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The Critical Control Point of *Aspergillus spp*. Aflatoxin Contamination in Smallholder Dairy Farms

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ABSTRACT

Feed and food contamination by toxigenic fungi and their aflatoxins is one of the main threats to animal and human health worldwide and in the agricultural and industrial sectors. This study evaluated the contamination magnitude by *Aspergillus* species in dairy farms, aflatoxin AFB1 in cow feeds, and aflatoxin AFM1 in milk and local cheese (Dangke). One hundred twenty-two swabs from farms, 12 roughage feeds, 16 concentrated feeds, 39 fresh cow milk, and six cheese samples were analyzed for *Aspergillus* spp., AFB1, and AFM1 contamination. *Aspergillus flavus* and *Aspergillus niger* were detected in 13.93% and 7.38% of the swab samples, respectively. The roughage feeds showed low levels of AFB1, detected in 8.33% with contamination of 7.32 µ/kg, while concentrated feed was detected in 37.5% of specimens, with contamination levels of 27.8 µg/kg. Aflatoxin AFM1 was detected in raw milk samples and represented approximately 69.2% of samples, with a mean of 7.31 µg/kg. All local cheese samples were free of AFM1. There were critical points regarding HACCP inside the farms, which play significant roles in contamination by fungi and aflatoxins. Fungal contamination and aflatoxins pose dangerous public health problems to humans, especially infants and older people. Therefore, monitoring programs for mycotoxins are critical in reducing contamination.

Keywords

AFB1, AFM1, Aspergillus spp, Food contamination, HACCP

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Abbreviations

AFB1: Aflatoxin B1
AFM1: Aflatoxin M1
HACCP: Hazard Analysis Critical Control Points
AFG: Aflatoxin Green
PDA: Potato Dextrose Agar
ELISA: Enzyme-Linked Immunosorbent Assay

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Introduction

Mycotoxins are poisonous materials formed by toxigenic fungi that attack agricultural products in the field or a storehouse in natural conditions, including bad storehouses, high moisture, high temperature, and insect infestation [1]. When set up in animal ration and feed ingredients, these contaminants might pose big trouble and risk to lactating cows when they exceed normal levels. Initially, they had a mischievous effect on animal health, such as decreased feeding efficiency, milk productivity, immunodeficiency, emaciation, laminitis, infertility, and abortion [2, 3]. Furthermore, they may affect the food supply chain when they transfer from animal feed to milk and milk products [4, 5, 6]. Fungal fossils are very resistant to high temperatures and humidity, which can disrupt milk production during processing[7]. Subsequently, people are exposed to these poisons using contaminated animal products, such as meat, milk, and dairy products. People's ex-posure to mycotoxins can have several adverse health effects, including chronic and acute diseases, as well as teratogenic, carcinogenic, and immunosuppressive effects. It might bring about death in critical cases, such as delayed chronic toxicity or high acute intoxication [8].

Considering adverse consequences on all creatures and humans, mycotoxins cause huge monetary misfortunes for some countries, especially non-industrial nations, due to the expenses posed on food safety [9]. The most common toxigenic fungi in agricultural products are species belonging to the *Aspergillus*, *Alternaria, Fusarium*, and *Penicillium genera*. *Fusarium, Aspergillus,* and *Penicillium* are considered considerable mycotoxin-producers in animal feed worldwide [10].

In addition, fungal poisons, namely aflatoxins (AFB1, AFB2, AFG1, and AFG2), shaped by fungal species in the genera Aspergillus are critical mycotoxins found routinely in all dairy rations worldwide [11]. In tropical and subtropical areas worldwide, the issue is more articulated as it relates to humidity and high climate temperatures that lean toward the growth and multiplication of fungi. Grains and plant protein sources utilized in animal diets are the principal sources of fungal contamination and aflatoxins. Aspergillus multiplicates, preferably, on commodities at 15% or higher moisture levels at 25°C-35°C. Moisture levels of more than 17.5% and temperature of 27°C-30°C are required for the highest aflatoxins production. Aflatoxins decrease the quality of ingredients by using the nutrients in the ingredients for digestion and spread [12].

There is developing proof to propose that seasonal and geological contrasts impact mycotoxins and aflatoxins formation in both food and feed [13]. Tragically, there is a need for more research in Indonesia in this regard, especially in dairy farms and animal feed parts, considering the hot and wet conditions that characterize this country as tropical. Previous studies in this region elucidated the occurrence of aflatoxins in dairy feeds and milk.

