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Identification of molds producing aflatoxin from raw materials of free-range chicken feed in East Nusa Tenggara, Indonesia

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Abstract. *Lengur EAR, Daten H, Nadut A, Lalong PRF. 2024. Identification of molds producing aflatoxin from raw materials of free-range chicken feed in East Nusa Tenggara, Indonesia. Biodiversitas 25: 1588-1595.* The assessment of aflatoxin is an important determinant of animal feed quality. This study aimed to isolate, characterize, and identify molds responsible for aflatoxin production within raw materials used in free-range chicken feed. The isolation process follows the standard "pour-plate method", with subsequent molecular characterization and sequencing using the Internal Transcribed Spacer (ITS) gene. Results indicated the highest concentration of mold cells in fish meal and moringa leaves, reaching 4.06 log CFU/g. Isolate KIJK1 exclusively appeared in fish meal, cornmeal, and moringa leaf meal, while isolate KIJKD1 was present in fish meal, cornmeal, moringa leaves, and bran meal. The colony morphology of isolate KIJK1 exhibited gray coloring with white edges, whereas, at five days old, isolate KIJKD1 displayed a green center with white edges. Isolate KIJK1 was further identified as *Aspergillus fijiensis* strain IHEM 18675, and KIJKD1 as *Aspergillus flavus* strain 2011F7, with both acknowledged aflatoxin producers. Mold contamination incidence hinges on substrate type, production processes, storage conditions, and nutritional composition. Consequently, periodic mold identification is recommended to maintain animal feed quality control.

Keywords: Aflatoxin, *Aspergillus*, feed raw material, free-range chicken, mold

INTRODUCTION

Free-range chickens are a common commodity in Indonesia, with East Nusa Tenggara being one of the provinces extensively engaged in rearing. According to the Central Statistics Agency of East Nusa Tenggara Province, the production of free-range chickens reached 10,191,289 individuals. This resulted in a total chicken meat production of 10,986,209.85 kg and a free-range chicken egg production of 9,416,751 by the end of 2022 (BPS 2023a; BPS 2023b; BPS 2023c). The number of free-range chickens in the last three years (2020-2022) has consistently increased, contributing to the overall surge in chicken meat and egg production. An effective husbandry system should support the quantity and quality of free-range chicken production. One crucial aspect of support being the provision of adequate chicken feed (Heines et al. 2022; Medina-Cruz et al. 2024).

The primary energy source required to ensure the survival of free-range chicken is their feed, which should contain sufficient concentrations of nutrients. Good chicken feed should adhere to specific quality standards, including a maximum water content of 13%, a maximum ash content of 9%, a minimum protein content of 20%, a minimum crude fat content of 4%, a maximum crude fiber content of 5%, a calcium content of approximately 0.70 to 1.20%, a minimum total phosphorus content of 0.50%, a minimum lysine amino acid content of 1.20%, and a minimum methionine content of 0.45% (INS 2023a). Meanwhile, specifications for late-stage chicken feed, the specifications include a maximum water content of 13%, a maximum ash content of 9%, a minimum protein content of 19%, a minimum crude fat content of 4%, a maximum crude fiber content of 6%, a calcium content between 0.60-1.10%, a minimum total phosphorus content of 0.45%, a maximum aflatoxin of 50 µg/kg, a minimum lysine amino acid content of 1.05%, and a minimum methionine content of 0.40% (INS 2023b). High-quality chicken feed is derived from equally high-quality raw materials.

