BRILL
WAGENINGEN
ACADEMIC

JOURNAL OF INSECTS AS FOOD AND FEED (2024) DOI:10.1163/23524588-00001286

Journal of Insects as Food and Feed
brill.com/jiff

RESEARCH ARTICLE

A preliminary study on the degradation of AFB1 by *Tenebrio molitor*, *Rhizopus oryzae* and *Trichoderma reesei*

E. Daniso, G. Pascon, C. Uboni and F. Tulli*

Department of Agricultural, Food, Environmental and Animal Science, University of Udine. via Sondrio 2/A, 33100 Udine, Italy; *enrico.daniso@uniud.it

Received 12 June 2024 | Accepted 8 October 2024 | Published online 25 October 2024

Abstract

Recently, genus *Aspergillus*, a fungus capable of producing aflatoxins, secondary highly toxic metabolites, has spread to new areas. These areas became suitable habitats due to the recent climate changes. The use of aflatoxin-contaminated crops is a cause of great concern in guaranteeing food safety and is responsible for major economic losses along the supply chain. For this reason, several strategies have been investigated to utilize these contaminated products as a possible food or feed resource by reducing or eliminating their aflatoxin content, but with limited relevant success. The presented study was aimed to evaluate a combination of biological processes to use aflatoxin B1 contaminated crops for their reintroduction into the production chain. The high tolerance to AFB1 and the apparent lack of accumulation in yellow mealworm larvae, reared on wheat bran substrates, spiked with increasing AFB1 concentration (0, 125, 250, 500 μg/kg) to obtain proteins of high biological value. Subsequently, the aflatoxin-degrading capacity of *Rhizopus oryzae* and *Trichoderma reesei* was applied to insect breeding waste (frass) in a fermentation process to ensure further utilization of biohazardous frass as soil conditioner. Individually, each process proven to be able to reduce the AFB1 present by about 80%, while the combination of the two approaches ensured the total degradation of aflatoxin B1-contaminated substrate and frass, which resulted in the possible production of biomass, that could be used for the feed and agricultural industry.

Keywords

AFB1 – aflatoxin degradation – bioremediation – protein production

1 Introduction

Aflatoxins are a group of polycyclic mycotoxins ubiquitously present in agricultural products. They are synthesised, as secondary metabolites, by various moulds of the genus *Aspergillus* of which *A. flavus* and *A. parasiticus* are the best-known ones. The production of these toxic secondary metabolites is closely related to fungal development and depends on specific climate conditions (Jamali *et al.*, 2012; Keller *et al.*, 2005; Yagudayev and Ray, 2023). Aflatoxins are among the most dangerous mycotoxins found in agricultural products, and climate change is expected to have a strong impact on the increasing presence of aflatoxigenic fungi in feed and food in the EU (Schrenk *et al.*, 2020). Four types of aflatoxins are significant in the agriculture and especially in the health sector: aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2). The diseases caused by exposure to aflatoxins are referred to as "aflatoxicosis", which could be acute or chronic. Acute aflatoxicosis,

 | DOI: 10.1163/23524588-00001286
 | ISSN: 2352-4588 (online)

contracted through the contaminated food and feed, poses a significant health risk to both humans and animals (Gong *et al.*, 2016). Aflatoxicosis in humans can lead to abdominal pain, vomiting, diarrhoea, pulmonary edema, cerebral edema, anorexia, hepatic steatosis, jaundice, depression and photosensitivity. In animals, it causes anorexia, reduced egg or milk production and increased susceptibility to infectious diseases (Gilbert-Sandoval *et al.*, 2020).

On the contrary, it is recognized that daily consumption of foods, contaminated with low levels of aflatoxin B1, will result in chronic aflatoxicosis, cause a growth retardation in children, immune suppression, and a reduced life expectancy (Dhakal *et al.*, 2022; Shephard, 2008). It is recognized as responsible for the majority of cases of hepatocellular carcinoma in the world's population (Claeys *et al.*, 2020; Gong *et al.*, 2016). In animals, low AFBs contamination levels in feed cause immunosuppression and susceptibility to infectious diseases, weight loss, reduced reproductive capacity, and increased livestock mortality (Macías-Montes *et al.*, 2020; Pinotti *et al.*, 2016; Popescu *et al.*, 2022). AFB1 carcinogenic properties in humans are mainly based on the genotoxic properties of its metabolite (AFB1-8,9-epoxide), which is highly reactive and binds to DNA, proteins and glutathione (Razzaghi-Abyaneh *et al.*, 2013). Aflatoxins have been shown to extensively contaminate maize, cotton, soya, peanuts and nuts during the pre-harvest, harvest and storage; which is mainly a result of poor processing conditions. Moreover, AFB1 present in contaminated feed is metabolized and could be released into milk in the form of aflatoxin M1, which is resistant to heat treatments (Sabina *et al.*, 2023), such as pasteurization and freezing, and classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC) (Bennett and Klich, 2003; Hong *et al.*, 2017).

