL + AFB_1, LMW + AFB_1	w	Pooled sample (n = 3) of the BSFL, HFL or LMW fed on a clean diet, subsequently spiked with AFB_1 prior to extraction
BSFL + 2NF, HFL + 2NF, LMW + 2NF	w	Pooled sample (n = 3) of the BSFL, HFL or LMW fed on a clean diet, subsequently spiked with 2NF prior to extraction
+	w	AFB_1 (final concentration in well 9.52E−02 *μg/mL)*

The net luminescence activity was calculated by dividing the RLU of sample by RLU of the untreated sample (Table 2) and expressed as a percentage of the total activity. The output of the untreated sample was considered as 100 % activity, while as based on the output of the inhibitor (120 μM itraconazole), an activity of ≤ 40 % was considered as major inhibition and an activity between 40 % and 60 % was considered as minor inhibition (Doshi and Li, 2011). Cytochrome P450 (CYP3A4, 3A5 and 3A7) activity was calculated per insect species, therefore an average activity was determined for the larvae (BSFL, HFL, LMW) fed on a clean diet and the AFB_1-spiked diet.

2.2.10. Bacterial reverse mutation test (Ames test)

According to OECD guideline 471, a non-GLP bacterial reverse mutation test (Ames) was performed by Charles River Laboratories Den Bosch BV (The Netherlands) on the extracts of HFL and LMW reared on 20 μg/kg spiked substrate and their controls. BSFL extracts were not tested in the Ames-test as, due to matrix interference observed with the Ames MPF™ assay (see Results section), they were not considered suitable. The test was performed using the *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and the *Escherichia coli* strain WP_2uvrA. DMSO was used as the negative control. The positive controls used with and without S9-mix can be found in Supplementary Table 2. The samples were tested in concentrations of 3, 4 and 5 μL/plate in triplicate in each strain, in the absence and presence of 5 % (v/v) S9-mix. The negative and positive control were also tested in the presence and absence of S9-mix and in triplicate.

The larval extracts were considered not mutagenic if the total number of revertants in tester strain TA100 or WP_2uvrA was not greater than two times the concurrent control, and unless the total number of revertants in tester strain TA1535, TA1537 or TA98 was not greater than three times the concurrent control. Otherwise, it was considered mutagenic in the test.

2.2.11. In vitro micronucleus assay

Due to the incompatibility of BSFL extracts for *in vitro* testing as determined with the Ames MPF™ assay, only HFL and LMW extracts were additionally evaluated by Charles River Laboratories Den Bosch BV (the Netherlands) for its ability to induce micronuclei in human lymphoblastoid TK6 cells (Cryovial 300357, Vital: 330357, CLS, Eppelheim, Germany) as a measure of DNA damage (non-GLP). This was done in the presence or absence of metabolic activation (rat S9-mix, Trinova Biochem GmbH, Giessen, Germany). The *In Vitro* MicroFlow® Kit (Litron Laboratories, Rochester, NY) was used, developed according to OECD guidelines 487. DMSO was used as the negative control. The positive control for the assay without the S9-mix was Mitomycin C (MMC-C; CAS No. 50–07–7, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) at a final concentration of 0.06 and 0.15 μg/mL for a 24 h exposure period. For the assay with the S9-mix, Cyclophosphamide (CP CP; CAS No. 6055–19–2; Sigma Aldrich Chemie GmbH, Schnelldorf, Germany) was used at a final concentration of 11.9 and 15.0 μg/mL for a 3 h exposure period. All positive controls were dissolved in Hanks' Balanced Salt Solution (HBSS) without calcium and magnesium (HBSS; Life Technologies, Bleiswijk, The Netherlands). No correction was made for the purity/composition of the HFL extracts. The pure extract was used as stock solution. The larval extracts were tested in triplicate using eleven spike solutions with and without 2 % (v/v) S9-mix during a 24 hour treatment period. The stock and spike solutions were diluted 25-fold with exposure medium. These solutions were diluted 4-fold with cell suspension in the assay resulting in final test concentrations of 0.03 – 1 %. The final concentration of the vehicle (DMSO) in the culture medium was kept at 1 % (v/v).

The larval extracts were considered not genotoxic in the MicroFlow® *in vitro* micronucleus Assay if none of the test concentrations exhibited a 3-fold increase in the number of micronuclei compared with the concurrent negative control and there was no concentration-related

increase. Otherwise, it was considered genotoxic.

