

RESEARCH ARTICLE

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Occurrence, diversity and toxigenicity of fungi isolated from the udder of lactating cows

Fungal isolates from the udder of lactating cows

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Abstract

Objectives: This study examines toxigenic fungi associated with the udder of lactating cows.

Methods: The teats of forty-three ($n = 43$) lactating cows in Olupoona, Southwest Nigeria, were swabbed using labelled sterile cotton-tipped swabs. The swabs were transported to the laboratory, individually streaked on Potato Dextrose Agar (PDA) and incubated at 25 °C for 72 hours. Distinct fungal isolates were sub-cultured on fresh PDA and subjected to molecular identification and phylogenetic analysis. The occurrence of each fungal morphotype was computed. Potentially toxigenic isolates were analysed for Aflatoxin B₁ (AFTB₁) production, using a High-Performance Liquid Chromatography protocol. Student's T-test was used to evaluate for a significant difference in AFTB₁ production between toxigenic isolates.

Results: Culturable fungi were detected in 11 (25.6%) out of the 43 samples. A total of 5 distinct fungal isolates were obtained and identified as *Penicillium oxalicum*, *Penicillium citrinum*, *Fusarium brachygibbosum*, *Mucor irregularis* and *Curvularia lunata*. *Penicillium citrinum* was the most occurring isolate (11.6%) while *Mucor irregularis* and *Curvularia lunata* were the least occurring (2.3% each). AFTB₁ was detected in the extract of *Penicillium citrinum* and *Fusarium brachygibbosum*. The amount of AFTB₁ detected in the extract of *F. brachygibbosum* (0.22 ± 0.04 ppm) was significantly higher than that detected in the extract of *P. citrinum* (0.08 ± 0.04 ppm).

Conclusion: This study raises potential public health concerns and emphasises the need to take proper hygiene measures during the milking process.

Keywords: Aflatoxin, Cattle, Fungi, Mycotoxins, Milk

Plain English Summary

Milk is considered a complete food for humans from birth because it contains almost all the nutrients required for body growth, in addition to its good sensory properties. However, milk's neutral pH, high water activity, and high nutrient content, which includes proteins, lipids, vitamins, minerals, and essential amino acids, make it a perfect place for a variety of microbes to grow and contribute to some food-borne illnesses. Though it is believed that milk in healthy udder cells is sterile, microbial contaminants (e.g. bacteria, fungi, etc.) from a variety of sources, such as the teat apex, milking equipment, air, water, feed, grass, soil, and other environments, eventually colonise the milk.

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Due to poor hygienic procedures throughout the milking process and technique, toxic fungi are common and have been discovered in raw cow milk that has been taken from the udder. The study of fungal contamination of raw milk from cows is an important research focus as it reveals the possibility of contamination of fresh milk even from the source, including human activity, surfaces, air, unsanitary circumstances, and the area around the milking process.

It is also of great importance as it determines the health of consumers, resulting in serious illnesses. In this study, toxic fungi linked to breastfeeding cows' udders are investigated.

Background

Milk is widely recognised as a complete food, rich in proteins, lipids, vitamins, minerals, and essential amino acids that support growth and health from birth (1). However, its neutral pH, high moisture, and nutrient density also make it an excellent medium for microbial growth, posing challenges to food hygienists who must ensure milk safety without compromising its nutritional value (2). While milk is initially sterile in the udder, it can easily become contaminated by microorganisms from the teat surface, milking equipment, water, feed, soil, and farm environments (3, 4). Poor hygiene during milking further increases the risk of microbial contamination (5, 6).

Among contaminants of concern, fungi are particularly important because they produce mycotoxins, secondary metabolites with severe health implications (7). Mycotoxins such as those produced by *Aspergillus*, *Penicillium*, and *Fusarium* species can contaminate feed and subsequently appear in milk, where they persist even after processing (8, 9, 10, 11, 12, 13, 14, 15). Their toxic effects, including immunosuppression, carcinogenicity, genotoxicity, teratogenicity, and neurotoxicity, make them a major public health concern worldwide (16).