Production of aflatoxins in *Aspergillus* spp. is highly variable and depends on genotype, substrate, geographic origin, abiotic factors, such as water activity and temperature, and agronomic practices (Perrone *et al.*, 2014; Romero Donato *et al.*, 2022). Despite the efforts to contain the spread of *Aspergillus* spp., outbreaks have been sporadically recorded even in areas previously unscathed (Alameri *et al.*, 2023; Kumar *et al.*, 2022). Since the presence of these toxins in feed and food has been frequently reported in several countries, the European Union has imposed AFB1 legal limit levels related to the considered matrix (Regulation (EC) 1881/2006) and strict controls measurements (Regulation (EC) 401/2006) (Battilani *et al.*, 2008; EC No. 1881/2006, 1881) The need to eliminate AF-contaminated agricultural products has a large economic and ecological impact affecting everyone involved in the production chain; such as farmers, grain producers, handlers and distributors, processors and consumers (Karlovsky *et al.*, 2016). Mycotoxin contamination of food and feed is considered unavoidable, but to minimise possible product contamination, different procedures have been implemented during the pre- and post-harvest phases to achieve mycotoxin degradation/detoxification (Awuchi *et al.*, 2021; Udomkun *et al.*, 2017). Among the most fruitful strategies for mycotoxin control in pre-harvest, such as the selection of resistant plant genotypes and insect management, biocontrol strategies using atoxigenic strains of *A. flavus* have also been exploited (Alaniz Zanon *et al.*, 2022; Logrieco *et al.*, 2021). The last method is based on the competition for substrate, potential production of inhibitory metabolites and the inability of atoxigenic strains to recombine with native toxigenic strains, which prevent the re-acquisition of aflatoxigenicity (H. K. Abbas *et al.*, 2011; Wu and Khlangwiset, 2010). The strategy's effectiveness in the biocontrol of the toxigenic strains has been demonstrated under field conditions in cotton (Cotty, 1997), peanuts (Alaniz Zanon *et al.*, 2013) and maize (H. Abbas *et al.*, 2006). The time of harvesting and the storage conditions, such as disinfection, pest exclusion and control of certain environmental parameters (relative humidity and temperature) are the main factors affecting the spread of mycotoxins in foodstuffs during these critical (Logrieco *et al.*, 2021). Despite the preventative measures, aflatoxin levels exceeding the legal limits can occasionally be detected in agricultural products in the post-harvest period. In order to reduce the level of aflatoxins in such products, various physico-chemical procedures have been investigated and developed. Both physical and chemical approaches implemented to date have some disadvantages, such as inefficiency of removal, lack of cost-effectiveness, etc. Moreover, both detoxification systems may lead to the undesirable alterations in food characteristics, such as sensory quality, safety and technological properties (Faucet-Marquis *et al.*, 2014; Nitzke *et al.*, 2016). A further aspect to be considered is the additional economic cost of treating the substrate after aflatoxins decontamination.

It has recently been evidenced that some insect species like black soldier fly (*Hermetia illucens*, *HI*), yellow mealworm (*Tenebrio molitor*, TM) and lesser mealworm (*Alphitobius diaperinus*) have a high AFB1 tolerance, which is approximately 20 times above the legal limit for a feed material in the EU (20 μg/kg) and do

not accumulate AFB1 in their body (Bosch *et al.*, 2017; Camenzuli *et al.*, 2018; Gützkow *et al.*, 2021; Zhao *et al.*, 2022; Heuel *et al.*, 2023). Heuel *et al.* reported a significant reduction in aflatoxin B1 (AFB1) concentration from feedstock to HI meal. While the substrate used to rear insects contained 842 μg/kg AFB1, the resulting HI meal exhibited a considerably lower AFB1 level of 1.22 μg/kg. Additionally, the AFB1 content in TM larvae reared on contaminated crops was approximately 10% of the European Union's legal limit and the gut contents contributed a relatively large part to the total AFB1 present in TM (Bosch *et al.*, 2017). A comprehensive overview of the effects of mycotoxin bio-transformation has been proposed by Niemans *et al.* (2021). This comprehensive overview suggests that aflatoxins-contaminated crops could be used as rearing substrates for these insect species, upgrading contaminated agricultural commodities into high-valuable proteins.

However, frass produced during the insect breeding still contains high levels of AFB1, so if this by-product is to be used in agriculture as a soil conditioner, it would require an additional decontamination step. Several studies reported the aflatoxin-degrading capacity of fungal and bacterial strains isolated from soil, faeces and crops. Lactone ring of coumarin, the basic structure of all aflatoxins, which is responsible for its toxicity, is cleaved from *Aspergillus niger*, thus allowing the aflatoxin B1 biodegradation (Zhang *et al.*, 2014). Similarly, in *Rhizopus oryzae* and *Trichoderma reesei* the degradation of aflatoxins B1, B2, G1, G2 and M1 is associated with the lactone ring cleavage, created by a set of enzymes that are recognised as generally safe (GRAS) by Food and Drug Administration (Hackbart *et al.*, 2014). *Armillariella tabescens*, an edible mushroom, has been proven as a powerful degrader of aflatoxins; as it is capable of producing enzymes exerting dual reactions: epoxide formation and epoxide hydrolysis to dihydrodiol. The dual reactions result in the cleavage of the bisfuran ring, a primary toxic structure of the aflatoxin (Kim *et al.*, 2017). Additionally, some bacteria, like *Bacillus subtilis* and *Myxococcus fulvus*, which are naturally present in the soil, are known to degrade aflatoxin B1, G1, and M1 Hackbart *et al.*, 2014).

Supported by the reported studies, the present study was aimed to present a strategy to valorised AFB1-contaminated crops in the food supply chain, combining the high tolerance to AFB1 and apparent lack of accumulation of farmed insect species and the aflatoxin-degrading capacity of fungal strains to improve food safety and bioremediation. Specifically, TM was reared on AFB1-contaminated substrates up to 400 μg/kg and the produced frass, still containing AFB1, was decontaminated using *Rhizopus oryzae* and *Trichoderma reesei*, which were reported to have 100% lactone ring cleavage activity (Kim *et al.*, 2017).

2 Material and methods

Experimental feeds

Experimental AFB1 contaminated wheat bran was used as a growth substrate for the test. Four solutions (50% water and 50% acetonitrile) of 5 ml, containing increasing concentrations of AFB1, were prepared in order to obtain 4 substrates (AFB1-0, AFB1-125, AFB1-250, AFB1-500) with the concentrations of 0-125-250-500 μg of aflatoxin B1 per kg of substrate. Each solution was sprayed onto 1 kg of wheat bran and, to achieve homogenous contamination of the substrate, the wheat bran was divided into 50 g batches and sprayed with 0.25 ml of the solution. The spiked substrates were left overnight to dry in a flow cabinet by air to avoid the possible proliferation of mould due to the water sprayed on the wheat bran, and the day after each growth substrate was homogenised using C-Tronic 15 Plus (Sirman, Pieve di Curtarolo, Italy). Each substrate was subsequently analysed to evaluate the actual AFB1 contamination level. Aflatoxin (AF) B1 and acetonitrile (purity ≥ 99.98% chromatographic grade) were purchased from Sigma Aldrich-Merck (Darmstadt, Germany).

