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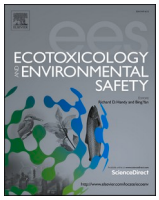

The role of larvae of black soldier fly and house fly and of feed substrate microbes in biotransformation of aflatoxin B_1

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ABSTRACT

Over the past few years, there has been growing interest in the ability of insect larvae to convert various organic side-streams containing mycotoxins into insect biomass that can be used as animal feed. Various studies have examined the effects of exposure to aflatoxin B_1 (AFB_1) on a variety of insect species, including the larvae of the black soldier fly (BSFL; *Hermetia illucens* L.; Diptera: Stratiomyidae) and the housefly (HFL; *Musca domestica* L.; Diptera: Muscidae). Most of these studies demonstrated that AFB_1 degradation takes place, either enzymatic and/or non-enzymatic. The possible role of feed substrate microorganisms (MOs) in this process has thus far not been investigated. The main objective of this study was therefore to investigate whether biotransformation of AFB_1 occurred and whether it is caused by insect-enzymes and/or by microbial enzymes of MOs in the feed substrate. In order to investigate this, sterile and non-sterile feed substrates were spiked with AFB_1 and incubated either with or without insect larvae (BSFL or HFL). The AFB_1 concentration was determined via LC-MS/MS analyses and recorded over time. Approximately 50% of the initially present AFB_1 was recovered in the treatment involving BSFL, which was comparable to the treatment without BSFL (60%). Similar patterns were observed for HFL. The molar mass balance of AFB_1 for the sterile feed substrates with BSFL and HFL was 73% and 78%, respectively. We could not establish whether non-enzymatic degradation of AFB_1 in the feed substrates occurred. The results showed that both BSFL and substrate-specific MOs play a role in the biotransformation of AFB_1 as well as in conversion of AFB_1 into aflatoxin P_1 and aflatoxicol, respectively. In contrast, HFL did not seem to contribute to AFB_1 degradation. The obtained results contribute to our understanding of aflatoxin metabolism by different insect species. This information is crucial for assessing the safety of feeding fly larvae with feed substrates contaminated with AFB_1 with the purpose of subsequent use as animal feed.

1. Introduction

As the global population grows and income levels rise, a higher demand for food and nutrition, especially for animal proteins, is expected (UN, 2015; Van Huis et al., 2013). These developments will increase the impact of food production on the environment due to the limited availability of global agricultural land required for animal feed production (Herrero et al., 2016). Consequently, there is an increasing demand for alternative and sustainable animal proteins, which has led to the rise of insect farming (Van Huis et al., 2013).

A variety of insect larvae can be used to convert different organic side-streams into insect biomass for feed (Smetana et al., 2016). Larvae of the black soldier fly (BSFL; *Hermetia illucens* L.; Diptera: Stratiomyidae) and the housefly (HFL; *Musca domestica* L.; Diptera: Muscidae) are able to recycle these organic streams efficiently (Gold et al., 2018). However, such organic side-streams can contain various contaminants including mycotoxins, which are agricultural contaminants affecting 60–80% of the food crops globally (Eskola et al., 2020). Mycotoxin contamination is not only associated with large economic losses and negative impacts on domestic and global trade, it also severely impacts the health of both animals and humans (Eskola et al., 2020). Many different mycotoxins are known today (Berthiller et al., 2007), but the aflatoxins have received most attention due to their widespread occurrence and toxicity. Aflatoxins are secondary metabolites produced by fungal species of the genus *Aspergillus* (Streit et al., 2012). Amongst the aflatoxins, aflatoxin B_1 (AFB_1) is most abundant in agricultural food crops (EFSA, 2004). Due to the carcinogenic, teratogenic and mutagenic effects and the immunosuppressive nature of AFB_1,

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maximum levels (mL) have been set for its presence in food (EC, 2006) and feed (EC, 2002) in Europe.

Multiple studies have examined the effects of AFB_1 exposure on a variety of insect species, among which BSFL and HFL (Bosch et al., 2017; Camenzuli et al., 2018; Gold et al., 2023; Meijer et al., 2019; Niermans, 2024). Most of these studies calculated a molar mass balance, and used this as a measurement for the fraction of the initially present toxic parent compound (or metabolites) in the substrate that had been recovered in the larvae and the residual material. In the studies using AFB_1-spiked substrates, the molar mass balance was incomplete (for both BSFL and HFL), indicating a missing, unrecovered fraction (Niermans, 2024). Interestingly, when performing a feeding study with BSFL and peanut press cake naturally contaminated with a mix of aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1), the molar mass balance was close to being complete (Niermans, 2024). Molar mass balance calculations performed in previous studies therefore showed inconsistent results, even when the same insect species were investigated. The distinguishing factor in the experimental setup across these studies was the choice of feed substrate. This observation suggests that the microorganisms (MOs) that colonise such feed substrates may play a role in the degradation of AFB_1.