Aflatoxins are among the most dangerous mycotoxins, with Aflatoxin B1 (AFB1) being the most potent. Classified as hepatotoxic, mutagenic, and carcinogenic, even trace dietary exposure to AFB1 can result in chronic health problems in both humans and animals (17). In dairy cattle, ingestion of contaminated feed leads to the biotransformation of AFB1 into Aflatoxin M1 (AFM1), which is secreted in milk (9, 10, 11, 12, 18, 19). Aflatoxin M1 resists heat treatments such as pasteurisation and sterilisation, thereby reaching consumers and increasing food safety risks (13, 14, 15). Moreover, AFB1 and its metabolites impair animal health by damaging the liver, suppressing immunity, reducing reproductive efficiency, and lowering productivity, factors that also contribute to significant economic losses (20).

Of particular concern is the possibility that calves may be directly exposed during suckling when the udder or teat surface is contaminated with toxigenic fungi (21). This highlights the udder not only as a route of fungal colonisation but also as

a potential source of aflatoxin contamination. Despite the recognised risks of aflatoxin in milk (21, 22, 23, 24), there is a paucity of information on the presence of toxigenic fungi directly in the udders of lactating cows. This study, therefore, seeks to isolate and identify fungi present in the udders of lactating cows in Southwestern Nigeria and to determine their potential to produce AFB1. By addressing this knowledge gap, the research will provide insights into improving udder health, ensuring safer milk production, and reducing both public health risks and economic losses associated with aflatoxin contamination.

Materials and Methods

Collection of samples, transport and storage

Sampling was carried out by swabbing the teats of lactating cows ($n = 43$) in the Olupoona area, using sterile cotton-tipped swabs previously moistened with 0.1% peptone diluent (25). A swab was used to rub the teats of each cow and labelled immediately. The samples were thereafter transported to the laboratory under cold conditions (4°C) and maintained at the same temperature in a refrigerator until processing (no longer than 24h after collection) (26).

Media preparation and culturing

Potato dextrose agar (PDA) was prepared by first dissolving 39 g of PDA powder in 1000 ml of distilled water and then subsequently performing sterilisation by autoclaving at 15lbs (121 °C for 15 minutes). The sterilised agar was poured into sterile petri plates and allowed to solidify under sterile conditions. For culturing, the cotton tip of each swab was streaked on an individual PDA plate and then incubated at 25 °C for 72 hrs (27). Distinct fungal colonies on the plates were sub-cultured on new PDA plates to obtain pure cultures.

Molecular identification and phylogenetic analysis of fungal isolates: DNA extraction

About 100 mg of fungal mycelia was macerated in a sterile mortar by adding 1 ml of DNA Extraction Buffer (DEB) containing proteinase K (0.05 mg mL⁻¹), and the extract was transferred into a 1.5 ml Eppendorf tube. 50 µl of 20% Sodium Dodecyl Sulphate (SDS) was subsequently added and incubated in a water bath at 65 °C for 30 min. The tubes were then allowed to cool to room temperature. 100 µl of

7.5M potassium acetate was added hereafter and mixed briefly. This mixture was centrifuged at 13,000 rpm for 10 min. The supernatant was transferred into a new sterile Eppendorf tube, and two-thirds of cold Isopropanol was added. The tube was capped and then inverted 3-5 times gently before incubation at -20 °C for 1 hr. Afterwards, the mixture was centrifuged at 13,000 rpm for 10 min, and then the supernatant was discarded. 500 µl of cold 70% ethanol was added to the remaining sample/mixture in the tube, and centrifugation was carried out for 5 min at 13,000 rpm. The supernatant obtained was carefully discarded while retaining the DNA pellet intact, and then the DNA pellet was dried at 37 °C for 30 min and re-suspended in 50 µl of sterile distilled water. DNA quantity and quality (which ranged from 423.4 to 1141.3 ng/µl concentration and 260/280nm value of between 1.6 and 2.1) were assessed on a Nanodrop spectrophotometer. In addition, further Agarose gel electrophoresis was carried out to further confirm DNA quality. An equal volume of stock DNA was mixed with 4 µl of loading dye and run on 1% agarose gel at 100 V for 50 min to determine the integrity and quality of the DNA, as evidenced by the presence of 600 to 700 bp size bands.