2.3. Data analysis

2.3.1. LC-MS/MS data

SCIEX-OS 2.2 software was used to analyse the aflatoxin concentrations from LC-MS/MS. The identification of peaks was performed by determining the signal to noise ratio. Mean recovery was within the range of 80–120 %, hence the analysed concentrations were not corrected for recovery (Pihlström et al., 2021). The concentrations detected in the LC-MS/MS analyses were adjusted based on the wet weight measured for each treatment. These concentrations were calculated by dividing the peak area of the sample by the difference between the area of the sample with standard addition and the area of the sample, and multiplying by the addition level of the respective mycotoxin.

2.3.2. Molar mass balance calculations

The amount of AFB_1 and its metabolites present was calculated in the larvae and residual material prior to and after the rearing experiment following the method described by Meijer et al. (2022). The molar mass balance was determined by dividing the total mycotoxin mass detected in both the larvae and residual material by the molar mass of the mycotoxin. This calculation was based on the initial wet weight of the substrate. The molar mass balance was separately calculated for the residual material on day seven (BSFL) day five (HFL), or day 25 (LMW).

All calculations (aflatoxin concentrations, Ames MPF™ assay, cytotoxicity etc.) were performed in Excel. All figures were made in GraphPad Prism v5.02.

3. Results

3.1. Insect rearing experiments

3.1.1. Aflatoxins in spiked and control substrates

The levels of aflatoxins (AFB_1, AFM_1, AFP_1, AFQ_1 and AFL) detected in the control substrate for HFL and LMW were all below the LOQ of the analytical method used (Supplementary Tables 3 and 4). Additionally, for the spiked substrates, the analysed concentrations of AFB_1 were within an acceptable relative standard deviation range of $\leq$ 20 % difference between the measured concentrations of the samples, confirming the homogeneity of the spiked AFB_1 in the substrates. The measured mean AFB_1 concentration in the BSFL spiked substrate was 22.5 ± 1.7 μg/kg (Shah et al., 2024), and 19.7 ± 1.0 μg/kg for the HFL substrate. For the LMW substrates the AFB_1 concentrations were 11.8 ± 1.0 μg/kg and 114.8 ± 12.0 μg/kg AFB_1 for the low and high spiked treatment, respectively, being 2-fold lower as the intended concentration. All spiked substrates were also analysed for the presence of AFM_1, AFP_1, AFQ_1 and AFL, and concentrations of these aflatoxins were found to be below their respective LOQ. However, the LOQ could not be determined for AFP_1 due to matrix-related signal noise. In addition, the presence of AFB_2, AFG_1, AFG_2 was also analysed in the substrates of BSFL and HFL, and concentrations of these aflatoxins were also found to be below their respective LOQs (data not shown).

3.1.2. Larval survival and biomass

No significant differences were seen in the survival and biomass between AFB_1-exposed BSFL and HFL and their controls. Survival of BSFL fed on the control diet was on average 94.0 ± 3.7 %, whereas survival after feeding on an AFB_1-spiked diet resulted in a survival of 90.5 ± 2.1 % (Shah et al., 2024). In the HFL experiment feeding on either the control diet or the AFB_1-spiked diet led to a survival of 66.5 ± 12.7 %, and 71.0 ± 10.2 %, respectively (Niermans et al., 2023) (Supplementary Table 5). The average number of survived LMW reared on te control substrate was 3817 ± 41. The average number of survived LMW reared on 20 μg/kg and 200 μg/kg AFB_1-spiked substrates was 3706 ± 222 and 3832 ± 231 respectively. Survival was in all cases not affected by exposure to the aflatoxin spiked substrates.

The average individual weight of each BSFL was 139.0 ± 10.8 mg when grown on the control diet and 137.3 ± 6.9 mg when feeding on the AFB_1-spiked diet (Shah et al., 2024). Average individual HFL weight was 14.8 ± 2.8 mg for the control substrate and 16.9 ± 1.9 mg for the contaminated substrate (Supplementary Table 5). Average individual weight of LMW reared on the control substrate was 30.0 ± 0.4 mg. The average individual weight of LMW reared on 20 μg/kg and 200 μg/kg AFB_1-spiked substrates was 31.1 ± 1.0 mg and 30.4 ± 1.8 mg, respectively (Supplementary Table 5). Biomass was in all cases not affected by exposure to the aflatoxin spiked substrates.