The raw materials commonly used to make chicken feed usually come from grains, leaves, and larvae. These ingredients include cornmeal, bran, fish, moringa leaves, and maggots. Raw materials with high nutritional value are also susceptible to mold contamination, which can significantly alter their nutritional composition and functionality. Mold can originate from the production process, including planting, harvesting, drying, storage, transportation, and marketing. Food commodities affected by decay are vulnerable to mycotoxin contamination with environmental support. Mycotoxins are toxic secondary metabolites produced by molds, posing a danger to humans and animals. These metabolites form the mold's response to various environmental signals for adaptation (Xue et al. 2022; Boudjaber et al. 2023). Mycotoxin, such as aflatoxin,

was used as a parameter for food quality due to its negative impact on health and the economy. For instance, the type Aflatoxin B1 (AFB1) is a natural carcinogen that can cause liver cancer (Kiran et al. 2016; Mukhtar et al. 2020; Boudjaber et al. 2023). The International Agency for Research classifies this group on Cancer (IARC) in Group 1, which includes carcinogenic molecules to humans and animals. Some examples of molds that produce aflatoxins include *Aspergillus flavus, Aspergillus niger, Penicillium verrucosum,* and *Fusarium* sp. (Kiran et al. 2016; Twaruzek et al. 2021; Boudjaber et al. 2023). One solution to this issue is implementing maximum tolerable standards in specific food categories.

Following the Indonesian National Standard (INS), the aflatoxin content serves as a key quality parameter for both raw materials and chicken feed. The maximum aflatoxin content starter and finisher chicken feed is 50 µg/kg (INS 8173-2 2023; INS 8173-2 2023). Exceeding these aflatoxin levels does not comply with the INS standard. Previous research only determined the type and amount of aflatoxin without knowing the species of mold (den Hollander et al. 2015). Subsequently, this research provides valuable data on the number and species of molds in raw chicken feed materials. This information facilitates the early prevention and inhibition of contamination by aflatoxin-producing molds, which are dangerous for chickens and result in economic losses for the poultry industry. This method demands a substantial investment of time and resources and yields specific information about the mold species responsible for aflatoxin production. Hence, the research aimed to isolate, characterize, and identify aflatoxin-producing molds from raw chicken feed ingredients. Identifying the specific mold species can facilitate the implementation of mold growth inhibition based on its type.

MATERIALS AND METHODS

Collection of raw feed material

The raw materials for free-range chicken feed include fish meal, moringa leaves, corn, bran, and maggots. Fish meal, moringa leaves, bran, and corn were collected from Kupang City in East Nusa Tenggara Province, and maggots from Bandung in West Java. Moringa leaf powder is made by drying it in the sunlight and grinding it using a milling machine. Meanwhile, bran, maggots, and corn were also ground before being used for mold isolation.

Isolation and characterization of mold

The mold isolation was conducted using the pour-plate method. A total of 25 grams of the sample (fish meal, moringa leaves, corn, bran, and maggots) were added to 225 ml of 0.85% NaCl. Subsequently, serial dilution was performed from 10^{-1} to 10^{-4}. One milliliter of the sample and Potato Dextrose Agar (PDA) medium containing 1% chloramphenicol antibiotic (30 mg/ml) were poured, following the method described by Gutleb et al. (2015) and Ollinger et al. (2020). The petri dishes were then incubated at 30°C for 4-7 days. Mold colonies were analyzed for colony size and physical characterization. The developed colonies were purified and observed under a microscope at 400x magnification. Morphological observations were performed, and lactophenol cotton blue was used as a staining agent for conidiophores following the method outlined by Omeiza et al. (2018).

Isolation, amplification, and sequencing of DNA

The DNA from the mold was extracted using the Tiangen Kit, following the instructions provided by the manufacturer. The isolated DNA was amplified using a total volume of 15 µL, comprising 2.5 µL of PCR Master Mix Nexpro®, 2.5 µL of DNA template sample (100 ng/µL), 7.5 µL of distilled water, and 2.5 µL of primers (10 pmol for each forward and reverse primer). The primers used were ITS1 (5'TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') as outlined by Mohamed et al. (2020) and Zubairi et al. (2021). The amplification protocol involved an initial pre-denaturation step at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 45 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 45 seconds. Subsequently, a post-elongation process was carried out at 72°C for 7 minutes. The PCR products are then subjected to gel electrophoresis on a 1% agarose gel (Iheanacho et al. 2014). The DNA sequences obtained from the PCR are determined through sequencing services provided by 1st BASE of Apical Scientific Sdn. Bhd. (Malaysia). Sequence identification is performed using the BLAST tool at the National Center for Biotechnology Information (NCBI). Subsequent, sequence alignment was carried out using the muscle algorithm, and a phylogenetic tree was constructed using the Neighbor-Joining method with the maximum composite likelihood substitution model in The Molecular Evolutionary Genetics Analysis (MEGA) version 11 software (Tamura et al. 2021; Zubairi et al. 2021; Arreguin-Perez et al. 2023).

