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Assessing the Biodetoxification Potential of *Enterococcus faecium* on Aflatoxin M1 in Fresh Raw Cow Milk

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The adverse effects of aflatoxins on the economy and public health have prompted research into strategies to prevent their contamination in food and feed. Biological methods have become popular because they are environmentally friendly and beneficial for health. This research aims to determine the bio-detoxification potential of E. faecium, on Aflatoxin M1 (AFM1) in raw cow milk. Biochemical and molecular methods were used to identify the E. faecium isolates obtained from locally fermented cow milk (Nono). Three (3) distinct sampling locations in the Zaria metropolis, Dan-Magaji, Kufena, and Gabari, were used to gather nine (9) fresh milk samples. A quick test kit was used to screen the samples for AFM1 contamination. To determine the level of contamination, AFM1-contaminated samples were analyzed using High-Performance Liquid Chromatography (HPLC) analysis. The isolates were injected into the tainted milk samples at a cell density of 1.5 x 10^8cfu/mL and 3.0x10^8cfu/mL for 30 and 60 minutes at 4 and 37 degrees Celsius. The amount of unbound AFM1 in the samples was measured using HPLC analysis and surface binding assay. "ANOVA" single factor and two ways were used to analyze the data. All collected raw milk samples were contaminated with aflatoxin M1 at concentrations exceeding the EU/NAFDAC limit of ≤0.05 µg/L. The isolates bound AFM1 at 35-45% rates at a cell concentration of 3.0x10^8 cfu/mL at 37°C for 60 minutes. This research suggests that E. faecium could be an effective option for reducing AFM1 contamination in milk samples due to its harmless nature and recognition as generally safe. Additionally, regulatory agencies should implement thorough monitoring to ensure AFM1 levels in milk and milk products remain below acceptable limits.
Keywords: Bio-detoxification, Aflatoxin M1 contamination, raw cow milk, E. faecium, Surface binding assay

INTRODUCTION

The Food and Agriculture Organization of the United Nations (FAO) reports that filamentous fungi spoil a quarter of the world's food crops, necessitating their rejection for food safety reasons. This issue exacerbates the strain on the food supply for an ever-growing global population. Over 250 types of mold produce especially problematic mycotoxins. Among the 300 known mycotoxins, aflatoxins are the most significant (Hans-George *et al.*, 2016). Food safety is a significant global issue, prompting numerous studies to explore ways to address consumer concerns regarding its various aspects (Nielsen *et al.*, 2009).

Milk is a fundamental food for people of all ages because of its high nutritional value (Zeluta *et al.*, 2009). AFM1 is heat-stable in raw, processed, and dairy products. It is not destroyed by pasteurization, sterilization, or other food processing methods (Oruc *et al.*, 2006).

According to Patterson and Lima (2010), mycotoxins are low-molecular-weight chemicals filamentous fungi produce during their secondary metabolism. Their chemical structures vary, ranging from basic C4 compounds to complicated molecules. Feed, food, and raw materials all include these natural pollutants. Mycotoxin-producing mold species are common and can grow on a range of substrates in various environmental circumstances, making them frequent in most agricultural goods sold globally (Bennet *et al.*, 2007). Even at low concentrations, several mycotoxins can cause allergic reactions and autoimmune disorders in vertebrates and other animals. Furthermore, Bennet et al. (2007) state that certain mycotoxins are mutagenic, carcinogenic, and teratogenic.

Aflatoxin M1 (AFM1) is a 4-hydroxy derivative of AFB1 ($C_{17}H_{12}O_7$), bio-transformed in the liver. This conversion is catalyzed by cytochrome P450 (CYP450) enzymes (Diaz *et al.*, 2010).

AFM1 is the most rapidly formed metabolite of AFB1 in cattle after ingesting the parent toxin in contaminated feed (Patterson *et al.*, 2008). Like other aflatoxins, AFM1 has been classified in Group 1 as carcinogenic to humans, with sufficient evidence for its hepatocarcinogenicity (IARC, 2002).