The HACCP system has long been presented worldwide to identify, assess, and control hazardous food safety factors. It is a coherent, fundamental, efficient food safety control system with a complicated structure intended to identify and control risks and critical circumstances. From one perspective, this system ensures the safety of products on the way of the pecking order from maker to the shopper, empowers recognizing all the critical points that can influence the security and safety of the final product, takes out unsafe factors, and controls the total production process [14]. The current research aims to isolate and identify the extent of *Aspergillus spp*. contamination and its toxin production in smallholder dairy farms and to determine critical control points of contamination in the environment, raw milk, and cheese processing units in Enrekang province, South Sulawesi, Indonesia.

Result

Socio-demographic of smallholder dairy farms in Enrekang Regency, Indonesia

The socio-demographic and household characteristics showed that the milking cows ranged from 1 to 14 heads, with a mean (SD) of 5 (0.78) per farm. Approximately 69% of the owners used elephant grass as roughage feed, 30.8% used rice bran as concentrated feed, and most had feed storage facilities. The owners had a chance to be educated, and most of them attended secondary school (about 84.61 %), of which a high percentage was male as described below (Table 1). Our results were similar to those in the Ethiopia [16].

Among all collected samples, about 39 (31.97%) of fungi were isolated and identified macroscopically as *Aspergillus* and segregated by colony color into subgenera *Flavi* (green colonies), *Nigri* (black colonies), *Fumigate* (blue colonies), and *Terrei* (brown colonies). Therefore, macroscopic characters alone are insufficient and inaccurate for identification. Colony color has been examined microscopically to identify some micro features of isolated samples, such as conidiophore, vesicle, and conidia. Molecular analysis is sometimes conducted to confirm the isolates and resolve the cultural limitations, but it was not carried out in the current study (Figure 2). Mycological

Table 1.
Socio-demographic features of smallholder dairy farms in Enrekang regency, Indonesia (N = 13)

Variables	Characteristics	Value
Gender	Men	11
	Women	*2*
Education	University	1
	Secondary	11
	Primary	1
Age	Mean (SD)	36 (0.64)
	30-40	3
	50-60	8
	60-70	2
Number of milking cows	Mean (SD)	5 (0.78)
Milk production/ litter/day	Mean (SD)	5 (0.86)
Type of roughage feeds	Elephant grass	9
	Elephant grass plus green corn	4
	Rice bran	4
	Soya by-products	3
Type of concentrated feed	Rice bran plus commercial concentrate	2
	Rice bran plus soya by-product	3
	Soya plus palm oil cake	1
Feed storage facilities	Yes	10
	No	3

SD: standard deviation

analysis in this study revealed that most of the samples were contaminated by diverse fungi, all of which were identified as mycotoxigenic fungi. Yeasts were also present in the samples.

A. flavus was the most frequently isolated among samples (N = 17, 13.93%), followed by A. *niger* (N = 9, 7.38%), A. *terreus* (N = 6, 4.91%), and A. *fumigatus* (N = 7, 5.74%) (Table 2). These results are similar to the report on the domination of *A. flavus* and *A. niger* in dairy animals and poultry feeds. The current study showed the contamination of 31.97% of samples by *Aspergillus* (Table 2) A high percentage was found in cows' udders (N = 12, 31.58%), followed by cage floor (N = 8, 66.67%), and milker hands (N = 5, 41.67). A low percentage was recorded in coconut mold (N=1, 8.33) and banana leaf (N = 1, 8.33%) as natural packaging.

Discussion

Socio-demographic of smallholder dairy farms in Enrekang Regency, Indonesia

The percentage of women in agricultural activity was less than men, sometimes working beside their husbands or just working was widowed or divorced. The farmers needed more information about fungi and aflatoxins, and their knowledge about contamination was fragile. All of them agreed on the storage process and stated that they stored their animal feeds in a specific place to avoid humidity and rain for less than one month. Therefore, the growth of *Aspergillus* species will be less than expected when we compare their knowledge and feed practices inside the farms.

Isolation of Aspergillus spp.

The main reason for the prevalence of these fungi is their ability to tolerate and live in a wide zone of temperatures. Much research has investigated contamination by *Aspergillus* in animal feed. Most findings concluded that *A. flavus* was the most frequent fungi, followed by other species of *Aspergillus*. No research has been published about the prevalence of *Aspergillus spp.* in cage floors, cow udder, milker hands, milk cans, hands of cheese workers, and cheese mold.