Statistical analysis

All experiments were performed in triplicate, and the values were represented by the mean ± SD. The results were analyzed by a one-way ANOVA test with SPSS version 23 software. The level of significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

The mold cell size in feed raw materials

The amount of mold cells can be seen in each feed composition illustrated in Figure 1. The cell size of mold in fish meal is 4.06 log CFU/g, bran meal is 3.90 CFU/g, moringa leaf powder is 4.06 CFU/g, cornmeal is 1.23 CFU/g, and maggots have no mold content. The cell size of the mold was also compared with that of raw materials. Some raw materials exhibit differences from each other. Here are the differences: The cell size of maggots was significantly different from that of cornmeal, moringa leaves, fish meal, and bran meal ($0.001<0.05$). Meanwhile, the cell sizes of fish meal, moringa leaves, and bran meal are not significantly different ($1>0.05$). Furthermore, the

cell sizes of mold in the cornmeal and maggots were not significantly different (Figure 1).

Colony and cell morphological characterization of the isolate

The KIJK1 isolate was exclusively identified in fish meal, corn, and moringa leaves, while the KIJKD1 isolate was present in fish meal, corn, moringa leaves, and bran. The colony morphology of KIJK1 and KIJKD2 differs. The KIJK1 colony exhibits a gray coloration with white edges, while the KIJKD1 colony is characterized by a green center with white edges. Despite these differences, both isolates share similar cell morphology, featuring metulae, phialides, vesicles, conidia, stipes, and unbranched hyphae, as detailed in Table 1.

Utilizing ITS gene sequencing for mold identification

Isolates KIJK1 and KIJKD1 were identified based on both phenotypic and phylogenetic characteristics. The internal transcribed spacer (ITS) locus is the most reliable region for identifying strains at the species level. This locus is an alternative, accurate molecular method for identifying *Aspergillus* strains up to the species level (Yan et al. 2022). Phylogenetic analysis revealed that isolate KIJK1 matched *Aspergillus fijiensis* strain IHEM 18675 with 99.83% similarity, while isolate KIJKD1 was identified as *Aspergillus flavus* strain 2011F7, also with 99.90% similarity (Figure 2). Although the colony morphology of the two isolates differs, their cell shapes exhibit similarities.

Discussion

Based on previous research, the mold cell size in corn ranges from 570-620 colonies/gram (Wen et al. 2021). Additionally, other studies mention mold counts in corn ranging from 4.3×10^2 to 6.2×10^5 cfu/m^3 and 46.7×10^3 cfu/g to 203.3×10^3 cfu/g. Interestingly, in the current study, the mold count in the raw materials of chicken feed was lower than that reported in previous studies (Wen et al. 2021). Moreover, there is no mold in maggots. Meanwhile, there is limited research on the detected mold counts in fish meal, moringa leaves, bran meal, and maggots. However, mold counts in dried fish products range from 1.07×10^2 to 4.58×10^4 cfu/g (Deng et al. 2020; Siyame et al. 2021). Mold contamination occurs during the processing of fish meal. Fish processed outdoors with exposure to sunlight during drying can lead to contamination by bacteria, molds, insects, and rodents. Additionally, contamination occurs due to unhygienic handling and the poor quality of equipment used in fish meal processing (Deng et al. 2020). Mold contamination in bran flour and moringa leaves also results from unhygienic drying and milling processes.