From previous research it is known that insects themselves possess the genetic machinery capable of metabolising mycotoxins (Berenbaum et al., 2021; Niermans et al., 2021). Furthermore, multiple studies showed that biotransformation of AFB_1 after incubation in soil occurs (Accinelli et al., 2008; Albert and Muñoz, 2022; Angle and Wagner, 1980; Juraschek et al., 2022), and it has been shown that biotransformation of AFB_1 in soil is mainly driven by soil-specific microorganisms (MOs; Accinelli et al., 2008). Other studies have also shown the potential of (soil-) MOs, especially bacteria, to enzymatically metabolise aflatoxins (Ji et al., 2016; Liu et al., 2022; Vanhoutte et al., 2016; Verheecke et al., 2016). The above mentioned studies clearly show that biotransformation of AFB_1 could take place in the insect substrate and that – in general – MOs are expected to play a role in this.

However, thus far no study investigated the role of the substrate MOs versus the insect larvae in aflatoxin breakdown and biotransformation. Therefore, we will investigate this in the current study. We distinguish between enzymatic and non-enzymatic degradation. Enzymatic degradation is caused by the action of enzymes produced by living cells which causes a chemical reaction to happen resulting in the formation of metabolites. In non-enzymatic degradation, chemical reactions occur but enzymes are not involved. Enzymatic and non-enzymatic degradation of AFB_1 in the feed substrates for BSFL and HFL rearing has not been examined yet. The aims of the current study were to I) investigate whether enzymatic and non-enzymatic degradation of AFB_1 in spiked feed substrates takes place, and to determine the role of II) the insect species BSFL and HFL, and III) the substrate-specific MOs (bacteria, fungi) in this process.

2. Materials and methods

2.1. Chemicals and standards

Mycotoxin standards were purchased from Romer Labs (Getzersdorf, Austria): AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1), and from Enzo Life Sciences (Brussels, Belgium): aflatoxicol (AFL) and from TRC (Toronto, ON, Canada): aflatoxin P_1 (AFP_1) and aflatoxin Q_1 (AFQ_1).

2.2. Insects

BSFL used in this study originated from the BSF colony of the Laboratory of Entomology, Wageningen University. Eggs were collected from the cardboard strips used as egg laying substrate, and larvae were reared on chicken feed (Kuikenopfokmeel, Kasper Faunafood, Woerden, the Netherlands) until seven days old. The colony is maintained in a controlled climate chamber (27°C, a relative humidity of 65% and a day/night rhythm of 16/8 h).

The HF eggs used in this study were taken from the HF colony reared at the Laboratory of Entomology, Wageningen University. This colony originates from the Faculty of Science and Engineering, University of Groningen. The HF rearing process was identical to that described in Niermans, (2024).

2.3. Spiked feed preparation

BSFL and HFL were exposed to a control feed substrate or a feed spiked with 20 μg/kg AFB_1. This selected concentration was based on the mL of AFB_1 allowed in all feed materials which is set in Directive 2002/32/EC (EC, 2002). Both feed substrate treatments were performed in quadruplicate. An overview of the included feed substrates can be found in Table 1.

To prepare the spiked feed substrate for BSFL, 1.9 mL of the AFB_1 spiking solution (in MeOH) was mixed with 618 mL water and 332 g chickenfeed (moisture content 65%; final concentration of 0.2% MeOH) to obtain a total of 950 mL spiked wet feed. The HFL spiked feed substrate was prepared by mixing 1.2 mL of the same spiking solution with 144 mL water, 216 mL Nipagin solution in water (final concentration: 0.9 mg/L; Merck, Darmstadt, Germany) and 240 g dry food mix (37% wheat bran, 56% wheat flour, 4% full fat milk powder (28.2 g fat/100 g) and 2% dry instant baker's yeast) to obtain a total of 600 mL spiked wet feed (moisture content 60%; final concentration of 0.2% MeOH). The control feed substrates were prepared in the same way, but without AFB_1, with MeOH in the same final concentration as added in the spiked feed substrates. In order to prepare the substrate for the sterilized treatment, the dry substrates for both the BSFL and the HFL were sterilized for 20 min at 121°C in a Vapour Line Lite Autoclave (VWR International, Radnor, Pennsylvania, US). The BSFL substrate underwent the sterilization procedure twice, as pilot experiments showed that autoclaving once did not result in a sterile substrate. The same spiking procedure applied for the sterilized and non-sterilized treatments, however, instead of tap water, autoclaved MilliQ water was used. The wet feed substrates were mixed manually for 15 min.

In order to determine homogeneity of the non-sterile spiked feed, eight samples were taken. Homogeneity was confirmed when the measured concentrations (relative standard deviation of the replicates) in the eight samples differed $\leq$ 20% from each other. Four samples taken from the control feed were analysed to confirm the absence of aflatoxins in these feed substrates.

2.4. Experimental set-up

The spiked feed substrates were either incubated with the presence of BSFL or HFL (Table 1; BSF+ or HF+) or without larvae (Table 1; BSF- or HF-). Spiked sterilised feed substrates were prepared in order to study the effect of non-enzymatic degradation of AFB_1. Spiked feed substrates with- and without insect larvae allow a distinction between the contribution of biotransformation caused by the insect larvae or substrate-specific MOs, respectively.