Polymerase chain reaction (PCR) amplification. Amplification was carried out using a PCR system thermal cycler (Applied Biosystem Inc., USA). The PCR reaction mixture consisted of 10 µl of 5x GoTaq colorless reaction; 3 µl of 25 Mm MgCl₂; 1 µl of 10 mM of dNTPs mix; 1 µl of 10 pmol each of forward primer ITS 1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and reverse primer ITS2 (5'-GCTGCGTTCTTCATCGATGC-3'); 0.24 µl of 0.3 units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water and 8 µl working DNA template (28). The thermal cycling conditions were set to an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, and a final extension at 72°C for 10 min.

Purification of amplified PCR products. This was done following the method of Niu *et al.* (29) with slight modifications. The amplified DNA fragments of target sequences in PCR were ethanol-purified to remove the PCR reagents. Briefly, 7.6 µl of Na acetate 3M and 125 µl of 95% ethanol were added to each of about 50 µl PCR amplified products in a new sterile 1.5 µl tube Eppendorf, mixed by simple inversion and stored at -20 °C for 30 min. The tubes were centrifuged for 10 min at 13000 g, followed by the removal of the supernatant. The pellets were washed by adding 500 µl of 70% ethanol, and the mixture was then centrifuged for 15 min at 7500 g and

4°C. The pellet was re-washed with 70% ethanol and centrifuged again. Tubes containing the pellets were inverted on paper tissue until air dried, and then further incubated at 37°C for 25 min. Subsequently, they were re-suspended with 25 µl of sterile distilled water and kept at -20°C. The presence or absence of the expected band size of the amplified target ITS gene sequence was checked when the purified fragment was run on a 2% agarose electrophoresis gel at a voltage of 100V for 1 hr and then viewed under UV light. Sanger sequencing and analysis. The purified PCR products were subjected to Sanger sequencing using the following reaction mixture: Amplified PCR Product of 10 µl, ExoSAP Mix of 2.5 µl. The mixture was vortexed well and incubated at 37 °C for 30 min. Subsequently, the reaction was stopped by heating the mixture at 95 °C for 5 min. Fragments were sequenced using the Nimagen, Brilliant Dye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000 according to manufacturer's instructions: <https://www.nimagen.com/products/Sequencing/Capillary-Electrophoresis/BrilliantDye-Terminator-Cycle-Sequencing-Kit>. The labelled products are then cleaned with the ZR-96 DNA Sequencing Clean-up Kit (Catalogue No. D4053):

<http://www.zymoresearch.com/downloads/dl/file/id/52/d4052i.pdf>. The cleaned products were injected on the Applied Biosystems ABI 3500XL Genetic Analyser with a 50 cm array, using POP7:

<https://www.thermofisher.com/order/catalog/product/4406016>. Sequence chromatogram analysis was performed using BioEdit analysis software.

Molecular phylogenetic analysis. Contig sequences generated were aligned in Mega 11 software with available sequences of *Fusarium brachygybbum*, *Curvularia lunata*, *Penicillium citrinum*, *P. oxalicum* and *Mucor irregularis* in the GenBank database using the MUSCLE algorithm (30). The ITS1-5.8S-ITS2 of rRNA gene sequences obtained were trimmed to 450 bp. Maximum-likelihood (MLK) tree constructed using the Kimura 2 parameter model (31) selected using the Akaike information criterion (AIC), with 1000 bootstrap pseudo-replications.

Evaluation of toxin production by fungal isolates

Three of the fungal isolates (namely BUOLC8, BUSLC9 and BUSLC6) projected to belong to toxigenic genera, based on their morphological characteristics, were screened for AFTB₁ production.

Fungal culture in liquid medium

Using a sterile inoculating loop, the pure culture of each isolate (i.e., isolate BUOLC8, BUSLC9

and BUSLC6) growing on a PDA plate was inoculated into 150 ml Yeast Extract Sucrose (YES) media containing 2% yeast extract and 20% sucrose and 75 µg/ml Ampicillin in triplicate. Yeast Extract Sucrose medium is known to facilitate the production of aflatoxins (32). The inoculation was carried out under aseptic conditions and was achieved by picking a loop full of an isolate in a region with conidia, inserting it into the YES medium, and then gently agitating. Each of the flasks containing inoculated YES medium was capped/covered with a sterile cotton plug and incubated at 25 °C for a duration of 7 days (33).