The mold found in raw materials is a facultative microorganism that can survive in tropical areas under various environmental conditions. This mold can survive from 28 to 37°C, with high relative humidity of about 95%. The mold growth temperature of 30°C was suitable for this research. Mold takes nutrients from substrates rich in carbohydrates and proteins, such as corn, legumes, bran, fish meal, and products from these materials. The mold also has the potential to produce secondary metabolites such as aflatoxin B1 and aflatoxin B2, aspergillic acid, nitropropionic acid, and kojic acid. However, only about 40-50% of strains are pathogenic. Subsequently, it is necessary to distinguish between toxic and non-toxic strains (Okayo et al. 2020).

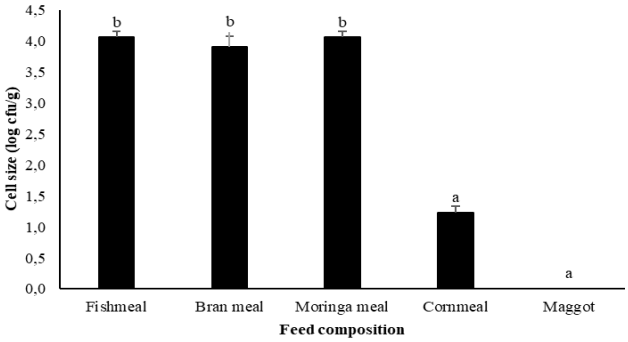


Figure 1. Cell size of aflatoxin-producing mold in raw materials for chicken feed (cfu/g). Note: The notation (a or b) describes the significant difference in each raw material (0.001<0.05)

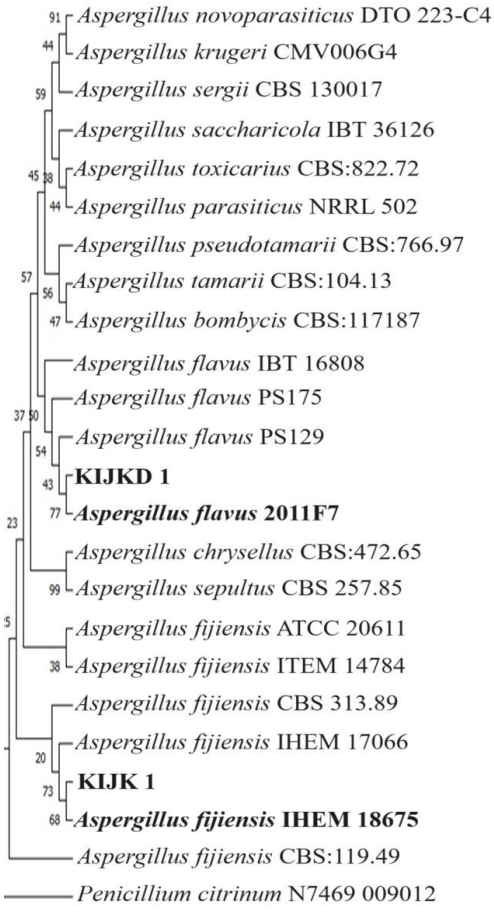
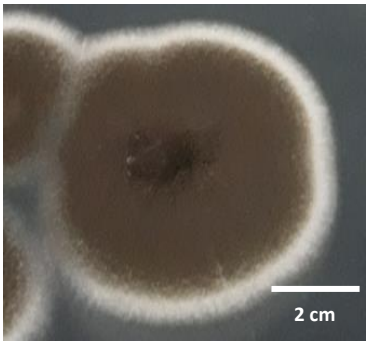
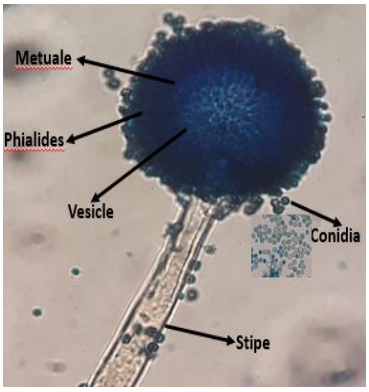
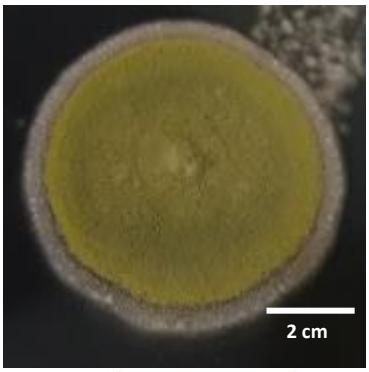
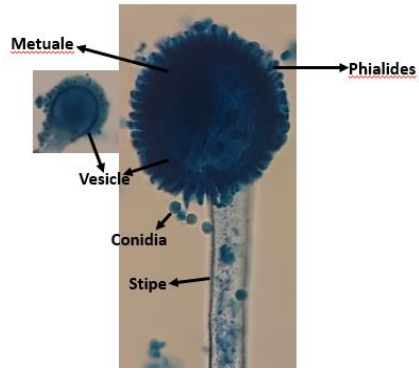