Among all isolated fungi, A. *flavus* was the most dominant, followed by A. *niger,* A. *terreus,* and A. *fumigatus*, all of which are known as mycotoxigenic fungi. Udder of cows and cage floors were highly contaminated, explaining that the floor is not properly cleaned, and when the cows slumber on the floor, the fungi transmit from one cow to another. On the other hand, the owners neglected the importance of hygiene and cleaning practices. Therefore, it is essential to practice safety systems to minimize contamination. We took samples from the milkers' hands immediately after milking, which could suggest that the fungi spread from the cow's udder to the hands or vice versa. The problem is

Isolation of Aspergillus spp.

Table 2.
Isolation and distribution of *Aspergillus* species in different swab *samplesregency*, Indonesia (N = 13)

Source of samples	No. of samples	*Aspergillus spp.*				Total
		A. flavus	*A. fumigatus*	*A. niger*	*A. terreus*	
Water	12	3	0	1	1	5
Cage floor	12	*4*	*0*	*2*	*2*	*8*
Cow udder	38	5	3	3	1	12
Milker hand	12	2	1	1	1	5
Milk cans	12	2	1	0	1	4
Cheese worker hand	12	1	1	1	0	3
Coconut mold	12	0	0	0	1	1
Banana leaf	12	0	0	1	0	1
Total	**122**	**17**	**6 (4.91%)**	**9**	**7 (5.74%)**	**39 (31.97 %)**

Figure 1.
Collection of samples in farm sites

that these fungi transmit contamination to milk and milk products directly or indirectly, and cause adverse health impacts on workers' and animals' health regularly inside farms [17]. In a study, these findings disagreed with the present results. The results of another study on the prevalence of *Aspergillus* in well water in dairy farms revealed that water was contaminated by A. *flavus*, A. *niger*, and A. *terreus*. These results were similar to our findings, and this similarity may be due to environmental conditions in the two countries.

Aspergillus was isolated from animal feeds in Indonesia in another place in Bogor, West Java. The results demonstrated the presence of A. *flavus* in feed samples [18]. Another study was conducted on *Aspergillus* in agricultural commodities in Indonesia, and their results revealed toxigenic *Aspergillus* [19]. Natural weather conditions, including high humidity, rainfall, and moderate temperature in Indonesia, support the growth of fungi, especially *Aspergillus* spp. In South Sulawesi, there was no previous study on the isolation of fungi from the feed of dairy farms or farm environment, making this study the first research in this area, which has considerable dairy farms. Investigations showed no contamination in the majority of roughage feed samples, with only one sample (8.33%) being positive for AFB1 and the mean of contamination being 7.32 µg/kg (Table 3), which is above legal limits set by the European Union (5 µg/kg). However, it is lower than the National Indonesian Standard (50 µg/kg). The present results supported those obtained in

Table 3.
Levels of AFB1 contamination in different animal feed types

	Animal feed type				
	Elephant grass	Rice bran	Soya by-products	Commercial concentrate	Palm oil cake
AFs (µg/kg)	7.32	32.81	-	44.08	6.3
EU limits 20 (µg/kg)	LOD	EOD	LOD	EOD	*LOD*
SNI 200 (µg/kg)	LOD	LOD	LOD	LOD	LOD

LOD: Low Limits of Detection, LOD is low in terms of the detection limits. EOD exceeds the limits of detection

Ethiopia, which reported that about 52% of feed samples were above EU limits. On the other hand, the findings here partially agreed with those obtained in Italy, showing a lower degree of AFB1 contamination [20].

However, the owners did not store roughage feed. They introduced it directly from the field to the animals, which explains that the fungi grow on feed in the field, not during storage. Mold growth needs suitable humidity and temperature. To prevent mold growth and contamination, we should focus on the natural and chemical methods that reduce the multiplication of fungi in the field during harvest, transportation, and storage.

Concentrated feeds in this study revealed low and high AFB1 contamination levels of 6.3 and 44.08 µg/kg, respectively. These incidents are different based on concentrated feed type. Palm oil cake showed low incidents, while rice bran and commercial concentrated feed showed high levels. High AFB1 in concentrate can be attributed to the proven and scientific facts that concentrated feed has high levels of fat, carbohydrate, and protein favorable for Aspergillus species multiplication and aflatoxin production [12]. These findings align with another study (Omeiza et al. 2018) in which the authors detected AFB1 in animal feeds with a concertation range of 10-20 µg/kg. Many factors can play a role in the contamination of dairy cattle feed, such as the type of feed, feed processing, storage and handling, geographic conditions, and owners' awareness of the risk of aflatoxin. The owners in the present study area had no idea about aflatoxins. Therefore, the lack of sufficient knowledge of aflatoxins might lead to their high occurrence in the feed of animals besides milk and milk products [16].