3.1.3. Fate and metabolism of AFB_1 during insect rearing

The presence of aflatoxins (AFB_1, AFM_1, AFP_1, AFQ_1 and AFL) were analysed in the larvae as well as in residual material of BSFL, HFL, and the LMW (day 25) and LMW (day 25 and 26). None of the aflatoxins were detected in concentrations above their respective LOQs in the BSFL, HFL and LMW, but were in some cases above the LOQ in the residual material. The molar mass balance calculations, for all insect species, were therefore completely based on the aflatoxin concentration in the residual material.

In the BSFL experiment, the total calculated molar mass balance was 50.0 ± 2.6 %, with AFB_1 accounting for 38.5 ± 1.5 %, and the aflatoxin metabolite AFP_1 contributing with 11.0 ± 1.8 %, as was published before by Niermans et al. (2024). In the HFL experiment, only AFB_1 contributed and the average calculated molar mass balance was 64.0 ± 3.5 %, as was published before (Niermans et al., 2024). For the LMW, the molar mass balance was 11.1 ± 0.8 % (only AFB_1 contributed) in the 20 μg/kg AFB_1 treatment. In the 200 μg/kg treatment the total molar mass balance was 13.1 ± 2.7 %, of which AFB_1 contributed 12.1 ± 2.7 % and AFM_1 1.0 ± 0.07 %. The majority of the initially spiked AFB_1 could thus not be recovered in all three insect experiments.

3.2. In vitro assessment of insects reared on AFB_1-spiked substrates

3.2.1. In-house testing of genotoxicity and cytotoxicity of larval extracts

Mutagenic properties of the insect extracts upon rearing the larvae on AFB_1-spiked substrate or control substrate were evaluated *in vitro*. The Ames MPF™ test was performed using TA98 and TA100 with and without S9 activation for all insect extracts, after evaluation of the cytotoxicity of all insect extracts. BSFL, HFL and LMW extracts did not induce a cytotoxic effect in the *S. typhimurium* strains.

The extracts (n = 3 per treatment) fed on both the control and AFB_1-spiked substrates were negative in all strains that were included in the Ames MPF™ assay (Fig. 1). Pooled extracts (n = 3) of HFL and LMW fed on clean substrate and spiked with AFB_1 prior to extraction were positive in the TA98 + and TA100 + strains, to the same extent as the positive control. However, the pooled extracts (n = 3) of the BSFL spiked with AFB_1 after control substrate treatment showed negative results in all strains, whereas this was expected to be positive. The negative, result was also observed in all strains with the pooled BSFL extracts (n = 3) spiked with 2NF after treatment with control substrate. These results suggest that the BSFL extracts may interfere with the Ames MPF™ assay, possibly due to a matrix effect. The pooled extracts (n = 3) of HFL fed on clean substrate and spiked with 2NF prior to extraction were positive in all strains. This was also true with the pooled LMW extracts (n = 3) spiked with 2NF after being fed on control substrate, in TA98 + and TA100 + strains. However, these extracts were negative in the TA98- and TA100- strains, also indicating a possible interference of the LMW extracts with the assay. The outcomes of all negative control samples (DMSO and chem bl.) and all positive control samples (chem bl.+AFB_1 and +) were negative and positive, respectively.

3.2.2. CYP450 (CYP3A4, 3A5 and 3A7) inhibition

The unexpected negative results observed for the control BSFL extracts (after spiking with AFB_1 and 2NF) and LMW extracts (after spiking