Figure 2. Neighbor-joining phylogenetic tree of the ITS sequences of the isolated fungal strains with the sequences of closely related strains. *Penicillium citrinum* N7469 009012 as the outgroup. The percentage of replicate trees in which the associated taxa clustered in the bootstrap test (1000 replicates)

Table 1. Characteristics of colony and cell morphology of mold from feed raw materials

Isolate	Characters	Description	Picture	Source
KIJK 1	**Macroscopic characterization:**			Fishmeal, cornmeal, moringa meal
	Colony size	The diameter ranges from 1.5 to 7 cm of 5-day-old culture		
	Colony growth pattern	Circular colonies: Growth in concentric circles		
	Colony texture	Flat and velvety		
	Colony color (top)	The texture is initially colorless and slimy, forming around the inoculum. It changes to white at the edges and gray in the center.		
	Colony color (bottom)	The color is gray to black, saturating with aging.		
	Microscopic characterization:			
		The characteristics include unbranched hyphae, upright conidiophores, the presence of a stipe, oval-shaped metulae, oval-shaped phialides, oval-shaped vesicles, and round conidia. Conidiophores ranged from 800 to 1200 µm, and vesicles ranged from 1800 to 2000 µm, conidia ranged from 250 to 450 µm, and hyphae 800 to 900 µm		
KIJKD 1	**Macroscopic characterization:**			Fishmeal, cornmeal, bran meal, moringa meal
	Colony size	The diameter ranges from 2 to 8 cm of 5-day-old culture		
	Colony growth pattern	Circular colonies: Growth in concentric circles		
	Colony texture	Flat and velvety		
	Colony color (top)	The texture is initially colorless and slimy, forming around the inoculum. It changes to white at the edges and green in the center.		
	Colony color (bottom)	The color is white to cream, and it saturates with aging.		
	Microscopic characterization			
		The characteristics include unbranched hyphae, upright conidiophores, the presence of a stipe, oval-shaped metulae, oval-shaped phialides, oval-shaped vesicles, and round conidia. Conidiophores ranged from 800 to 1200 µm, and vesicles ranged from 1800 to 2000 µm, conidia ranged from 250 to 450 µm, and hyphae 1000 to 1100µm		