Regarding the analysis of the factors associated with aflatoxins, many factors showed a

Table 4.
Logistic regression analysis of factors associated with aflatoxins

Factors	AFs			
	≤ 5 µg/kg	≥ 5 µg/kg	OR	P value
Level of education				
Primary	1	0		
Secondary	0	5	0.0	0.99
University	0	1		
Gender				
Female	0	2		
Male	0	0	0.0	0.99
Type of roughage feed				
Elephant grass	0	0	0.0	0.99
Type of concentrated feed				
Rice bran	7	3		
Soya by-products	1	0		
Commercial concentrate	0	2	69.95	0.99
Palm oil cake	0	1		
Feed Storage				
On floor	1	2		
On Special place	3	7	0.0	1.00

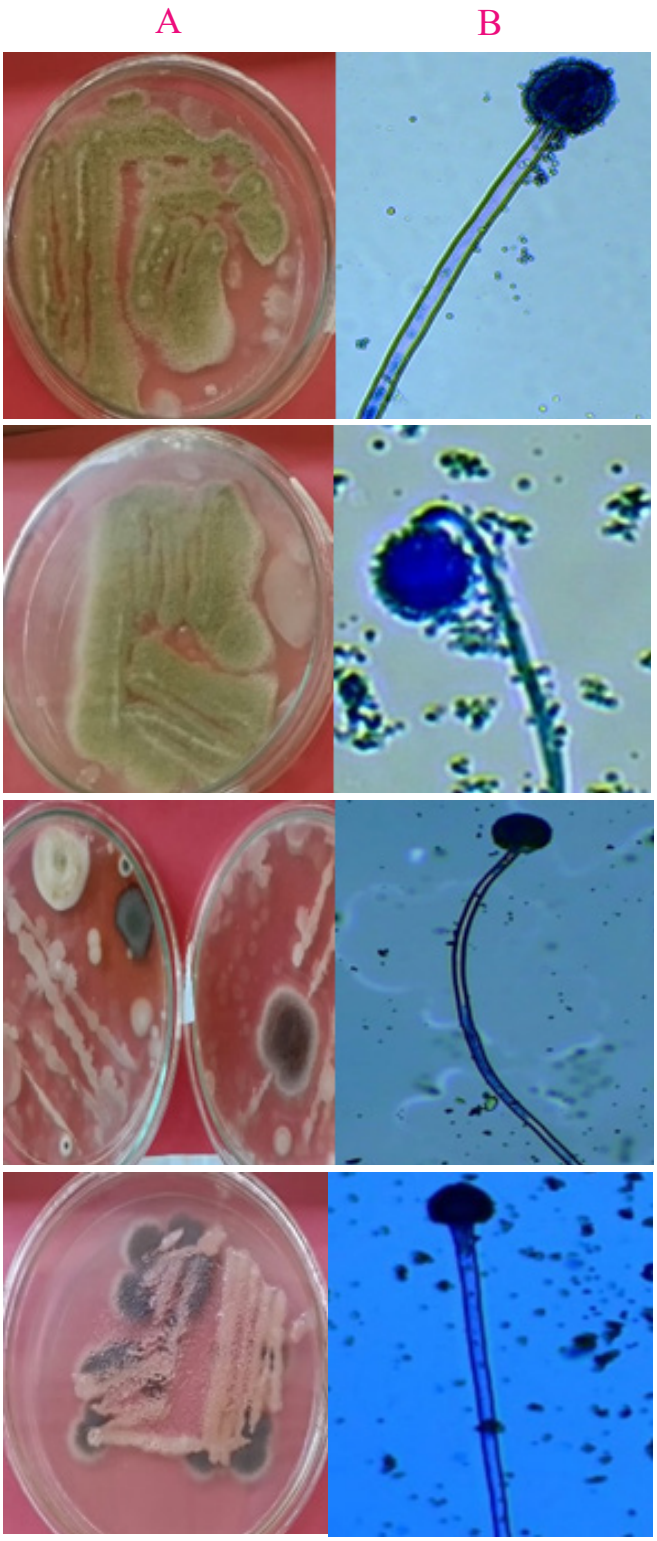

Figure 2.
(A) macroscopic characters and (B) microscopic characters of *Aspergillus spp.*

strong relationship with aflatoxin. This study showed that only concentrated feed positively impacts aflatoxin, and its aflatoxin content is about six times more than roughage (Table 4). These results partially support those who reported that concentrated feed has increased aflatoxin seven times more and disagree with those regarding education level, gender, feed storage, and type of roughages. None of these factors had a positive effect on aflatoxin content.