T. molitor *larvae*

Tenebrio molitor (TM) larvae used for the experiment were obtained from 2 batches of adult specimen (500 g/batch) and kept in plastic containers measuring 40 × 60 × 7 cm. They were provided with 1 kg of wheat bran (Mulino di Giusto, Italy). The broodstock was kept under the controlled environmental conditions (darkness: 24 h; temperature: 25 °C; relative humidity: 50-60%) and every other day, a potato slice (2 g) was added for hydration and the uneaten potato was removed to avoid mould proliferation. The spawners were left in these conditions for 4 days for oviposition before being removed. The hatching of the eggs took place in the same containers in which oviposition was carried out, and the larvae were kept for 60 days in the same rearing conditions described above.

Trial of **T. molitor** *breeding on contaminated substrate*

TM larvae were separated from the first common growth substrate, visually examined to discard specimens het-

erogeneous in size and 100 larvae were individually weighed (Sartorius Entris II series BCE, Lemgo, Germany) to register the initial body weight of 11 ± 2 mg. For each level of experimentally spiked substrate, 150 g of wheat bran was added in the food-grade plastic containers (40 × 60 × 7 cm) with 3 g of larvae (corresponding to 272 larvae per replicate), according to a single-factorial experimental design with 3 replicates per treatment.

The 12 prepared trays were kept in a chamber under the controlled conditions (darkness: 24 h; temperature: 25 °C; relative humidity: 50-60%); 2 g of potato was administered every 3 days in each tray for the water supply needed by the larvae. The trays were moved randomly every 3 days to avoid any position affecting the experiment. With the appearance of the first pupa after the 44 days, larvae were separated from the growth medium using tweezers, mortality was recorded per each tray and the individual weights of 30 specimens per tray/replicate were registered. At the end of the experimental period, the larvae were not fasted after harvesting but were immediately suppressed by freezing (−20 °C) and stored at the same temperature. Frass was separated from the unconsumed feed by sieving (10 μm mesh size).

Inoculum preparation

The micro-organisms *R. oryzae* (CCT7560) and *T. reesei* (QM9414) were obtained from a culture collection provided by the André Tosello Foundation (Campinas, SP, Brazil). After rehydration, the cultures were maintained at 4-8 °C in Potato Dextrose Agar (PDA) medium (Oxoid, Rodano, Italy). The culture was carried out according to the procedures of Hackbart *et al.* (2014). The spores were spread on an aqueous emulsion of Tween 80 (0.2%) solution, scraped with a nickel-chrome spatula and inoculated again in 4% PDA. The cultures were incubated for 7 days at 30 °C until complete sporulation. The spore suspensions used for the trial were obtained by adding 50 ml of Tween 80 aqueous emulsion to the fungal culture and by releasing the spores after scraping with a spatula. The spore concentration obtained following this protocol was estimated by enumeration in quadrant C with a Neubauer chamber. Determination of spore concentration was performed according to

$$C = (M \times 1000 \times dil)/0.004$$

where: M is the average number of spores counted in quadrant C of the Neubauer chamber; 1000 is the Neubauer chamber correction factor per ml; dil is the dilution factor equal to 10 (9 ml of water + 1 ml of spore solution); 0.004 is the Neubauer chamber correction factor per 1 ml.

Trials of microbial action at aflatoxin concentration

Both the sterilized broth medium and the TM frass were utilized as the growth substrate for RO, TR and RO-TR combination at increasing AFB1 levels. The spore concentration (4×10^6 spores g^{-1}) proposed by Hackbart *et al.* (2014) was used to culture the microorganisms. Two trials were carried out. In the first trial 14 ml of 2% Malt Extract Broth (ME Broth) (Oxoid, Rodano, Italy) and 1 ml of spore suspension were placed into a falcon, fortified with an AFB1 content equivalent to either 0, 62.5, 125, 250 or 500 μg/kg, 10 g of uncontaminated frass, 1 ml of spore suspension and 14 ml of water were placed into a falcon fortified with the AFB1 content mentioned above. The additional concentration of 62.5 μg/kg was added because it was equivalent to the frass concentration obtained at the end of the TM breeding trial. RO and TR were tested individually and in combination (RO-TR).

In the second trial, the frass derived from the breeding TM trial was used as substrate for RO and TR and their combination (RO-TR) culture.

All the experimental treatments were tested in triplicate. The cultures were incubated in sterile falcon tubes at 30 °C for 5 days (120 h) on a shaker plate. The tubes were then placed in a thermostatic bath at 70 °C for 10 min to interrupt the metabolic process. The concentrations of aflatoxin residues were calculated using the kit AgraQuant®Aflatoxin B1 Assay kit (2-50 ppb) (Romer Labs, Tulln an der Donau, Austria), and the liquid parts of these samples were used as the aqueous part for the dilution of pure methanol to obtain the 70% methanol extraction solution.

AFB1 content determination

For each sample, 10 g was weighed into a falcon and 50 ml of the 70/30 (v/v) methanol/water extraction solution was added, resulting in a ratio of 1:5 (w:v) between the sample and the extraction solution. Using T25 Ultra Turrax (IKA, Staufen, Germany) the samples were homogenized for 3 min and then centrifuged for 5 min at 10,000 *g*. The supernatant was filtered using a 0.45 μm nylon filter. The pH of the filtrate was measured and corrected if necessary to be between 6 and 8, because excessively alkaline or acidic conditions may affect the test results. The filtrate was diluted to 1:2 with the assay buffer provided in the AgraQuant®Aflatoxin B1 Assay kit (2-50 ppb). Subsequently, the samples

were tested following the manufacturer's procedures. The concentrations of AFB1 in the samples were calculated by interpolating OD values with the standard curve, using the Romer®Log/Logit spreadsheet provided (free of charge) on request. Samples with the AFB1 concentrations higher than 50 ppm were diluted and processed again.

Data analysis

Data were subjected to analysis of variance (ANOVA) according to a single-factor or two-factors design and if appropriate, the means were compared using Tukey's test. Capital letters were used for $P < 0.001$ and minuscule letters for $P < 0.05$. Data were analysed using the SPSS package (Release17.0, SPSS Inc., Chicago, IL, USA).