Mold contamination depends on various conditions, such as substrate type, harvest period, storage methods, and the chemical nature of the substrate (Kedia et al. 2014). Additionally, moisture content and pH are crucial factors influencing the number of microorganisms and their reproduction in raw feed materials (Suleiman et al. 2013; Kedia et al. 2014). In this study, the mold isolates' cell characteristics and species were the same as in previous studies. Based on morphological characteristics, the KIJK 1 and KIJKD 1 isolates belong to the *Aspergillus* group. This genus is widely distributed in nature, especially in tropical climates. The *Aspergillus* group was identified based on

macroscopic and microscopic characteristics. Some macroscopic characters used for characterization include conidia, conidiophore, mycelial color, colony reverse color, colony diameter, exudate production, and sclerotia and cleistothecia formation. Meanwhile, microscopic character characterization includes vesicle shape, size, seriation, stipe, hulle cell formation, and cleistothecium. Previous studies have also found morphological characteristics similar to those of the KIJKD isolate (Thathana et al. 2017; Zulkifli and Zakaria 2017; Okayo et al. 2020). Mold sporulation begins after five days from the center and develops radially, covering the colony surface. The produced conidia are yellowish to green. As sporulation spreads outward, it forms a characteristic white line surrounding the sporulating mycelium. The white border eventually closes as the entire mycelium sporulates, producing more conidia by the tenth day. Along with the increasingly rapid growth of the colony, it becomes slightly raised as the mycelium accumulates, and its central part becomes clustered and rough (Odhiambo et al. 2013; Okayo et al. 2020; Khan et al. 2020).

Based on previous studies, the morphological characteristics of the KIJK isolate are that the fungal colony exhibits radial grooves, and the mycelium has a dense and smooth texture. The color of the mature colony gradually deepens, turning into an earthy gray. The colony diameter ranges around 8.37 cm after five days of incubation, and its sporulation reaches 3.86 x 10^8 conidia/mL after ten days of incubation. Conidiophores possess podia with straight-shaped, measuring 200-1100 µm x 8-16 µm, and enlarge into a round uniseriate vesicle with a 20-60 µm diameter at the top. The conidia are roughly ellipsoidal to somewhat fusiform with a 3-5 µm diameter and are connected in a chain (Yan et al. 2022). Based on the size and color of the mold colonies described by the previous researcher, they show similarities with the isolates found in this study. Morphologically, the colony diameter of isolate KIJKD 1 ranges from 2-8 cm, and isolate KIJK 1 ranges from 2-7 cm. Meanwhile, the colony color of isolate KIJK 1 is brown with white on the edges, while isolate KIJKD 1 is green with white on the edges (Table 1).

The two isolates were confirmed to belong to the genus *Aspergillus* by the presence of conidiophores, which is a primary characteristic of *Aspergillus*. However, the observed conidiophores in this study have a rough texture and are unbranched. Vesicles are found in subglobose to globose shapes with varying diameters, and sterigmata are biseriate or filiate that spread from all sides. The similarity in morphological characteristics among *Aspergillus* cells complicates the identification for researchers up to the species level, resulting in inaccurate identifications. Additionally, morphological identification methods are laborious, time-consuming, and require skilled microbiologists. Therefore, molecular approaches were considered a suitable strategy for mold identification at the species level (Fakruddin et al. 2015; Ren et al. 2020; Okayo et al. 2020; Rahimi et al. 2021).

Results from phylogenetic analyses that study the evolution of species according to their biological characteristics are frequently structured as phylogenetic trees. The tools prepared to construct a phylogeny tree for mold isolates are the target gene (ITS region), isolate DNA sequence, comparison sequence, and outgroup sequence. The outgroup sequence was *Penicillium citrinum* N7469 009012. The ITS (Internal Transcribed Spacer) region has been widely regarded as a universal fungi barcode marker for successfully identifying the broadest range of mold. Compared to 18S, ITS is more variable and, hence, more suitable as the genetic marker for measuring intraspecific genetic diversity. Sequencing results showed that the ITS gene were 549 bp (Yan et al. 2022). One of the most widely used methods for reconstructing them is the distance-based method known as the Neighbor-Joining (NJ). Neighbor-joining method is proposed for reconstructing phylogenetic trees from evolutionary distance data. The principle of this method is to find pairs of operational taxonomic units (OTUs [= neighbors]) that minimize the total branch length at each stage of clustering of OTUs starting with a starlike tree. The branch lengths, as well as the topology of a parsimonious tree, can quickly be obtained by using this method (Fernández et al. 2023). Based on phylogenetic analysis, the two isolates were identified as belonging to the *Aspergillus* group.