Aflatoxin AFM1 was found in 69.2% of raw milk samples with contamination levels from 6.14 to 10.02 µg/kg with a mean of 7.31 µg/kg. These results disagree with those recorded in Albania, which showed about 0.022 to 1 µg/kg of AFM1 in milk [21]. A study in the Amazon region found AFM1 of about 0.06 µg/kg, which is less than our results [22]. These differences are related to feed, weather, and animal physiological status as the concentration of AFM1 in milk was higher during the early lactation period and decreased during the late lactation stage in dairy cows. All of the tested local cheese Dangke samples were free from aflatoxin AFM1, which might be due to the method of cheese processing that uses the natural plant enzyme papain extracted from the papaya tree as a coagulant. This finding disagrees with many researchers reporting the presence of AFM1 in different kinds of cheese fields [23, 24].

Regarding HACCP, these results identified many critical points and risks inside the farm that affect the quality of milk and, subsequently, the health of both animals and humans. Cows' udder contained high *Aspergillus spp*. followed by cage floor, milker hands, milk cans, and cheese worker hands. All of these are critical control points, and it is possible to control them to minimize the magnitude of biological and chemical hazard risks in the food supply chain and ensure food safety by implementing good management practices in dairy farms. The point is that milk quality starts from milking until the final product. Many studies have been conducted on implementing the HACCP program in dairy companies and dairy products [25].

In conclusion, the results of the present study indicated the high contamination of the farm environment by mycotoxigenic fungi, especially in cow's udder, cage floor, water, milker hands, milk cans, and cheese worker hands, especially *Aspergillus spp.* Considerable levels of contamination by aflatoxins AFB1 and AFM1 in animal feeds and milk were recorded. We found that concentrated feed type was significantly associated with high aflatoxin contamination levels. All positive feed and milk samples are subject to Indonesian legislation. It is essential to focus on implementing good practices for feed production from the field during cultivation, harvest, transport, processing, storage, and feeding procedures.

The farmers should be trained and educated about the health risks of aflatoxin for their animals and humans, and how to control and manage by implanting the HACCP program, in addition

to adopting hygiene practices and cleaning milker hands, farm floor, and cow udder and sanitation before and after milking to reduce contamination levels and produce clean milk. Therefore, further research and investigations are needed on aflatoxigenic fungi in dairy farms and their feed and produced milk to provide a more comprehensive approach to one health program strategy.

Materials and Methods

Study area

The study areas were intentionally chosen to serve the research aims. The site was located about 1300 m above sea level, with a day-to-day typical temperature of approximately 27°C-34°C. The climate of this area is a tropical rainstorm described by the rainy season from November to June and the dry season from July to November. Dairy cows are mostly kept in a zero-grazing system called 'stall feeding,' and are supplemented with concentrated feeds. The expected milk production in this area was estimated to be around 10-15 liter/head/day.

Sampling

Thirty-nine raw milk samples and six local cheese samples were collected from smallholder farms from August to September 2023. Simultaneously, different types of concentrated and roughage feeds were collected, from which the roughage (N = 12), commercial concentrate (N = 2), soya bean (N = 4), palm oil meal (N = 1), and rice bran (N = 9).

At the same time, a total of 122 swabs from water, cage floor, milker hands, cow udders, milk cans, cheese maker hands, coconut mold, and banana leaf samples were collected (Figure 1). The samples were kept in a cool ice box at 4°C, transported to the laboratory, and stored until analysis. A structured questionnaire was used to assess farmers' knowledge, their practices of animal feeds, and farmers' experience with aflatoxin and fungi in feed, in addition to animal feed handling and storage. The samples were taken from all farms around the research area, and the results were represented and generalized to all communities there.