3 Results and discussion

Evaluation of the contaminated substrates

After spiking the wheat bran with the AFB1 solutions, the actual concentration of AFB1 in the substrate was verified. A discrepancy between the theoretical value and the analytical determination of AFB1 resulted in the experimental contamination of the wheat bran. As it can be seen in Table 1, the content of AFB1 in the substrate differed by approximately 20% from the expected value in all the tests in which the toxin was inoculated. This discrepancy can be attributed to systematic errors such as a non-uniformity of the contamination carried out in the laboratory (part of the toxin sprayed on the wheat bran could have adhered to the surface of the chamber used for the contamination) and/or to other factors that could have affected the extraction procedures (i.e. solvent, pH, and incubation temperature, low recovery, etc.) (Haskard *et al.*, 2001; Niermans *et al.*, 2024). Deviations between intended and analytical spiked concentration have also been registered in Camenzuli *et al.* (2018) without an apparent reason. The analytical values were utilized for mass balance calculation. Furthermore, the analytical content of aflatoxins in the AFB1-0, used as a reference diet, reported a low-level AFB1 contamination of 3.23 ± 0.68 μg/kg. It is important to note that this level of contamination is below regulatory limits for commercialization and does not preclude the use of this diet as a control.

TABLE 1 Theoretical and analytical concentrations (μg/kg) of AFB1 in the substrates, used for the experiment. Analyses conducted on the wet weight basis

	Theoretical	Analytical
AFB1-0	0	3.23 ± 0.68
AFB1-125	125	97.86 ± 2.20
AFB1-250	250	187.44 ± 8.88
AFB1-500	500	390.69 ± 19.91

Bioconversion of the AFB1 contaminated wheat bran by **T. molitor** *larvae*

The test ended after 44, as soon as the first pupa appeared independently from the test treatment. The effect of the AFB1 concentrations on the growth performance and mortality is shown in Table 2. At the end of the test, the larvae went from an initial average weight of 0.011 ± 0.0026 g to an average value for the four tests of 0.117 ± 0.006 g, with the highest value in the AFB1-250 test (0.125 ± 0.027 g). Significant differences in the specific growth rate were observed (6.47 vs 6.20, $P < 0.05$). A similar finding was also noted by Bosch *et al.* (2017) who reported that larvae reared in a substrate containing 200 μg/kg of AFB1 had a significantly higher growth rate than those reared on substrates with AFB1 levels of 100 μg/kg and 500 μg/kg. The mortality observed in the present study ranged from 6.54 to 12.22% with the highest value ($P < 0.05$) observed in the larvae fed on AFB1-0. These differences between the individuals from different trials should not be surprising, as it was found that larval growth is not uniform even in the replicates of the same trial, due to an interaction between the individuals, which has not yet been clearly explained (Rumbos *et al.*, 2021). TM larvae confirmed to be quite tolerant to AFB1 as already reported in Niermans *et al.* (2021) where only 5% mortality was observed for TM exposure up to 415 μg/kg.

At the end of the experiment, *T. molitor* larvae were separated from the frass and both were analysed using the kit mentioned above to assess the AFB1 content. The AFB1 content data for the four experimental trials are shown in Table 3. A value of 4.10 ± 0.91 μg/kg AFB1 was detected only in the TM larvae reared on the AFB1-500 substrate, whereas for the other three trials, the AFB1 content resulted below the detection value of the commercial test used (2 μg/kg). The AFB1 concentration detected in the frass resulted in 0 μg/kg, 16.99 ± 1.68 μg/kg, 32.99 ± 0.49 μg/kg and 67.39 ± 5.19 μg/kg for AFB1-0, AFB1-125, AFB1-250 and AFB1-500, respectively.

The data show that only with the highest contamination level (AFB1-500) a detectable residual AFB1 concentration (1.2%) has been found in the TM larvae. About 17.4 ± 0.4% of the initially inoculated AFB1 was detected in the frass, thus reflecting an AFB1 degradation capacity of TM equivalent to 81.4 ± 0.6%. It must

TABLE 2 Effects of the AFB1 concentrations on final weight, specific growth rate (SGR) and mortality of TM larvae at the end of the 44-day breeding trial

	AFB1-0	AFB1-125	AFB1-250	AFB1-500	*P*-value
Final weight (g)	0.119	0.110	0.125	0.116	0.211
SGR (%)	6.20^{b}	6.19^{b}	6.47^{a}	6.35^{ab}	0.024
Mortality (%)	12.22^{a}	6.54^{b}	7.28^{ab}	7.90^{ab}	0.044

SGR(specific growth rate) = [ln(initial weight) – ln(final weight)]/44 days ∗ 100.

TABLE 3 Levels of AFB1 (μg/kg) detected in *T. molitor* larvae, frass and estimated percentage of degraded AFB1 compared to initial concentration. Values are the mean of the biological triplicates ± standard deviation. Analyses conducted on the wet weight basis

	AFB1 in TM	AFB1 in Frass	AFB1 degraded by TM
AFB1-0	ND	ND	-
AFB1-125	ND	16.99 ± 1.68	17.3
AFB1-250	ND	32.99 ± 0.49	17.6
AFB1-500	4.10 ± 0.91	67.39 ± 5.19	17.2

ND = Non-detectable.

be remarked that other AFB1 metabolites have not been taken into account and that larvae were not fasted after harvesting but immediately frozen, not allowing the gastrointestinal tract to be emptied of any toxins present (Bosch *et al.*, 2017).

The mass balances of aflatoxin B1 at the end of the breeding trial are presented in Figure 1. The average mass balance for AFB1 in TM ranged between 16.9 and 19.9% among the different treatments. Only in TM larvae, reared with the highest AFB1 contamination level, a 0.35% of the overall mass balance of AFB1 was recovered. The data confirm a high bioconversion capacity (from 80.1 to 83.1%) of TM larvae for AFB1. Zhao *et al.* (2022) found a similar (87.9%) conversion rate of AFB1 by TM larvae and identified the formation of 13 metabolites in frass and 3 metabolites in the larvae.