Aflatoxin B1 extraction from fungal biomass
Extraction was carried out according to the method of Huq *et al.* (34). Briefly, 100 ml of distilled water was added to 50 g biomass (of each isolate). 25 ml of acetone was then added to produce an acetone-to-water ratio of 1:4. The mixture was shaken on a mechanical shaker for 30 min and then filtered through Whatman No. 1 filter paper on a conical flask. 10 ml of methanol and 1 ml of lead acetate were added to 10 ml of the filtrate samples of each isolate in 250 ml measuring cylinders, and the mixture was then made up to 150 ml with distilled water.

Extract purification and HPLC analysis of Aflatoxin B1

This was carried out according to the procedure of Hossain *et al.* (35) with slight modification. Briefly, nine SPE Cartridges were conditioned by passing 15 ml of methanol followed by 15 ml of water under vacuum after adding 1 g methanol-washed celite. Afterwards, the different 150 ml filtrate sample solutions prepared in the step above were each passed through separate cartridges under vacuum at the rate of 10 ml/min.

Each cartridge was washed with 10 ml of water and allowed to dry by allowing air to pass through for about 5 min. Exactly 4 ml of chloroform was added to each cartridge at the rate of 0.5 ml/min to elute Aflatoxin B1 present. The eluents were allowed to dry in air and then reconstituted with 1 ml of methanol and water (1:1) for HPLC analysis.

For HPLC analysis for the detection and quantification of AFTB1, an Agilent HPLC system equipped with series 1100 solvent delivery pumps and oven, 1200 series Fluorescence detector, manual injector and cobra cell for post-derivatisation, and Agilent ChemStation software. A C18, 250 mm (L) x 4.6 mm (ID) column was used for the isocratic separation of Aflatoxin B1, and the mobile phase consisted of 630 ml water, 220 ml methanol, 150 ml acetonitrile, and 120 µl of conc. HNO₃ and 100 mg of KBr. The injection volume and flow rate were set to 20 µl and 1 ml/min, respectively, while the total run time was 15 min. Also, the column oven temperature was set to 30 °C, while the excitation wavelength and emission wavelength were set to 365 nm and 464 nm, respectively. The concentration of Aflatoxin B1 in each sample was determined using a suitable five-point calibration curve (consisting of 0.01, 0.05, 0.10, 0.20, 0.30 and 0.40 ppb levels). The linear regression was 0.92. Concentrations of the Aflatoxin B1 standard used for the calibration were prepared in a 1:1 solution of methanol and distilled water.

Results

The diversity of fungi isolated from the udder of lactating cows in Oluponna was: *Curvularia lunata*; *Penicillium oxalicum*, *Penicillium citrinum*, *Fusarium brachygibbosum* and *Mucor irregularis* (Figure 1; A-E).

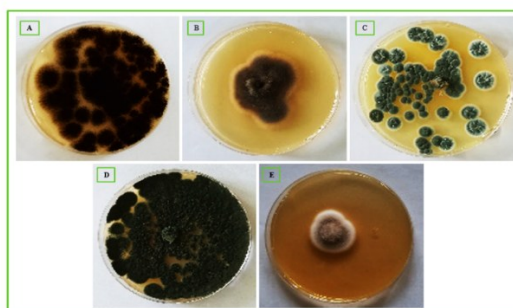


Figure 1: Fungal isolates from the udder of lactating cows

(A) *Fusarium brachygibbosum*; (B) *Curvularia lunata*; (C) *Penicillium citrinum*; (D) *Penicillium oxalicum*; (E) *Mucor irregularis*

The percentage occurrence of fungal isolates from the udder of lactating cows is presented in Figure 2. Out of 43 lactating cows sampled, 11 (26%) of them were positive for fungal growth, while the remaining 32 (74%) were negative. The

most prominent species was *Penicillium citrinum* (n = 5), followed by *Fusarium brachygibbosum* (n = 3), while *Curvularia lunata* (n = 1), *Penicillium oxalicum* (n = 1) and *Mucor irregularis* (n = 1) were the least frequent isolates.