Aspergillus fijiensis (*A. fijiensis*) is one of the molds that causes bronchitis, along with invasive aspergillosis, and its colony characteristics include a gray color with white at the edges (Perrone et al. 2013). This mold typically produces concentrations of aflatoxin B1 below 30 µg/kg, and some strains, such as *A. fijiensis* GDIZM-1, are non-aflatoxigenic. Therefore, further testing of the mold strains in this study is needed to detect the types and concentrations of aflatoxins produced. This mold can be found in soil, water, and fresh fruits (Perrone et al. 2013; Hassan et al. 2019; Sheikh and Awad 2022). *Aspergillus fijiensis* also produces the enzymes endo-polygalacturonase and endo-1,3(4)-b-glucanase, which can pose food safety issues in large quantities. Both enzymes play a role in the depolymerization of pectic acid and the successive breakdown of cellulose. Additionally, this species also produces the enzyme β-fructofuranosidase. *Aspergillus fijiensis* can also reduce the proximate content of raw materials (Coetzee et al. 2020; Silano et al. 2022).

Aspergillus flavus (*A. flavus*) is a mold that can survive in various environments. It is saprophytic (Ling et al. 2022) and can contaminate food and feed commodities, reducing their quality and quantity (Zhang et al. 2023). This mold is classified as a Group 1 carcinogen by the International Agency for Research on Cancer, posing a threat to human and animal health and causing a decrease in economic value. *A. flavus* is a pathogenic group causing both invasive and non-invasive aspergillosis, leading to carcinogenic diseases, keratitis, respiratory and skin infections, and disruption of the immune system (Gao et al. 2023; Hu et al. 2023; Karami-Osboo et al. 2023). *A. flavus* produces various secondary metabolites, including the toxic and carcinogenic aflatoxins (AFs) and Aflatoxin B1 (AFB1), which are toxic compounds. If these compounds interact with the hepatitis B virus (HBV), it can increase the occurrence of chronic liver disease or hepatic cell carcinoma (HCC) (Pan et al. 2023).

Aflatoxin is one of the most common mycotoxins in corn, a crucial feed grain. Based on surveys of mycotoxin levels in feed samples and feed raw materials, almost 90% of samples contain at least one type of mycotoxin, and 64% are contaminants with several mycotoxins (Pena et al. 2018; Gruber-Dorninger et al. 2019; Nasaruddin et al. 2021; Wen et al. 2021). Contamination by aflatoxin-producing molds causes the loss of nutritional value of feed raw materials, leading to acute diseases with high mortality rates, thus reducing animal productivity. The level of toxicity depends on the type of toxin present, dosage, exposure duration, and other factors such as the type of animal, age, and nutrition (Wen et al. 2021; Dhanamjayulu et al. 2023). Aflatoxin-producing molds usually do not reach levels high enough to cause diseases but can disrupt various metabolic processes. Consumption of feed containing aflatoxin-producing molds supports the formation of free radicals and induces oxidative damage to DNA, proteins, and fats (Wen et al. 2021).