While it is possible that other microorganisms can contribute to the AFB1 degradation within the substrate as reported by Niermans *et al.* (2024), the relatively low humidity (10.5%) of the substrate utilized in the present study may have limited the microbial growth and activity. In comparison, the humidity used for black soldier fly (*Hermetia illucens*) rearing was 65% (Niermans *et al.*, 2024). These findings demonstrate the ability of TM to proliferate on the tested substrates without apparent inhi bition by aflatoxin B1 up to a concentration of 500 μg/kg. However, an additional process is needed to further decontaminate the frass from AFB1 to allow its utilization.

The bioconversion of the AFB1 by microorganisms

The additional biotransformation process considered the capacity of RO and TR to metabolize AFB1. Significant differences in the degradation capacity of the studied microorganisms (RO, TR and RO-TR) were observed on laboratory-contaminated samples, as reported in Table 4. Significant differences ($P < 0.01$) were observed among the concentration of the AFB1 detected after the exposure to the microorganisms considered. RO has proven to be more effective than TR for almost all the concentrations considered. The combination of RO and TR has always resulted in the lowest AFB1 concentration levels. It was also observed that the growth substrate could affect the degradation capacity of the applied microorganisms; significantly lower values have been observed when frass was used as growth substrate. Only for the toxin concentration of 250 μg/kg no differences were reported. These data underline the importance of evaluating the growth substrate, especially if the scaling up of the process is considered, because the solid part of the frass could interfere with the capacity of RO and TR to reach the target toxin, reducing the degradation performance.

As shown in Figure 2, the combination of the two microorganisms has always provided the highest degradation capacity (92%) in comparison with the one performed by using the microorganism singularly (RO 82%, TR 78%), demonstrating the synergy of RO and TR in the toxin's degradation in the studied system.

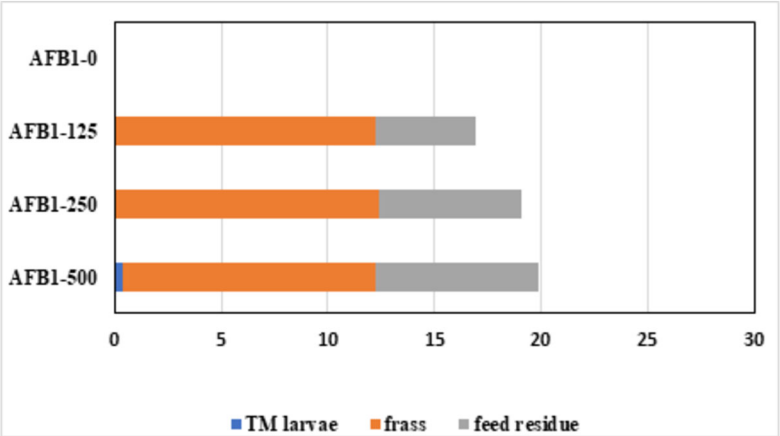

FIGURE 1 Mass balances (% of spiked substrate) of aflatoxin B1 in *T. molitor* larvae, frass and feed residues at the end of the breeding trial. Mass balance (dry matter basis) was calculated as: (sum of AFB1 in larvae, frass and feed residue / amount of AFB1 in the substrate) * 100.

TABLE 4 Effect of the microorganism exposure (RO, TR and RO-TR), and of the growth substrate (broth or frass) on the concentration (µg/kg) of detectable aflatoxin B1. Data are presented as mean values ± SD of 3 biological replicates

µg/kg of AFB1	Microorganism			Growth substrate	
	RO	TR	RO-TR	ME Broth	Frass
0	ND	ND	ND	ND	ND
62	2.28 ± 2.28^{B}	3.25 ± 3.59^{C}	ND^{A}	ND^{A}	3.68 ± 2.97^{B}
125	38.41 ± 4.86^{B}	41.54 ± 9.58^{B}	8.97 ± 3.47^{A}	26.52 ± 12.90^{a}	32.77 ± 19.38^{b}
250	51.69 ± 3.36^{B}	67.68 ± 4.79^{C}	24.48 ± 3.08^{A}	49.04 ± 20.50	46.79 ± 17.80
500	74.27 ± 10.31^{B}	93.68 ± 10.05^{C}	38.90 ± 4.60^{A}	62.46 ± 21.52^{A}	75.42 ± 27.25^{B}

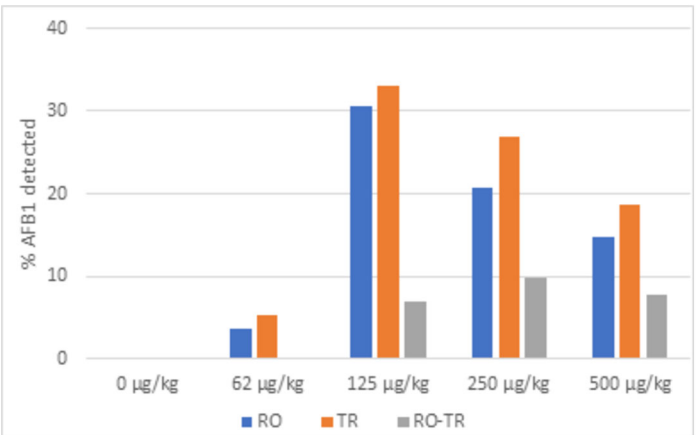

FIGURE 2 Evaluation of the degradation capacity of the two microorganisms (RO, TR and RO-TR). Data are presented as mean values ± SD of 3 biological replicates.