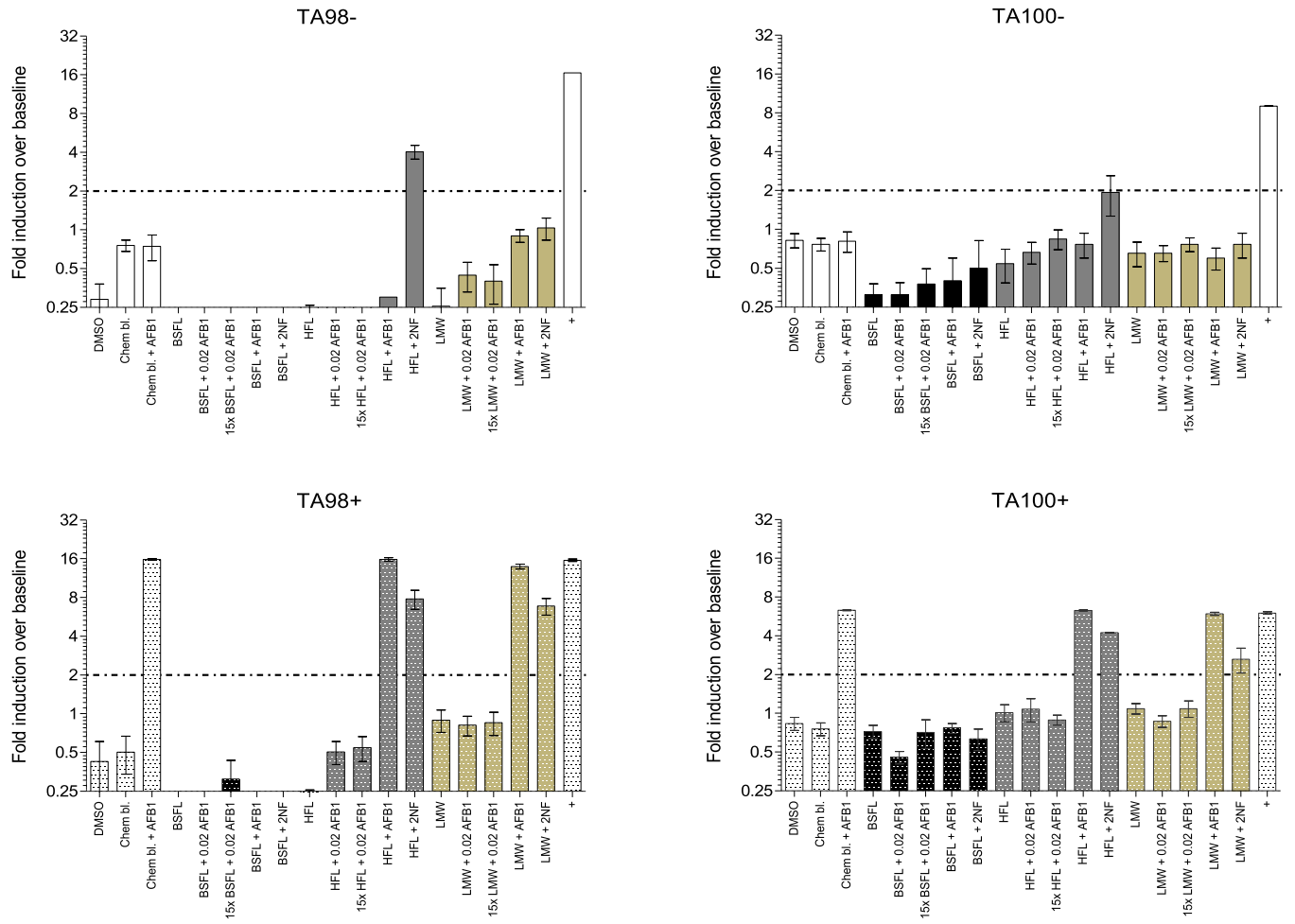

Fig. 1. Fold induction over baseline (chemical blank) for all insect samples tested in two *S. typhimurium* strains with (+) and without (-) the addition of a 30 % induced rat liver S9 mix. Fold induction < 2 is negative, whereas a fold induction ≥ 2 is positive (indication for mutagenicity).

with 2NF) raised suspicion of interference with the S9 activation step in the Ames MPF™ assay. To investigate this possible interference of the insect extracts on the assay, a CYP450 inhibition assay was performed. Suppression of CYP450 (CYP3A4, 3A5 and 3A7) enzymes was seen with all three insect extracts, with BSFL showing the greatest suppression and HFL the lowest (Fig. 2). AFB_1 or 2NF in DMSO and the chem bl. or DMSO showed the same level of activity (61.5–65.5 %).

3.2.3. *Genotoxicity and mutagenicity of larval extracts: Ames test and in vitro micronucleus assay*

Due to the interference observed in the Ames MPF™ assay caused by the control BSFL extracts (after spiking with AFB_1 and 2NF) and the clear