For the treatments in which larvae were feeding on the substrate, 150 seven-days-old BSFL, with an individual average weight of 22.41 (± 0.19) mg were placed on 100 g of either the wet control- or wet AFB_1-spiked feed in a 480 mL rearing cup (BugDorm insect pots purchased from MegaView Science Co., Ltd., Taichung, Taiwan). The rearing cups were placed in a climate room at 27°C, relative humidity of 65% and a day/night rhythm of 12/12 h for seven days. 0.012 g HF eggs (corresponding to 200 eggs) were placed on 60 g of either the wet control- or wet spiked feed in a 480 mL rearing cup (BugDorm insect pots purchased from MegaView Science Co., Ltd., Taichung, Taiwan). The rearing cups were placed in a climate cell at 25°C, relative humidity of 65% and a day/night rhythm of 12/12 h for five days. At day seven, all BSFL were separated from the residual material (a mixture of left-over substrate and frass), washed, dried, weighed and transferred to clean (non-spiked)

Table 1
Overview of experimental feed substrates.

Species	Treatment code	With or without larvae	Type of matrix	Feed substrate	Replicates	Intended mycotoxin conc. (μg/kg ww)	Samples taken on day
BSFL	BSFC	With	Substrate	Control	4	0	1, 7, 9, 11, 13
	BSF+	With	Substrate	Spiked (AFB_1)	4	20	1, 7, 9, 11, 13
	BSF-	Without	Substrate	Spiked (AFB_1)	4	20	1, 3, 5, 7, 9, 11, 13
	BSFS	Without	Sterile substrate	Spiked (AFB_1)	3	20	1, 5, 9, 11, 13
HFL	HFC	With	Substrate	Control	4	0	1, 5, 7, 9
	HF+	With	Substrate	Spiked (AFB_1)	4	20	1, 5, 7, 9
	HF-	Without	Substrate	Spiked (AFB_1)	4	20	1, 3, 5, 7, 9
	HFS	Without	Sterile substrate	Spiked (AFB_1)	3	20	1, 3, 5, 7, 9

feed on the seventh day of exposure. This procedure was repeated on day eight of exposure after which the larvae were stored at −20°C until further analyses. The same procedure applied for HFL, however, here exposure ended after five days and larvae were fed on clean feed for ± 5.5 hours. For the feed substrates without larvae, samples of the residual material were taken at multiple timepoints (at least five) during the experiment. The same was done for the treatments with larvae, however, during the time that the larvae were feeding on the substrate no samples were taken in order to not interfere with the experiment.

The experimental set-up of the sterile substrate was adjusted slightly. For both the BSFL and HFL, only one batch of sterile spiked feed was prepared after which 5.8 (± 1.8) g was divided over fifteen tubes (sterile 50 mL centrifuge tubes, Greiner Bio-One GmbH, Alphen aan den Rijn, Netherlands). The caps of the tubes were sealed with Parafilm® and incubated under the same conditions as mentioned previously. At each of the five timepoints (see Table 1), three tubes were collected, tested for sterility (see next section) and stored at −20°C until LC-MS/MS analysis.

2.5. Confirmation of sterility

Sterility of the substrate and residual samples collected for LC-MS/MS analysis was confirmed for each replicate at all sample collection days (see Table 1 for the exact days). Sterility was confirmed by plating sample extract on Luria-Bertani (LB) agar plates (Naveed et al., 2014; Zhang et al., 2011) and Sabouraud 4% Dextrose Agar (SDA; Millipore; Merck KGaA, Darmstadt, Germany (Kusari et al., 2012). The LB agar consisted Bacto™ Tryptone (final concentration 10 g/L; ThermoFisher Scientific Inc., Waltham, USA), Bacto™ Yeast Extract (final concentration 5 g/L; ThermoFisher Scientific Inc., Waltham, USA), NaCl (final concentration 5 g/L; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), Difco™ Agar (final concentration 15 g/L; Becton, Dickinson and Company, Franklin Lakes, USA) and MilliQ water. The SDA agar was prepared by mixing SDA (final concentration 65 g/L) and MilliQ water. Both were sterilized for 20 min at 121°C in a Vapour Line Lite Autoclave (VWR International, Radnor, Pennsylvania, US). After the agar cooled down to 60°C, the plates were poured and stored at 4°C until further use. One g of each of the collected samples at all timepoints was dissolved in two mL sterile PBS, vortexed for 30 s and set aside for 10 min to let the sediment settle. One-hundred μL of supernatant was pipetted on an agar plate (in duplo), spread with L-shaped sterile spreaders (Heathrow Scientific, Vernon Hills, US) on the agar plates. LB plates were incubated aerobically for 24 h at 27°C (Feizi et al., 2023) while the SDA plates were incubated aerobically at 25°C and were checked after 72 h and after 120 h (Fasuan et al., 2022). When the LB agar plates and SDA were free of colonies after incubation, samples were considered sterile (photos can be found in Figures S1 and S2).