TABLE 5 Concentration (μg/kg) of AFB1 in the frass (as it is) obtained from the TM breeding trial after culturing with RO, TR and RO-TR. Data are presented as mean values ± SD of 3 biological replicates. Analyses conducted on the wet weight basis

	Initial level	Final level		
		RO	TR	RO-TR
AFB1-0	ND	ND	ND	ND
AFB1-125	16.99 ± 1.68	ND	ND	ND
AFB1-250	32.99 ± 0.49	ND	ND	ND
AFB1-500	67.39 ± 5.19	3.12 ± 1.79	4.70 ± 2.35	ND

Initial results demonstrated the potential of the two microorganisms, which could be interesting as the next phase of the study, as observed by Kim *et al.* (2017). These microorganisms were applied to frass samples obtained from the TM farm trial, which contained various levels of the AFB1 (Table 3). These microorganisms efficiently degraded the aflatoxin B1 (AFB1); in fact, the analysis revealed undetectable AFB1 levels in all treated samples, except those involving initial AFB1 concentrations of 67.39 ± 5.19 μg/kg (AFB1-500) and treated with RO and TR individually. In these cases, the residual AFB1 concentrations of 3.12 ± 1.79 μg/kg and 4.70 ± 2.35 μg/kg, respectively, were observed (Table 5). In contrast, combined treatment with RO and TR successfully eliminated AFB1. This underlines what has already been observed with the laboratory contaminated samples, where the combination of the two microorganisms provided a better degradation of the AFB1. Consistent with previous research (Bosch *et al.*, 2017; Zhao *et al.*, 2022), the TM strain exhibited tolerance to high concentrations of the AFB1, up to ten times the regulatory limit, without compromising the growth. While TM demonstrated the capacity to degrade the AFB1, a complete elimination was not achieved. However, subsequent treatment of the resulting rearing biomass (frass) with RO and TR microorganisms resulted in the complete removal of the detectable AFB1, suggesting a synergistic effect in the decontamination process of the initial AFB1 presence.

4 Conclusion

The use of a single proposed approach for toxin degradation can lead to a maximum reduction by 80% of the AFB1. A combination of a first decontamination step performed by TM, resulting in an important AFB1 bioconversion (80-83%); with a second decontamination step of the frass by RO and TR could envisage the possibility to obtain a high-value AFB1-free protein source for the preparation of feed, and conditioner for agriculture using products that would otherwise have to be disposed of. However, a comprehensive analysis is required to assess the potential formation of alternative toxic byproducts during the degradation processes. Moreover, additional factors, including the presence of indigenous microorganisms within the growth substrate and potential experimental variables, that may affect the AFB1 degradation and the secondary metabolite formation, should be investigated. Consequently, the current preliminary study represents a premise for a further research.

Acknowledgement

Authors are sincerely grateful to Elisabetta Orecchia for her helpful support in the microorganism tests.

Funding

This work was supported by the University of Udine in the framework of the Strategic Plan 2022-25 Interdepartmental Research Project *CibiAmo*.

References

Abbas, H., Zablotowicz, R., Bruns, H.A. and Abel, C., 2006. Biocontrol of aflatoxin in corn by inoculation with non-aflatoxigenic *Aspergillus flavus* isolates. Biocontrol Science and Technology 16: 437-449. https://doi.org/10.1080/09583150500532477

Abbas, H.K., Zablotowicz, R.M., Horn, B.W., Phillips, N.A., Johnson, B.J., Jin, X. and Abel, C.A., 2011. Comparison of major biocontrol strains of non-aflatoxigenic aspergillus flavus for the reduction of aflatoxins and cyclopiazonic acid in maize. Food Additives and Contaminants – Part A Chemistry, Analysis, Control, Exposure and Risk Assessment 28: 198-208. https://doi.org/10.1080/19440049.2010.544680

Alameri, M.M., Kong, A.S.Y., Aljaafari, M.N., Ali, H.A., Eid, K., Sallagi, M.A., Cheng, W.H., Abushelaibi, A., Lim, S.H.E., Loh, J.Y. and Lai, K.S., 2023. Aflatoxin contamination: an overview on health issues, detection and management strategies. Toxins 15: 246. https://doi.org/10.3390/toxins15040246

Alaniz Zanon, M.S., Bossa, M., Chiotta, M.L., Oddino, C., Giovanini, D., Cardoso, M.L., Bartosik, R.E. and Chulze, S.N., 2022. Pre-harvest strategy for reducing aflatoxin accumulation during storage of maize in Argentina. International Journal of Food Microbiology 380: 109887. https://doi.org/10.1016/j.ijfoodmicro.2022.109887

Alaniz Zanon, M.S., Chiotta, M.L., Giaj-Merlera, G., Barros, G. and Chulze, S., 2013. Evaluation of potential biocontrol agent for aflatoxin in Argentinean peanuts. International Journal of Food Microbiology 162: 220-225. https://doi.org/10.1016/j.ijfoodmicro.2013.01.017

Awuchi, C.G., Ondari, E.N., Ogbonna, C.U., Upadhyay, A.K., Baran, K., Okpala, C.O.R., Korzeniowska, M. and Guiné, R.P.F., 2021. Mycotoxins affecting animals, foods, humans and plants: Types, occurrence, toxicities, action mechanisms, prevention and detoxification strategies-a revisit. Foods 10: 1279. https://doi.org/10.3390/foods10061279

Battilani, P., Pietri, A., Barbano, C., Scandolara, A., Bertuzzi, T. and Marocco, A., 2008. Logistic regression modeling of cropping systems to predict fumonisin contamination in maize. Journal of Agricultural and Food Chemistry 56: 10433-10438. https://doi.org/10.1021/jf801809d

Bennett, J.W. and Klich, M., 2003. Mycotoxins. ASM Journals Clinical Microbiology Reviews 16: 497-516. https://doi.org/10.1128/CMR.16.3.497

Bosch, G., Van der Fels-Klerx, H.J., De Rijk, T.C. and Oonincx, D.G.A.B., 2017. Aflatoxin B1 tolerance and accumulation in black soldier fly larvae (*Hermetia illucens*) and yellow mealworms (*Tenebrio molitor*). Toxins 9: 185. https://doi.org/10.3390/toxins9060185

Camenzuli, L., van Dam, R., de Rijk, T., Andriessen, R., van Schelt, J. and van der Fels-Klerx, H.J.I., 2018. Tolerance and excretion of the mycotoxins aflatoxin B1, zearalenone, deoxynivalenol, and ochratoxin A by *Alphitobius diaperinus* and *Hermetia illucens* from contaminated substrates. Toxins 10: 91. https://doi.org/10.3390/toxins10020091

Claeys, L., Romano, C., De Ruyck, K., Wilson, H., Fervers, B., Korenjak, M., Zavadil, J., Gunter, M.J., De Saeger, S., De Boevre, M. and Huybrechts, I., 2020. Mycotoxin exposure and human cancer risk: a systematic review of epidemiological studies. Comprehensive Reviews in Food Science and Food Safety 19: 1449-1464. https://doi.org/10.1111/1541-4337.12567

Cotty, P.J., 1997. Aflatoxin-producing potential of communities of *Aspergillus* section Flavi from cotton producing areas in the United States. Mycological Research 101: 698-704. https://doi.org/10.1017/S0953756296003139

Dhakal, A., Hashmi, M.F. and Sbar, E., 2022. Aflatoxin toxicity. StatPearls Publishing.