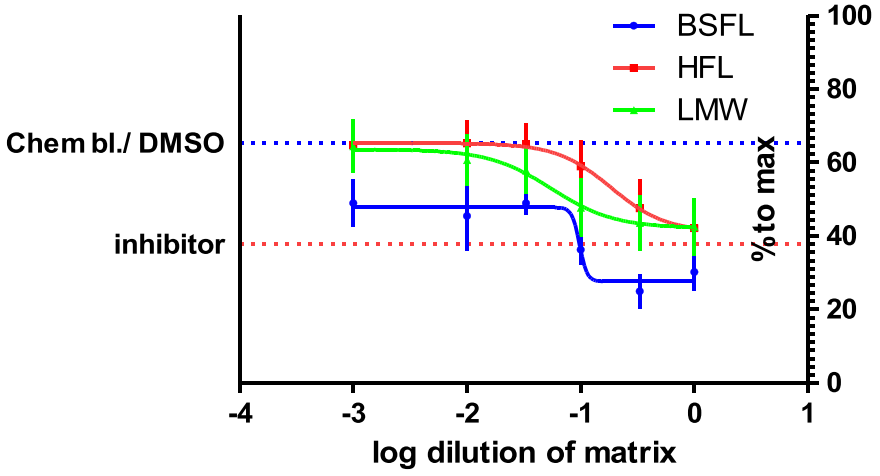

Fig. 2. Average (and SD) cytochrome P450 (CYP3A4, 3A5 and 3A7) PPXE-luciferin activity of undiluted and diluted BSFL, HFL and LMW extracts as compared to the max (100 %). Luminescence of the untreated sample was considered as 100 % activity, whereas ≤ 40 % indicated major inhibition. An explanation of treatments can be found in Table 2.

suppression of CYP450 enzymes, they were deemed unsuitable for further testing, and were thus excluded from further analysis. The outcomes of the Ames test and *in vitro* micronucleus assay performed by Charles River Laboratories Den Bosch BV (the Netherlands) on the HFL extracts confirmed the results found in the Ames MPF™ assay. The larval extracts did not induce a dose-related increase in the number of revertant (His^+) colonies in each of the four *S. typhimurium* tester strains and in the number of revertant (Trp^+) colonies in the *E. coli* strain, both in the absence and presence of S9-metabolic activation. In addition, the HFL extracts did not induce an increase in the number of micronuclei in TK6, compared to the vehicle control in the absence and presence of the S9-mix in the *in vitro* MicroFlow® Assay.

The Ames test also showed no significant dose-related increases in the number of revertants in the *S. typhimurium* strains TA98, TA100, TA1535 and *E. coli* strain WP2uvrA by the LMW extracts. However, the LMW extracts did induce up to a 14-fold (LMW control) and 4.8-fold (LMW AFB_1-spiked substrate) dose-related increase in the number of revertants in the tester strain TA1537 in the presence of S9-mix. Outcomes of the *in vitro* micronucleus assay on the LMW extracts showed no induced formation of micronuclei in TK6 cells compared to the vehicle control in the absence and presence of S9-mix at non-cytotoxic concentrations.

4. Discussion

Insects used for food or feed can be exposed to AFB_1 through contaminated substrates, especially in the view of circular food systems. Our study aimed to further explore safety aspects of the potential valorisation of AFB_1-contaminated crops by utilizing them as insect feed. Whether this affects the food and feed safety of these edible insects, remains uncertain as multiple studies reported incomplete mass balances, which indicate metabolism of AFB_1. To assess the safety, we conducted *in vitro* testing to examine the cytotoxicity, mutagenicity and genotoxicity of BSFL, HFL, and LMW larvae that were reared on AFB_1-spiked substrate.

It must be noted that in the present study, measured AFB_1 concentrations in the spiked substrates used for LMW rearing were almost 2-fold lower than the intended concentrations of 20 μg/kg and 200 μg/kg AFB_1. Measurements of selected AFB_1 metabolites in these substrates were all below LOQ and could therefore not explain breakdown of AFB_1 into these metabolites. Additional measurements of the AFB_1 stock solution in MeOH learned that crystallisation of AFB_1 accounted for the lower concentration of AFB_1 in the solution. Spiking of substrate was therefore performed with less AFB_1 than intended. All in all, the higher spiked concentration of the LMW substrate (intended 200 μg/kg) was still ~10-fold higher than the lower spiked concentration (intended 20 μg/kg) and above the EC maximum limit of AFB_1 in feed materials set at the time this study was conducted (EC, 2002). Hence, not affecting the intended experimental conditions of LMW rearing in this study.