2.6. Aflatoxin analyses

The extraction procedure for the substrate and residual material was performed as described by Camenzuli et al., (2018). Extraction of the larval samples was performed with 0.5 g sample material, thus the extraction procedure *e.g.* volume of water (1.5 mL) and extraction solvent (2 mL) and magnesium sulphate (0.8 g) were adjusted accordingly. Aflatoxin concentrations in the samples were quantified by means of standard addition, therefore, each extract was prepared with- and without the addition of a standard mix containing eight aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFL, AFP_1, AFM_1 and AFQ_1). Two-hundred μL sample 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 file (Mini-UniPrep, Whatman, Marlborough, MA). The files were capped, vortexed and placed in the refrigerator for 30 min after which the vials were closed and stored at 4°C until LC-MS/MS analyses.

The LC-MS/MS system consisted of a Waters Acquity injection and pump system (Waters, Milford, MA) and an AB Sciex QTRAP 6500 triple quad system equipped with an electrospray ionization (ESI) source. LC-MS/MS analyses were performed exactly as described in Niermans (2024). Aflatoxin analyses were also solely performed in positive ESI mode (instrumental MS/MS parameters of the mycotoxins analysed are shown in Table S1). LC separation was performed with an Acquity UPLC HSS T3 1.8 μm 100×2.1 mm column (Waters, Milford, MA).

2.7. Fungal identification

Identification of the present fungal species was done via MALDI-ToF analysis (Bruker MALDI Biotyper microflex LT-SH) after which the obtained spectra were matched against the specific MBT Filamentous Fungi module (MBT Fil. Fungi Library V5.0) and the regular MBT Compass Library Revision K (2022) inc. SR library: BTyp2.0Sec.Library 1.0.

2.8. Data analysis

LC-MS/MS data were analysed with SCIEX OS-MQ v2.1.6 software (Sciex, Framingham, MA). Analysed concentrations were corrected for recovery percentages of analysed aflatoxins per matrix (Table S2). Detected concentrations from the LC-MS/MS analyses were adjusted for the wet weight measured for each of the treatments. Concentrations were calculated by dividing the peak area of the sample by the (area of the sample with standard addition minus the area of the sample) and multiplied by the addition level of the respective mycotoxin. Kruskal-Wallis tests (significance level α: 0.05) were performed in SPSS (IBM® SPSS® Statistics 28, New York, USA) to determine whether the AFB_1 concentration in the residue samples per timepoint differed significantly between treatments. Significance was calculated for the two insect species separately. All figures were made in GraphPad Prism v5.02. In the current study, statistical analyses were performed using the non-parametric Kruskal-Wallis test. This means that significance of a possible interaction between the AFB_1 concentration over treatment and time was not tested.

Molar mass balance calculations were performed for all AFB_1-spiked feed substrates. Detected concentrations from the LC-MS/MS analyses were adjusted for the wet weight measured for each of the treatments. Mycotoxin (metabolite) concentrations below the LOQ were set to zero. The concentrations (μg/kg) of the mycotoxins determined in larval biomass and mass of residual substrate by LC-MS/MS analysis were

multiplied by the total wet weight of larval biomass or residual substrate mass, respectively, to yield the total amount of mycotoxin recovered, including the amounts of metabolites analysed. The molar mass balance was calculated by dividing the sum of the mycotoxin (metabolite) mass detected in the larvae and residual material by the mycotoxin molar mass, including that of metabolites quantified, present in the initial substrate on a wet weight basis. The molar mass balance was calculated for the residual material on either day seven (BSFL) or day five (HFL).

3. Results

3.1. Control feed substrates and sample homogeneity

None of the mycotoxins included in the analyses were detected (< LOQ) in the control feed substrates. For all feed substrates, the analysed corrected concentrations were within an acceptable deviation range of ± 20%, confirming the homogeneity of the spiked substances in the substrate (Table 2, Appendix G).

3.2. Aflatoxin metabolism

At day seven, samples of the BSF experiment were collected and a mass balance was calculated. The total aflatoxin (including AFB_1, AFL, AFP_1, AFM_1, AFQ_1) concentration in the BSFL was < LOQ, and the molar mass balance was therefore completely based on the aflatoxin concentration in the residual material. The total calculated molar mass balance in the BSFL experiment, with larvae (BSF+) was 50 ± 2.6% of which the aflatoxin metabolite AFP_1 contributed 11 ± 1.8%. The calculated molar mass balance for the treatment without BSFL (BSF-) was comparable as for the treatment with BSFL (60 ± 8.4%), and here not AFP_1, but AFL contributed (5 ± 2.5%) only to the overall mass balance (Fig. 1). The molar mass balance for the sterile feed substrate (BSFS) was 73 ± 11.9%, and no AFB_1 metabolites were detected. An overview of the average contribution of the aflatoxins to the overall molar mass balance can be found in Table S5. The molar mass balance was significantly different ($P < 0.05$) when BSFL were present in the substrate (BSF+) as compared to the sterile substrate (BSFS).