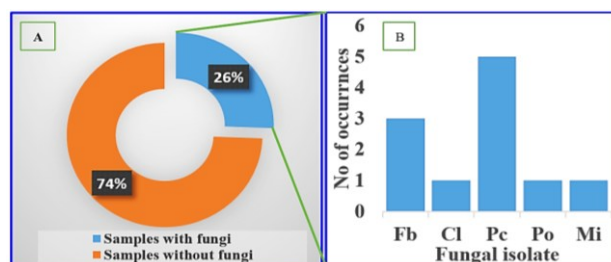


Figure 2: Occurrence of fungi in the udder of sampled lactating cows

(A): Prevalence in samples; (B): Frequency of the isolated fungi

Fb- *Fusarium brachygibbosum*, Cl -*Curvularia lunata*, Pc- *Penicillium citrinum*, Po-*Penicillium oxalicum*, Mi- *Mucor irregularis*

In Figure 3, the results of the phylogenetic relationship showed that the sequences of the isolates show high similarity to others. For example, *Penicillium oxalicum* BUSLC6

(OP801677), *Penicillium citrinum* BUSLC9 (OP801680) and *Mucor irregularis* BUOLC5 (OP801678) clustered with the ones from the Genbank database having 100% similarity.

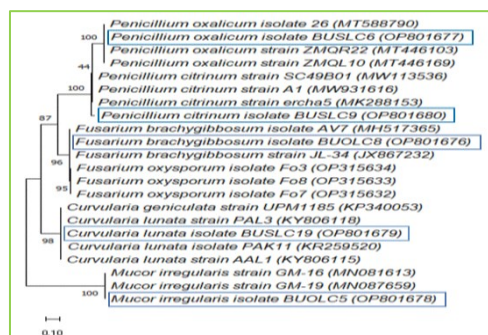


Figure 3: ITS phylogeny showing the relationship between isolated fungi and other related fungal species as indicated by maximum likelihood analysis

Fusarium brachygibbosum produced the highest concentrations of AFB1, while lower concentrations were found in *Penicillium*

citrinum, but none was found in *Penicillium oxalicum* (Table 1).

Table 1. Aflatoxin B1 was determined in the solvent extract of selected fungal samples.

	<i>Fusarium brachygibbosum</i>	<i>Penicillium citrinum</i>	<i>Penicillium oxalicum</i>
Aflatoxin B ₁ (ppm)	0.22 ± 0.04 ^a	0.08 ± 0.04	BDL ^b

^aSignificantly higher at $p \leq 0.05$; ^bBDL implies below the detection limit of 0.01ppm

Discussion

This study revealed a diversity of culturable fungi associated with the udders of lactating cows in Oluponna, Southwestern Nigeria, including *Fusarium brachygibbosum*, *Curvularia lunata*, *Penicillium citrinum*, *Penicillium oxalicum*, and *Mucor irregularis*. Although milk within the udder is generally assumed to be sterile, our findings corroborate earlier reports that opportunistic fungi from soil, feed, water, and farm environments can colonise teat surfaces and subsequently gain access to milk (4, 36). The presence of such fungi in the udder highlights the vulnerability of milk to fungal contamination and the consequent risk of mycotoxin production, as emphasised in the introduction.

Fusarium species are widely recognised as important mycotoxigenic fungi with significant impacts on food safety, livestock health, and

agricultural productivity. *Fusarium brachygibbosum*, isolated in this study, has been previously reported as a soil-borne plant pathogen (37) and a contaminant of diverse environments, including marine sediments (38) and date palm fruits (39). While its primary toxins include beauvericin, diacetoxyscirpenol, neosolaniol, and trichothecenes (40, 41). We demonstrate here that it is also capable of producing appreciable levels of AFB1, a potent hepatocarcinogen. This expands current understanding of its toxigenic potential and underscores the risk of dietary exposure to aflatoxins through contaminated milk.