EC No. 1881/2006, 1881. Commission regulation (EC) No. 1881/2006 of 19 December 2006. Setting maximum levels for certain contaminants in foodstuffs (Text with EEA relevance). Official Journal of European Commission 364.

Faucet-Marquis, V., Joannis-Cassan, C., Hadjeba-Medjdoub, K., Ballet, N. and Pfohl-Leszkowicz, A., 2014. Development of an in vitro method for the prediction of mycotoxin binding on yeast-based products: case of aflatoxin B1, zearalenone and ochratoxin A. Applied Microbiology and Biotechnology 98: 7583-7596. https://doi.org/10.1007/s00253-014-5917-y

Gilbert-Sandoval, I., Wesseling, S. and Rietjens, I.M.C.M., 2020. Predicting the acute liver toxicity of aflatoxin B1 in rats and humans by an in vitro-in silico testing strategy. Molecular Nutrition and Food Research 64: 2000063. https://doi.org/10.1002/mnfr.202000063

Gong, Y.Y., Watson, S. and Routledge, M.N., 2016. Aflatoxin exposure and associated human health effects, a review of epidemiological studies. Food Safety 4: 14-27. https://doi.org/10.14252/foodsafetyfscj.2015026

Gützkow, K.L., Ebmeyer, J., Kröncke, N., Kampschulte, N., Böhmert, L., Schöne, C., Schebb, N.H., Benning, R., Braeuning, A. and Maul, R., 2021. Metabolic fate and toxicity reduction of aflatoxin B1 after uptake by edible *Tenebrio molitor* larvae. Food and Chemical Toxicology 155: 112375. https://doi.org/10.1016/j.fct.2021.112375

Hackbart, H.C.S., Machado, A.R., Christ-Ribeiro, A., Prietto, L. and Badiale-Furlong, E., 2014. Reduction of aflatoxins by *Rhizopus oryzae* and *Trichoderma reesei*. Mycotoxin Research 30: 141-149. https://doi.org/10.1007/s12550-014-0202-6

Haskard, C.A., El-Nezami, H.S., Kankaanpää, P.E., Salminen, S. and Ahokas, J.T., 2001. Surface Binding of aflatoxin B1 by lactic acid bacteria. Applied and Environmental Microbiology 67. https://doi.org/10.1128/AEM.67.7.3086-3091.2001

Heuel, M., Kreuzer, M., Gangnat, I.D.M., Frossard, E., Zurbrügg, C., Egger, J., Dortmans, B., Gold, M., Mathys, A., Jaster-Keller, J., Weigel, S., Sandrock, C. and Terranova, M., 2023. Low transfer of cadmium, lead and aflatoxin B1 to eggs and meat of laying hens receiving diets with black soldier fly larvae reared on contaminated substrates. Animal Feed Science and Technology 304: 115733. https://doi.org/10.1016/j.anifeedsci.2023.115733

Hong, E., Lee, S.Y., Jeong, J.Y., Park, J.M., Kim, B.H., Kwon, K. and Chun, H.S., 2017. Modern analytical methods for the detection of food fraud and adulteration by food category.

Journal of the Science of Food and Agriculture 97: 3877-3896. https://doi.org/10.1002/jsfa.8364

Jamali, M., Ebrahimi, M.A., Karimipour, M., Shams-Ghahfarokhi, M., Dinparast-Djadid, N., Kalantari, S., Pilehvar-Soltanahmadi, Y., Amani, A. and Razzaghi-Abyaneh, M., 2012. An insight into the distribution, genetic diversity, and mycotoxin production of *Aspergillus* section Flavi in soils of pistachio orchards. Folia Microbiologica 57: 27-36. https://doi.org/10.1007/s12223-011-0090-5

Karlovsky, P., Suman, M., Berthiller, F., De Meester, J., Eisenbrand, G., Perrin, I., Oswald, I.P., Speijers, G., Chiodini, A., Recker, T. and Dussort, P., 2016. Impact of food processing and detoxification treatments on mycotoxin contamination. Mycotoxin Research 32: 179-205. https://doi.org/10.1007/s12550-016-0257-7

Keller, N.P., Turner, G. and Bennett, J.W., 2005. Fungal secondary metabolism – from biochemistry to genomics. Nature Reviews Microbiology 3: 937-947. https://doi.org/10.1038/nrmicro1286

Kumar, P., Gupta, A., Mahato, D.K., Pandhi, S., Pandey, A.K., Kargwal, R., Mishra, S., Suhag, R., Sharma, N., Saurabh, V., Paul, V., Kumar, M., Selvakumar, R., Gamlath, S., Kamle, M., Enshasy, H.A.E., Mokhtar, J.A. and Harakeh, S., 2022. Aflatoxins in cereals and cereal-based products: occurrence, toxicity, impact on human health, and their detoxification and management strategies. Toxins 14: 687. https://doi.org/10.3390/toxins14100687

Logrieco, A., Battilani, P., Leggieri, M.C., Jiang, Y., Haesaert, G., Lanubile, A., Mahuku, G., Mesterhazy, A., Ortega-Beltran, A., Pasti, M., Smeu, I., Torres, A., Xu, J. and Munkvold, G., 2021. Perspectives on global mycotoxin issues and management from the mycokey maize working group. Plant Disease 105: 525-537. https://doi.org/10.1094/PDIS-06-20-1322-FE