Data from the HFL experiment were collected on day five. The total aflatoxin concentration in the HFL was < LOQ. Additionally, none of the included AFB_1 metabolites contributed to the molar mass balance in the HFL experiment. Therefore, the molar mass balance was completely based on the recovery of AFB_1 in the residual material as compared to the concentration spiked in the feed substrate. The average calculated molar mass balance in the HFL experiment was similar for the treatments with (64 ± 3.5%) and without (69 ± 6.2%) larvae (Fig. 1). The molar mass balance for the sterile feed substrate was 78 ± 8.4%, and again solely based on AFB_1 itself. Similar to what was observed in the BSFL experiment, the molar mass balance was significantly less complete ($P < 0.05$) when HFL (HF+) were present in the substrate as compared to the sterile feed substrate (HFS).

3.3. Enzymatic vs. non- enzymatic degradation of AFB_1

The AFB_1 concentration in the BSFL feed substrates decreased over time finally resulting in a decrease of 76 ± 2% and 54 ± 5% for the non-sterile feed substrates with- and without BSFL, respectively, on the last day of the experiment (day 13). In the sterile feed substrate (BSFS), the

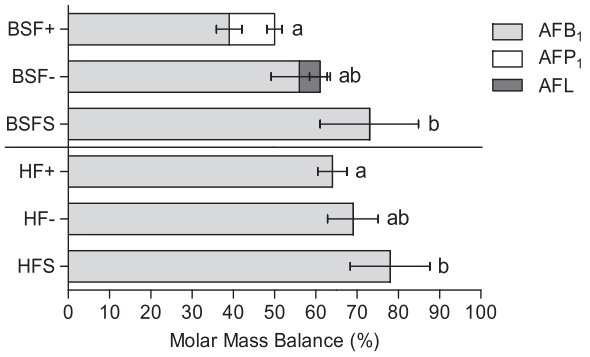


Fig. 1. Molar mass balance of AFB_1 for BSFL and HFL fed on a AFB_1 spiked feed substrate with larvae (BSF+, HF+), for the spiked feed substrates on which no larvae had grown (BSF-, HF-) and for the sterile feed substrate (BSFS, HFS). Error bars represent the SD as a measure of variability between the replicates. Significance was tested separately for BSF or HF. Treatments with different letters differ significantly (Kruskal-Wallis, P = < 0.05).

AFB_1 concentration decreased with 24 ± 7% by the end of the experimental period. A significant decrease ($P < 0.05$) in AFB_1 concentration in the sterile feed substrate (BSFS) only occurred between the start of the experiment and day 5, afterwards the AFB_1 concentration remained unchanged until the end of the experiment ($P > 0.05$; Figure S3). The presence of BSFL in the feed substrate resulted in a lower AFB_1 concentration as compared to that in the sterile feed substrate (BSFS) from day seven, and in feed substrates without BSFL (BSF-) from day nine onwards ($P < 0.05$; Fig. 2, Figure S4). The AFB_1 metabolite AFP_1 was formed in the BSF+ treatment and the absolute amount (μg) of AFP_1 – which was first found on day seven - decreased during the days that followed (Table 3). As the BSFL were removed from the residual material on day seven this could indicate that conversion of AFB_1 into AFP_1 stopped when the larvae were removed from the feed substrate. In the treatment without BSFL no AFP_1, but AFL was present from day seven onwards.

< LOQ: below limit of quantification. Table with overview of LODs and LOQ per respective matrix can be found in Table S4.

The AFB_1 concentration in the HFL feed substrates changed over time finally resulting in a decrease of 56 ± 5% and 51 ± 2% on day nine for the non-sterile feed substrates with- and without HFL, respectively. In the sterile feed substrate (HFS) the AFB_1 concentration decreased with 24 ± 3% by the end of the experimental period (day nine). The presence of HFL did not affect the time course of AFB_1 concentration ($P > 0.05$; all days) as compared to the non-sterile feed substrate without HF (HF-; Table 1, Figure S4).