The toxigenic strains of *A. flavus* and *A. fijiensis* indicate that commodities are vulnerable to aflatoxin contamination if environmental conditions support it, such as humidity and pH (Kiran et al. 2016). Aflatoxin is an example of a mycotoxin. Mycotoxins are contaminant secondary metabolites produced by toxigenic mold strains. Among all known mycotoxins, Aflatoxin B1 (AFB1) produced by *Aspergillus flavus, Aspergillus fijiensis, Aspergillus parasiticus,* and *Aspergillus nomius* is the most toxic and classified as a Group 1 carcinogen for humans (Natarajan et al. 2022). Due to its thermos-stable nature and widespread occurrence, AFB1 contamination is considered an unavoidable food contaminant by the WHO. Furthermore, it has been reported that AFB1 contamination induces the formation of free radicals and lipid peroxidation. Free radical-mediated oxidation not only negatively impacts food quality but is also responsible for various chronic and degenerative health conditions such as cancer, aging, cardiovascular diseases, and neurodegenerative disorders (Devi et al. 2014; Omeiza et al. 2018; Abd El-Hack et al. 2023). The commonly produced levels of aflatoxin by *A. flavus* are above 50 µg/kg to 800 µg/kg (Yin et al. 2015; Hassan et al. 2017; Ogara et al. 2017; Wen et al. 2021; Natarajan et al. 2022), thereby not meeting the requirements of the Indonesian National Standard (INS) for free-range chicken feed. Therefore, the raw materials should not contain these two types of molds to obtain good-quality food (Omeiza et al. 2018; Li et al. 2021; Oduola et al. 2022). This research also demonstrates the importance of identifying mold species for preventing and controlling aflatoxin contamination, facilitating the search for potential targets for controlling aflatoxin-producing molds.

The feed raw materials used include fish meal, maggot, bran meal, cornmeal, and fish meal because these contain high nutrients. The nutritional content of cornmeal consists of a water content of 9.29%, an ash content of 1.37%, a crude fiber content of 7.46%, a crude fat content of 4.95%, a crude protein content of 8.63%, and a carbohydrates content of 68.30% (Siyame et al. 2021). The nutritional composition of bran includes a water content of 4.3%, a protein content of 17.50%, a fat content of 13.10%, a crude fiber content of 7.85%, a carbohydrates content of 52.33%, and an ash content of 4.92% (Park et al. 2017; Manzoor et al. 2023). The nutritional content of fishmeal includes a water content of 7.02%, a crude protein content of 50.5%, a crude fat content of 8.70%, an ash content of 10.10%, a lysine amino acid content of 0.70%, and a methionine content of 0.28% (Niu et al. 2019; Psofakis et al. 2020). The nutritional content of moringa flour includes a protein content of 27.10%, a fat content of 2.30%, a carbohydrates content of 38.20%, a fiber content of 19.20%, and an ash content of 10.38% (Gopalakrishnan et al. 2016; Sultana 2020; Gonzalez-Burgos et al. 2021). One of the raw materials added to chicken feed is maggots. Maggots are the larvae of the black soldier fly (*Hermetia illucens*). These maggots were fed with organic waste. Maggots are insect larvae with a high protein content. The nutritional content of maggots includes a crude protein content of 47.10%, a fat content of 25.30%, a crude fat content of 7.50%, an ash content of 6.25%, a water content of 86%, a fiber content of 5.89%, a lysine amino acid content of 6.04%, and a methionine content of 2.28% (Witono 2023). Based on this research, maggots can be used as a raw material for chicken feed because mold is not detected.

In summary, among all raw materials examined, fishmeal and moringa leaves are the most significant contributors to the aflatoxin-producing molds, whereas maggots were found to be mold-free. The aflatoxin-producing molds identified in these raw materials were KIJK1 and KIJKD1, which belong to the *Aspergillus* group. Both of these molds have different colony morphologies but relatively similar cell morphologies. The KIJK isolate was identified as *A. fijiensis* IHEM 18675, while KIJKD was identified as *A. flavus* 2011F7, both belonging to the group of aflatoxin-producing molds. A recommendation for future work is to periodically identify molds by randomly and continuously sampling to analyze and control aflatoxin-producing molds in free-range chicken feed. Additionally, further work is needed to inhibit aflatoxin-producing molds by implementing biological control methods such as using plant extracts and essential oils and applying probiotics to create an environmentally friendly and sustainable chicken farming environment.

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