Macías-Montes, A., Rial-Berriel, C., Acosta-Dacal, A., Henríquez-Hernández, L.A., Almeida-González, M., Rodríguez-Hernández, Á., Zumbado, M., Boada, L.D., Zaccaroni, A. and Luzardo, O.P., 2020. Risk assessment of the exposure to mycotoxins in dogs and cats through the consumption of commercial dry food. Science of the Total Environment 708: 134592. https://doi.org/10.1016/j.scitotenv.2019.134592

Niermans, K., Hoek-van den Hil, E.F., van der Fels-Klerx, H.J. and van Loon, J.J.A., 2024. The role of larvae of black soldier fly and house fly and of feed substrate microbes in biotransformation of aflatoxin B1. Ecotoxicology and Environmental Safety 279: 11644. https://doi.org/10.1016/j.ecoenv.2024.116449

Niermans, K., Meyer, A.M., van den Hil, E.F.H., van Loon, J.J.A. and van der Fels-Klerx, H.J., 2021. A systematic literature review on the effects of mycotoxin exposure on insects and on mycotoxin accumulation and biotransformation. Mycotoxin Research 37: 279-295. https://doi.org/10.1007/s12550-021-00441-z

Nitzke, J.A., Cladera, F., Thys, R., da Cruz, F.T. and Martinelli, S., 2016. 5th food safety and security symposium: food and health. Food Science and Technology 36. https://doi.org/10.1590/1678-457X.0057

Perrone, G., Gallo, A. and Logrieco, A.F., 2014. Biodiversity of *Aspergillus* section Flavi in Europe in relation to the management of aflatoxin risk. Frontiers in Microbiology 5. https://doi.org/10.3389/fmicb.2014.00377

Pinotti, L., Ottoboni, M., Giromini, C., Dell'Orto, V. and Cheli, F., 2016. Mycotoxin contamination in the EU feed supply chain: a focus on cereal byproducts. Toxins 8: 45. https://doi.org/10.3390/TOXINS8020045

Popescu, R.G., Rădulescu, A.L., Georgescu, S.E. and Dinischiotu, A., 2022. Aflatoxins in feed: types, metabolism, health consequences in swine and mitigation strategies. Toxins 14: 853. https://doi.org/10.3390/toxins14120853

Razzaghi-Abyaneh, M., Saberi, R., Sharifan, A., Rezaee, M.B., Seifili, R., Hosseini, S.I., Shams-Ghahfarokhi, M., Nikkhah, M., Saberi, I. and Amani, A., 2013. Effects of *Heracleum persicum* ethyl acetate extract on the growth, hyphal ultrastructure and aflatoxin biosynthesis in *Aspergillus parasiticus*. Mycotoxin Research 29: 261-269. https://doi.org/10.1007/s12550-013-0171-1

Romero Donato, C.J., Cendoya, E., Demonte, L.D., Repetti, M.R., Chulze, S.N. and Ramirez, M.L., 2022. Influence of abiotic factors (water activity and temperature) on growth and aflatoxin production by *Aspergillus flavus* in a chickpea-based medium. International Journal of Food Microbiology 379: 109841. https://doi.org/10.1016/j.ijfoodmicro.2022.109841

Rumbos, C.I., Adamaki-Sotiraki, C., Gourgouta, M., Karapanagiotidis, I.T., Asimaki, A., Mente, E. and Athanassiou, C.G., 2021. Strain matters: strain effect on the larval growth and performance of the yellow mealworm, *Tenebrio molitor* L. Journal of Insects as Food and Feed 7: 1195-1206. https://doi.org/10.3920/JIFF2021.0035

Sabina, A.O., Uzoamaka, G.-O. and Chinedu, O.S., 2023. Evaluation of the thermal stability profile of aflatoxins from fungal isolates from dried fishes. International Journal of Biochemistry and Biophysics 11: 17-26. https://doi.org/10.13189/ijbb.2023.110202

Schrenk, D., Bignami, M., Bodin, L., Chipman, J.K., del Mazo, J., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L., Leblanc, J.C., Nebbia, C.S., Nielsen, E., Ntzani, E., Petersen, A., Sand, S., Schwerdtle, T., Vleminckx, C., Marko, D., Oswald, I.P., Piersma, A., Routledge, M., Schlatter, J., Baert, K., Gergelova, P. and Wallace, H., 2020. Risk assessment of

aflatoxins in food. EFSA Journal 18: e06040. https://doi.org/10.2903/j.efsa.2020.6040

Shephard, G.S., 2008. Impact of mycotoxins on human health in developing countries. Food Additives and Contaminants – Part A 25: 146-151. https://doi.org/10.1080/02652030701567442

Udomkun, P., Wiredu, A.N., Nagle, M., Müller, J., Vanlauwe, B. and Bandyopadhyay, R., 2017. Innovative technologies to manage aflatoxins in foods and feeds and the profitability of application – a review. Food Control 76: 127-138. https://doi.org/10.1016/j.foodcont.2017.01.008

Wu, F. and Khlangwiset, P., 2010. Evaluating the technical feasibility of aflatoxin risk reduction strategies in Africa. Food Additives and Contaminants – Part A 27: 658-676. https://doi.org/10.1080/19440041003639582

Yagudayev, E. and Ray, S.D., 2023. Aflatoxin. In: Wexler, P. (ed.) Reference module in biomedical sciences. Academic Press, Cambridge, MA, USA, pp. 193-200. https://doi.org/10.1016/B978-0-12-824315-2.00179-2

Zhang, W., Xue, B., Li, M., Mu, Y., Chen, Z., Li, J. and Shan, A., 2014. Screening a strain of *Aspergillus niger* and optimization of fermentation conditions for degradation of aflatoxin B_1. Toxins 6: 3157-3172. https://doi.org/10.3390/toxins6113157

Zhao, S. and Fernald, R.D., 2005. Comprehensive algorithm for quantitative real-time polymerase chain reaction. Journal of Computational Biology 12. https://doi.org/10.1089/cmb.2005.12.1047