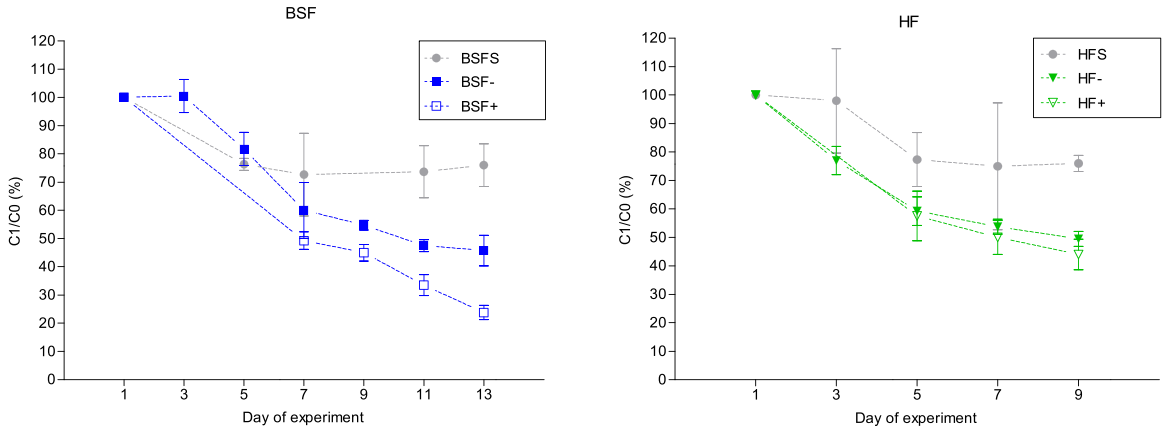
4. Discussion

This study investigated whether enzymatic and/or non-enzymatic degradation of AFB_1 spiked into feed substrates takes place, and looked into the role of the larvae of two insect species, BSF and HF, and substrate-specific MOs (bacteria, fungi) in this process. In order to do so, we calculated a molar mass balance and tracked metabolism and degradation of AFB_1 over time in sterilised feed substrates and in non-sterilised feed substrates with or without larvae over time. In the current study no distinction can be made between the role of the insect

Table 2
Overview of the analysed AFB_1 concentration in the spiked feed substrates (mean ± standard deviation) at the start (day 1) of the experiment.

Treatment code	BSFC	BSF+	BSF-	BSFS	HFC	HF+	HF-	HFS
AFB_1 concentration (μg/kg ww)	< LOQ	20.2 ± 1.1	19.2 ± 0.7	18.5 ± 0.9	< LOQ	22.8 ± 2.3	22.2 ± 1.2	24.6 ± 3.3

Individual measurements for the replicates (n=4 for BSFC, BSF+, BSF-, HFC, HF+, HF-; n=3 for BSFS and HFS) can be found in Table S3. ww: wet weight; < LOQ: below limit of quantification. Table with overview of LODs and LOQ per respective matrix can be found in Table S4.



	Day 5	Day 7	Day 9	Day 11	Day 13
BSF+	-	a	a	a	a
BSF-	a	ab	b	b	b
BSFS	a	b	-	b	b

	Day 3	Day 5	Day 7	Day 9
HF+	-	a	a	a
HF-	a	a	ab	ab
HFS	a	b	b	b

Fig. 2. Percentage of total aflatoxin concentration (mean ± SD) over time as compared to the starting concentration (Day one; 100%) in the spiked feed substrate with larvae (BSF+ or HF+), the spiked feed substrates on which no larvae had grown (BSF-, HF-) and the sterilized feed substrate (BSFS, HFS). Larvae were removed from the feed substrate on either day seven (BSFL) or day five (HFL). A complete overview of the percentual decrease of AFB_1 for all feed substrates can be found in Table S6. Significance was tested separately for BSF and HF, and was done by comparing the treatments on each day. Treatments with different letters differ significantly (Kruskal-Wallis, $P = < 0.05$).

Table 3
Average and standard deviation of absolute amount (μg) of total aflatoxins, AFB_1, AFP_1 and AFL over time in the spiked feed substrate with BSFL and for the spiked feed substrates on which no larvae had grown.

		Day 1	Day 7	Day 9	Day 11	Day 13
BSF+	AFB_1	2.03 ± 0.11	0.78 ± 0.06	0.72 ± 0.04	0.54 ± 0.05	0.38 ± 0.04
	AFP_1	< LOQ	0.21 ± 0.04	0.20 ± 0.05	0.13 ± 0.02	0.10 ± 0.01
BSF-	AFB_1	1.92 ± 0.07	1.07 ± 0.17	0.98 ± 0.09	0.84 ± 0.03	0.81 ± 0.12
	AFL	<L OQ	0.08 ± 0.06	0.07 ± 0.05	0.07 ± 0.05	0.07 ± 0.05

metabolic systems and their gut MOs as only intact feeding insects were used. Therefore, the current study considers the insect and their gut MOs as inseparable entities, namely the insect system.

The calculated molar mass balance was not different for the treatments with BSFL (BSF+) and without (BSF). Around half of the initially present AFB_1, and a clear contribution of AFP_1 was found when calculating the molar mass balance in the BSF+ experiment. Total recovery of AFB_1 was slightly higher in Niermans (2024), but the contribution of AFP_1 was similar. As AFP_1 was not formed in the treatment without BSFL (BSF-), we conclude that the BSFL caused its formation. Total recovery of aflatoxin was much lower in other studies (Bosch et al., 2017; Camenzuli et al., 2018) as compared to the current study. In the study of Camenzuli et al. (2018) the concentration of AFP_1 was below the LOQ in the larvae and could not be determined in the residual material due to matrix interferences. In the study of Bosch et al. (2017) AFP_1 was not analysed, so no comparison between the AFP_1 contribution to the molar mass balance in the current study and those studies can be made. In the above two referred studies, BSFL were exposed to the AFB_1-spiked feed substrate for ten days, whereas in Niermans (2024) and the current study, exposure lasted for seven days. Based on the collected data, it can be concluded that a longer exposure time to AFB_1 results in a higher fraction that is enzymatically metabolised by the larvae as well as the substrate MOs and, therefore, a more incomplete molar mass balance. The average calculated molar mass balance in the HFL experiment was similar for both treatments (with- and without larvae).