Similarly, *Curvularia lunata*, though less prevalent in our samples, poses potential risks due to its ability to produce bioactive metabolites such as cytochalasin B, racidin, and related toxins known to impair neural and renal function

in animals (42, 43, 44, 45). Although rarely studied in the context of milk contamination, its presence in cow udders may reflect environmental exposure from contaminated forage or soil, suggesting another underexplored route of entry for mycotoxins into the dairy chain. The *Penicillium* species isolated (*P. citrinum* and *P. oxalicum*) are of particular concern because of their established roles in food spoilage and mycotoxin production. *Penicillium citrinum* is known for producing citrinin, a nephrotoxic compound (46), and has been implicated in opportunistic infections such as pneumonia in immunocompromised individuals (47, 48, 49). Interestingly, while *Penicillium citrinum* in this study produced low levels of AFB1, *Penicillium oxalicum* did not produce detectable amounts, aligning with previous reports that aflatoxin biosynthesis is strain- and species-dependent (50). These findings highlight the importance of molecular and metabolic profiling in determining the true toxigenic potential of fungi isolated from milk sources.

Although *Mucor irregularis* was detected in only one sample, its identification is noteworthy. *Mucor* spp. are commonly associated with the spoilage of dairy products and can occasionally cause mucormycosis in humans (51, 52). While their aflatoxin-producing capacity is less established compared to *Aspergillus* spp., their presence indicates potential quality and safety concerns in milk, particularly under poor hygienic conditions.

Importantly, the detection of AFB1 at concentrations ranging from 0.08 to 0.22 µg/kg in isolates from this study, while within Codex Alimentarius tolerable limits for milk (0.5–15 µg/kg depending on commodity) (53), remains a public health concern. AFB1 is classified as a Group 1 carcinogen by the International Agency for Research on Cancer (54), and studies have shown that even chronic exposure to trace amounts can induce hepatotoxic, immunosuppressive, and mutagenic effects (55, 56, 57, 58). Moreover, in dairy cattle, ingested AFB1 is metabolised into AFM1, which persists in milk despite pasteurisation or sterilisation (59). Thus, while the levels detected here are below regulatory thresholds, the cumulative effect of dietary exposure, especially in populations with high milk consumption, cannot be overlooked.

Conclusion

The isolation of aflatoxin-producing fungi directly from the udder of lactating cows provides novel evidence that the udder environment itself can serve as a reservoir for potential milk contamination, in addition to the well-known dietary routes. This finding bridges a key knowledge gap highlighted in our justification:

while most previous research has focused on fungal contamination of feed and processed milk, the role of the cow udder as a direct source of toxigenic fungi has received little attention.

Given the health risks associated with aflatoxin B1 and its metabolite AFM1, our study underscores the need for improved hygienic practices in dairy farms, regular monitoring of fungal contaminants, and targeted interventions to reduce fungal colonisation of cow udders. In addition, molecular surveillance and toxin profiling of fungal isolates should be integrated into dairy quality assurance programs. Ultimately, addressing both environmental and udder-associated fungal contamination is essential to safeguard milk safety, protect public health, and minimise economic losses in the dairy sector.

List of abbreviations

AF: Aflatoxins
 AFTB₁: Aflatoxin B₁
 PDA: Potato dextrose agar
 YES: Yeast Extract Sucrose

Declarations

Ethical approval and consent to participate

Ethical approval was obtained from the Ethical Committee of Bowen University (BUTH/REC-27581), and consent to participate is not applicable as no human subjects were involved in this work. No animal experimentation. The researchers took concrete actions to ensure that their methodologies adhered to acceptable standards. Verbal consent was secured from farm owners, and sampling procedures were conducted with minimal disturbance to the lactating cows. The study was performed in accordance with the University's guidelines for research involving animals and potential biohazards.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed in this study are included in this published article

Competing interests

The author(s) declare that there are no conflicts of interest

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Authors' contributions

All the authors contributed equally to the work (the conceptualisation of the work, the practical work and the writing of the manuscript). All authors reviewed the manuscript and approved the submitted final version.

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