Fungal analysis

Equipment and selected media were correctly autoclaved before use. Culturing and isolation of the swabs were completed in sanitized conditions, and laboratory windows and doors were kept shut. Two plates of PDA were utilized for each swab sample; subsequently, the media was placed in 9 cm plates and left to solidify at room temperature. Each swab sample was spread on the surface of the plates in duplicate, and each plate was continuously labeled with the code name of the farm from which the swab was taken and the swab name. After the culture process, the dishes were incubated at 30^{0}C for 1-4 days, and until the third day, changes were noted and recorded each day.

Controls were prepared using two sterile PDA dishes, which were used to test the general conditions and environment of the laboratory. *Aspergillus* species were identified based on their colonial morphology and colony color after incubation. As described, the microscopic characteristics of isolates were examined using the lactophenol cotton blue staining [25]. One drop of the dye was placed on a prepared slide, and a small piece of the culture was taken and set in the decline of the dye using a mounting needle. The same needle was used to spread the culture. A cover slip was then delicately and gently put on the spread culture with delicate pressure to remove air bubbles. Afterward, the slide was mounted and observed under the ×40 objective lens. Identifying *Aspergillus spp.* depends on septate hyphae and rough and colorless conidiophores that end in vesicles with the whole surface covered with either uni- or biseriate sterigmata.

Isolation of aflatoxins B1 and M1 from animal feed, milk, and cheese

The samples were analyzed for AFB1 and AFM1 in animal feeds, milk, and cheese using a specific ELISA kit (Romer Labs, AgraQuant total Aflatoxin, Austria). Five mL from each raw milk sample was incubated for 30 min at 4°C and centrifuged at 3000 g for 10 min. Next, the serum of milk under the fat layer was taken and then immediately assayed for AFM1 using a specific ELISA kit. A volume of 5 g of ground samples of cheese and feeds (roughage and concentrated) was taken separately in a clean pitcher, and 25 ml of 70% methanol extraction solution (extraction ratio of 1:5 of sample to extraction) was added. Raw milk samples were prepared as described above.

Analysis of aflatoxins B1 and M1 in samples by competitive ELISA

All samples were analyzed for AFB1 and AFM1 in animal feeds, milk, and cheese using a specific ELISA kit (Romer Labs, AgraQuant total Aflatoxin, Austria). The maximum and minimum amounts were 4 and 40 ppb, respectively, for AFM1 and AFB1 with high specificity and sensitivity. The kit materials were stored at 2°C-8°C. Before starting the test, the materials were incubated for 1 h at room temperature. The kit test materials were used according to the manufacturer's instructions: About 200 µL of the conjugate solution was pipetted and moved into the dilution wells (supplied with the kit). Next, the samples (100 µL) were pipetted into all dilution wells (100 µL/well/sample). Standard samples were pipetted in duplicate (100 µL/well/standard). The solution was mixed well, 100 µL was moved from the dilution wells into antibody-coated wells, and the plate containing the samples was incubated at room temperature for 15 min. The unbound conjugate was washed using a washing solution five times (supplied with the kit). After the washing step, the washed wells were gently dried.

The aflatoxin substrate solution was added to the antibody-coated wells, and the plate was incubated again at room temperature for 5 min. The reaction was allowed to proceed in the dark, at the end of which a blue color developed. The reaction was stopped by adding 100 µL stop solution to the antibody-coated wells, and the color changed from blue to yellow. The absorbance was measured at 450 nm with a differential filter at 630 nm using an ELISA plate reader, and the absorption intensity was found to be inversely proportional to the aflatoxin concentration in samples. The aflatoxin soft worksheet program supplied with the kit was used to calculate the AFB1 and M1 concentrations in the samples.

Statistical analysis

Data were expressed as mean ± standard deviation by descriptive statistics, and the feed samples were calculated as a percentage using the SPSS software (version 26). In addition, logistic regression analysis was conducted, and an odd ratio with 95% confidence intervals was used to test the relationship between predictors and expected or outcome variables. Differences were considered statistically significant at $p < 0.05$.

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Authors' Contributions

S.Y.M.S., and R.M. conceptualize the manuscript. S.Y.M.S., and R.M. wrote the original draft. S.Y.M.S., R.M., S.B., J.M., and R.W.K. revised the original draft. S.Y.M.S., R.M., S.B., J.M., and R.W.K. wrote, reviewed, and edited the manuscript. R.M. per-formed supervision. All authors read and approved the final manuscript for publication.

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Competing Interests

The authors declare that they have no conflict of interest.

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