In the current study, the concentration of AFB_1 in both BSFL and HFL treatments (with- and without larvae) decreased over time. Metabolism of AFB_1 was more efficient in the presence of larval- and substrate MO-enzymes (BSF+) as compared to the substrate MOs alone (BSF-) from day nine onwards. AFB_1 was differently metabolised in treatments with- (AFP_1) and without BSFL (AFL), which indicated that both BSFL as well as substrate-inhabiting Mos seem to play a role in AFB_1 metabolism. However, for HFL, the absence of aflatoxin metabolites in the residual material, and the fact that the percentual decrease of AFB_1 over time was not affected by the presence of HFL in the feed substrates indicate that the HFL themselves do not have a role in AFB_1 metabolism.

In general, it can be stated that the substrate is the main determinant of the BSF larval gut bacterial community (Schreven et al., 2022). In addition, the larvae also alter the composition of the substrate bacterial community over time by inhibiting certain bacteria, changing the population sizes of resident bacteria, and by introducing gut bacteria into the substrate (Schreven et al., 2021). Schreven et al. (2022) showed that the presence of BSFL in chicken feed, used as a substrate for BSFL rearing, caused a change in the microbial composition of the substrate. While the chickenfeed was rich in *Curtobacterium* and *Pantoea* at the start of the study, *Pediococcus*, *Lactobacillus*, and *Weissella* were more dominant after five days of incubation with BSFL (Schreven et al., 2022). As chickenfeed was also used as a substrate in the current study, we assume that these shifts in MO community took place in the experiments reported here. We therefore assume that the treatment 'BSF-' did not trigger the same change in MO composition of the substrate as expected in the 'BSF+' treatment and might therefore have resulted in a lower degree of biotransformation by substrate MOs ($P < 0.05$; from day nine onwards) as compared to the treatments with BSFL. As the BSF larvae were removed from their feed substrate (BSF+) on day seven, this hypothesised shift in MO community in the feed substrate before day seven is likely responsible for the observed difference in the biotransformation of AFB_1 as compared to the BSF- and BSFS treatments from day nine onwards. The 'BSF-' treatment, however, clearly indicated the role of substrate-inhabiting MOs since AFL was formed.

Such a role of MOs was also observed in other matrices like soil, peanuts, cow's milk and yoghurt (Albert and Muñoz, 2022; Ndiaye et al.,

2022). Microorganisms, especially bacteria, have been studied for their potential to either metabolise mycotoxins or reduce their bioavailability (Abbès et al., 2013; Albert and Muñoz, 2022; Ben Taheur et al., 2019; Moretti et al., 2018; Ondiek et al., 2022; Peltonen et al., 2001; Topcu et al., 2010; Verheecke et al., 2016). For example, inoculation of 0.24 mg/kg AFB_1 with *L. plantarum PTCC* 1058 (37°C, 4–7 days, 9×10^9 CFU/mL) resulted in an AFB_1 reduction efficacy of 77% in corn (Verheecke et al., 2016). Additionally, Peltonen et al. (2001) examined the AFB_1 binding potential of twelve *Lactobacillus* strains and found that AFB_1 was rapidly bound (17.3–59.7%) by the respective bacteria (Peltonen et al., 2001). It is speculated that, for certain species of lactic acid bacteria, binding - rather than biodegradation - occurs as the primary mode of AFB_1 removal (Ondiek et al., 2022). Other authors hypothesised that AFB_1 can bind to the peptidoglycans and polysaccharides in the cell wall of lactic acid bacteria (Ben Taheur et al., 2019; Topcu et al., 2010). When AFB_1 is bound to the cell wall, the efficiency of its extraction can be influenced. It has, for example, been demonstrated that the extraction efficiency of AFB_1, when bound to *L. rhamnosus*, was dependent on the various conditions of the extraction method used, including the extraction solvent, pH, and incubation temperature, among others (Haskard et al., 2001).

Previous (vermicomposting) studies with HFL showed the potential of this insect species to alter the abundance and structure of the bacterial community in the feed substrate (Li et al., 2019; Zhang et al., 2012). However, in the current study no difference in AFB_1 reduction was observed for the HFL treatments. Schreven et al. (2022) showed that the impact of BSFL on microbial composition of the substrate is substrate-dependent and that bacterial communities in larvae and substrates can differ in composition depending on the feed substrate (Schreven et al., 2022). Furthermore, the influence of habitat and feed substrate on the internal microbiota was also shown in HF over five developmental stages (Voulgari-Kokota et al., 2022). The use of the different feed substrates therefore result in different substrate-inhabiting and insect gut-associated MOs and possibly different AFB_1 metabolic pathways.