No significant differences in survival or biomass were observed among the BSFL, HFL, and LMW after feeding on AFB_1-spiked substrate as compared to the control group. This is in line with previous findings (Bosch et al., 2017; Camenzuli et al., 2018; Gold et al., 2023; Niermans et al., 2023). Moreover, the concentrations of aflatoxins in the BSFL, HFL, and LMW after rearing were below the LOQ, suggesting that these toxins did not bioaccumulate in the larvae. This observation aligns with previous research findings (Bosch et al., 2017; Camenzuli et al., 2018; Gold et al., 2023; Niermans et al., 2023; Purschke et al., 2017). The calculated molar mass balances clearly indicated species-specific differences in AFB_1 biotransformation. In the HFL experiment, approximately 64 % of the initially spiked AFB_1 was recovered. In contrast, the BSFL and LMW experiments yielded around 50 % and 11–13 %, respectively. The molar mass balance observed for the HFL was slightly lower compared to the previous findings (50 %; (Niermans et al., 2023)). In contrast, Camenzuli et al. (2018) reported a much lower molar mass balance of AFB_1 in BSFL (11–18 %), while they found a higher molar mass balance for LMW (56–80 %).

In the BSFL experiment the formation of the AFB_1 metabolite AFP_1 was observed as this compound was detected in the residual material, while the LMW experiment showed the formation of AFM_1 as was detected in the residual material. None of the analysed AFB_1 metabolites were detected in the HFL experiment (larvae, substrate and residual materials). These results are consistent with data from previous studies. An earlier study involving HFL fed on an AFB_1-spiked diet consistently reported no formation of AFB_1 metabolites (Niermans et al., 2023), while research with BSFL typically demonstrates the formation of AFP_1 (Gold et al., 2023; Meijer et al., 2019; Niermans et al., 2024). Similarly, previous investigations involving LMW have also confirmed the formation of AFM_1 (Camenzuli et al., 2018), which is consistent with the findings in our study. However, the majority of the initially spiked AFB_1 was not recovered in all three insect experiments. Nevertheless, the absence of bioaccumulation of AFB_1 or its analysed metabolites suggests promising prospectives on the safe consumption of BSFL, HFL and LMW reared on AFB_1-contaminated substrates.

Spiking of insect material with AFB_1 prior to extraction (serving as a quality control) was done to evaluate possible matrix effects and inhibition of the insects extracts on the Ames MPF™ assay. The positive result (in TA98 + and TA100 +) of the HFL and LMW extracts spiked with AFB_1 indicated no matrix effect and no inhibition of the assays, which was as expected. For HFL, these outcomes were confirmed with the conventional Ames assay. Both LMW extracts (control and spiked treatment) also tested negative in the *in vitro* micronucleus assay. However, they tested positive in one (out of five) tester strain of the conventional Ames assay. Matrix interference of BSFL extracts spiked with AFB_1 and 2NF were suspected from the negative results in all strains of the Ames MPF™ assay. Notably, 2NF is a positive control for the TA98- strain and it does not require metabolic activation to show mutagenicity (Hakura et al., 2005; Kitchin et al., 1988). Interestingly, LMW extracts spiked with 2NF also showed negative results in the TA98- and TA100- strains, which raises a new hypothesis on the need of metabolic activation of 2NF in insect matrices and the possible presence of inhibiting factors therein.

The current study showed that the development of more robust insect extraction methods for bioassay *in vitro* testing is needed, in particular because of the suppression of CYP450 enzymes by insect matrices in *in vitro* assays and how this can be prevented. BSFL exhibited the greatest suppression of CYP450 enzymes (specifically CYP3A4, 3A5, and 3A7), while HFL demonstrated the lowest suppression, consistent with the observations from the Ames MPF™ assay. The incubation step with chitinase during the preparation of insect extracts indicated no effect on the CYP450 inhibition by the BSFL extracts with all tested chitinase concentrations (data not shown). Nevertheless, compared to previously performed CYP450 assays with the BSFL extracts in this study, a lower inhibition-effect was seen with no chitinase (data not shown). The potassium-phosphate buffer, the heating step, and the cold extraction solvent are suspected for the diminished matrix effect. However, it remains unclear how the adjusted pre-processing steps of BSFL extracts affects aflatoxins potentially present in the extracts, and requires further investigation. The putative inhibitory effect of fatty acids from insect matrices on *in vitro* toxicity assays could also be explored considering the potential of mono- and polyunsaturated fatty acids to inhibit human CYP450 enzymes. Notably, Yao et al. (2006) showed complete inhibition of CYP3A4 and other CYP enzymes by polyunsaturated fatty acids at 200 μM.

No prior studies (to authors' knowledge) have investigated *in vitro* cytotoxicity and mutagenicity testing with extracts of BSFL, HFL, and LMW fed on an AFB_1-spiked diet. Hence, comparing these results to other relevant literature involving the same insect species and mycotoxin is not possible. One study did test extracts of yellow mealworm (*Tenebrio molitor* L.) fed on an AFB_1-spiked diet in a much higher concentration (10,700 μg/kg) in another *in vitro* model, namely the γH2Ax assay (Gützkow et al., 2021). In this assay histone H2Ax

phosphorylation in human liver cells (HepaRG) was analysed as an indicator for genotoxicity. The authors concluded that extracts from exposed larvae did not demonstrate increased toxicity. Despite lingering uncertainties related to undetected transformation products, the mutagenic potential of the edible larvae seemed to be minimal (Gützkow et al., 2021). The authors did not report on any suspected matrix dependent interferences.

In our current study, we assessed the cytotoxicity and genotoxicity of larval extracts from insects fed on a control diet or a diet spiked at either 1 or 5-times (only LMW) the maximum limit of AFB_1 in animal feed. However, in practice aflatoxin concentrations in food or feed commodities often surpass legal thresholds. For instance, maize has been found to contain up to 3760 μg/kg total aflatoxins (Jallow et al., 2021; Sserumaga et al., 2020). Given the potential high contamination, it might be necessary to explore the impact of even higher AFB_1 concentrations in the larval substrate on cytotoxicity and genotoxicity. In addition, it is also interesting to investigate whether similar outcomes arise when the insect larvae are fed on naturally contaminated substrates, to also consider the effect of larval exposure simultaneous to multiple mycotoxins (Sulyok et al., 2020; Warth et al., 2012). For comprehensive safety evaluation, we propose conducting additional *in vitro* studies using extracts from BSFL, HFL and LMW fed diets containing other mycotoxins or mycotoxin mixtures. This should be performed after further optimization of insect extraction methods for bioassay *in vitro* testing. Moreover, it is crucial to select *in vitro* assays that align with the expected mode of action of the specific mycotoxin.

5. Conclusion

This study offers a positive perspective on the safe utilization of HFL reared on an AFB_1-spiked diet. Notably, we observed no bioaccumulation of AFB_1 or metabolites, no matrix effects during *in vitro* testing and no cytotoxic or genotoxic effects in HFL. Thus, incorporating HFL into the valorisation of AFB_1-contaminated crops appeared to align with circular food systems. Moreover, in-house testing of LMW extracts with the Ames MPF™ assay, indicated the absence of cytotoxic and mutagenic effects, which was confirmed with the *in vitro* micronucleus assay. However, matrix effects with the LMW extracts was seen with the conventional Ames test. Based on the current available data, we concluded that the Ames MPF™ assay, the 'regular' Ames and the micronucleus test were not suitable for testing the genotoxic potential of BSFL extracts. Therefore, further research is necessary to assess the genotoxic potential of BSFL reared on AFB_1-contaminated substrate. The additional use of other genotoxicity tests than performed in this study is recommended to better understand the presence of matrix-induced discrepancies between *in vitro* models of insect extracts in general.

CRediT authorship contribution statement

Kelly Niermans: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Winnie C.W. Tao:** Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **H.J. van der Fels-Klerx:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Hoek-van den Hil Elise F.:** Writing – review & editing, Supervision, Funding acquisition. **Nathan Meijer:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Katja C.W. van Dongen:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Yoran B.N. Weide:** Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

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

Acknowledgements

We would like to thank Kristian van Zadelhoff for helping with the LMW rearing experiment and Marlou Bosch from Ynsect for providing the LMW neonates and helping with the start-up of the insect experiment. Ed Boers is thanked for performing the LC-MS/MS analyses and data analyses, as well as Meike van der Zande, Geert Stoopen and Karsten Beekmann for their input on the experimental set-up of the Ames MPF™ and Cytochrome P450 inhibition assay and data-interpretation. This study has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement n° 861976 and Top Sector Agri & Food, the Netherlands under project number EU-20021. Additional funding from the Dutch Ministry of Agriculture Fisheries, Food security and Nature for the Wageningen University & Research Knowledge Base Program KB34 "Circular & Climate Neutral Society" (KB-34–002–024) and KB37 "Healthy & safe food systems" (KB-0037–002–038) is acknowledged.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2025.104724.

Data availability

Data will be made available on request.

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