In the current study, AFB_1 enzymatic and non-enzymatic degradation was higher in the non-sterile substrate with insect larvae (BSF+, HF+) as compared to the non-sterile substrate without insect larvae (BSF-) and the sterile substrate (BSFS, HFS) on the final day of the experiment. The AFB_1 concentration in the sterile feed substrates (BSFS, HFS) was around 24% lower at the end of the experiment as compared to the starting concentration, which seems to indicate that non-enzymatic degradation took place in the sterile substrates. Aflatoxins are known to be photosensitive, however, their non-enzymatic degradation efficiency depends on UV intensity (Liu et al., 2010; Stanley et al., 2020) and the spiked matrix e.g. soil type (Albert and Muñoz, 2022). During the incubation step in the experiment, samples were not exposed to daylight and the UV-intensity of the fluorescent tubes present in the incubator was negligible. We therefore assume that, in the current experiment, UV exposure did not play a role in the non-enzymatic degradation of AFB_1. Furthermore, we cannot exclude that the AFB_1 which was spiked to the feed substrate bound to *e.g.* the matrix of plant DNA, proteins and other (macro)molecules present in the feeding substrates during incubation. Resulting from this, extraction of AFB_1 in the residual material may have been incomplete with the extraction method used. This unextracted AFB_1 could account for a part of the missing fraction in the molar mass balance. Furthermore, we cannot exclude that conjugated metabolites were formed during the experiment. The used extraction method might not be optimal for extracting AFB_1 metabolites conjugated with *e.g.* glutathione. Addition of a hydrolysis step or enzymatic treatment to liberate bound AFB_1 and/or conjugated metabolites during extraction, and/or analyses of the water phase obtained from the QuEChERS partitioning step might be essential. Additionally, possible degradation of AFB_1 - an alteration of the molecular structure which includes metabolization and mineralization (complete decomposition into CO_2) - over time could explain this difference.

In the current study, sterile BSFL and HFL substrates were prepared. However, even though the BSFL substrate was autoclaved (20 min at 121°C) twice, growth of fungal colonies were still observed in the LB- and SDA agar plates. We attempted to identify the present fungal species via MALDI-ToF analysis, however, no match could be found using the available database. Fungal growth was not observed in all replicates collected on a day and neither on all days of the experiment. As the AFB_1 concentration did not differ much between replicates (with and without fungal growth), and because the remaining percentage of AFB_1 did not significantly differ between day seven, eleven and thirteen in the BSFL experiment we believe that this provides enough evidence that the presence of the fungal species did not have a role in AFB_1 breakdown in the current study.

Other studies have also shown the potential of MOs for biotransformation of other mycotoxins *e.g.* fumonisin B_1 (Liu et al., 2022), deoxynivalenol (Ji et al., 2016; Liu et al., 2022), T-2 toxin (Ondiek et al., 2022) and zearalenone (Gari and Abdella, 2023; Ji et al., 2016; Król et al., 2018; Liu et al., 2022; Yi et al., 2011). As the current study only focused on AFB_1, we advise to perform a similar study in which the role of the insect larvae and the substrate-specific MOs and their potential mycotoxin degrading capacities are investigated for other mycotoxins.

Overall, the current study showed that both the BSFL system and substrate-specific MOs play a role in the biotransformation of AFB_1 as well as in conversion of AFB_1 into AFP_1 and AFL, respectively, whereas the HFL system did not seem to be involved. Different AFB_1 metabolism/degradation patterns can be expected for different feed substrates and/or insect species. To get a complete picture of AFB_1 enzymatic and non-enzymatic degradation in insect feed substrates, spiking with isotopically labelled standards and tracking the formation of labelled CO_2 is advised. Additionally, investigation of enzymatic and non-enzymatic degradation of AFB_1 and the role of both substrate specific MOs and insects in naturally contaminated materials might provide valuable insights. Furthermore, examination of the extraction efficiency of AFB_1 bound to the cell wall of substrate MOs, plant-derived matrix, and conjugated metabolites with an optimized extraction method might be essential. In conclusion, this study showed that substrate specific MOs as well as the BSFL play a role in enzymatic degradation of AFB_1 while occurrence of non-enzymatic degradation of AFB_1 in the feed substrates cannot be confirmed. HFL themselves do not have a role in AFB_1 metabolism.

CRediT authorship contribution statement

K. Niermans: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **E.F. Hoek-van den Hil:** Writing – review & editing, Supervision, Conceptualization. **H.J. van der Fels-Klerx:** Writing – review & editing, Supervision, Conceptualization. **J.J.A. van Loon:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: J. J.A. van Loon, E.F. Hoek- van den Hil, H.J. van der Fels-Klerx report that financial support was provided by Nederlandse Organisatie voor Wetenschappelijk Onderzoek Utrecht. H.J. van der Fels-Klerx reports was provided by Netherlands Ministry of Agriculture, Nature and Food Quality. 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

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.116449.

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