Enterococcus faecium is a type of Lactic Acid Bacteria (LAB) commonly found in the gastrointestinal tract of humans and animals. It is a gram-positive, non-spore-forming bacterium that can appear as cocci, coccobacilli, or rods. They are generally non-respiratory and lack catalase. These bacteria ferment glucose to lactic acid, or lactic acid, CO_2, and ethanol. Despite the absence of catalase, they possess superoxide dismutase and use peroxidase enzymes to detoxify peroxide radicals (Belletti *et al.*, 2009). The bacteria are typically harmless and even beneficial, playing a role in maintaining a healthy gut microbiome, boosting the immune system, and producing vitamins. They can degrade pollutants and heavy metals and be used as starter culture in fermentation, helping preserve food and enhance its nutritional value (Ahlberg *et al.*, 2015).

It has been reported that approximately more than a quarter of the world's agricultural products are contaminated with mycotoxins, with aflatoxin being the most significant (Muthoni *et al.*, 2011). An estimated 5 billion people in developing countries risk chronic exposure to aflatoxins through contaminated foods (Williams *et al.*, 2004).

Internationally, mycotoxins have caused significant losses for Nigeria by reducing crop yields and contributing to food shortages. This impacts the quantity of produce available for export and is compounded by the perceptions and policies of buyer nations. The cumulative effect of fungal infestation on farmland and stored produce tarnishes the international reputation of Nigeria's agricultural products. This leads to reduced demand for the country's agricultural produce or, in some cases, total bans, resulting in economic losses (Bankole and Adebanjo, 2003).

Dietary exposure to AFM1 is a significant risk factor for hepatocellular carcinoma, the fifth most common cancer globally, and can suppress the immune system, particularly in individuals who are positive for the hepatitis B virus (Williams and Windham, 2015).

Researchers are actively pursuing new strategies for preventing and detoxifying this hazardous toxin to ensure the safety of products intended for human consumption (Ben Salah-Abbes *et al.*, 2015). Utilizing selected microorganisms for controlling aflatoxins and postharvest diseases has gained significant attention, offering an appealing alternative for removing toxins and safeguarding food and feed quality (Wambacq *et al.*, 2016).

Biological control methods provide safe means to eliminate aflatoxins from food sources (Fan *et al.*, 2013). Numerous studies have demonstrated the efficacy of various microorganisms in degrading or removing aflatoxins from food and feed (Hathout and Aly, 2014). Lactic acid bacteria (LAB) stand out as particularly suitable for reducing aflatoxin bioavailability due to their unique characteristics; they are Generally Recognized As Safe (GRAS) by the USFDA, and some strains exhibit beneficial probiotic effects on health (Fan *et al.*, 2013).

The European Union (EU) has established a maximum limit of 5 ng/kg for aflatoxin B1 (AFB1) in feed for dairy cattle. For aflatoxin M1 (AFM1) in milk, the EU limit is 50 ng/kg. In Nigeria, these limits have been adopted by the National Agency for Food and Drug Administration and Control (NAFDAC) as 50 ng/L for general milk consumption and 25 ng/L for milk intended specifically for infants. The Codex Alimentarius sets a limit of 500 ng/kg (European Commission, 2002). This research work aims to determine the potential of *E. faecium* to detoxify Aflatoxin M1 (AFM1) in fresh raw cow milk.

MATERIALS AND METHODS

The study was undertaken in the Zaria local government area of Kaduna State. *Enterococcus faecium* was isolated from locally fermented cow milk. The isolates were identified using cultural, morphological, physiological, biochemical, and molecular approaches, the bacterial DNA was extracted, and the target 16S rRNA gene was amplified. The PCR product was purified and sent to INQABA Biotech for sequencing.

Sample collection and Screening for AFM1

For AFM1 screening, three (3) fresh raw cow milk samples from each of the Fulani communities in Zaria, including Dan-magaji, Gabari, and Kufena, were collected and labeled. The samples were shipped to Multiuser Science Research Laboratory ABU Zaria in ice packs to be analyzed. Samples were screened using a rapid test kit designed specifically for milk samples in accordance with the manufacturer's instructions (Ring Biotechnology Co., Ltd. Art no.:100004-96T). The kit uses a monoclonal antibody with a high affinity for AFM1, making it simple to detect contamination in milk. Its AFMI detection limit can meet both the EU and USA permissible levels.

High-Performance Liquid Chromatography (HPLC) Conditions

The standard for aflatoxin M1 was acquired from R-Biopharm located in Darmstadt, Germany. Water, acetonitrile, and methanol of HPLC grade were purchased from Fisher Scientific Company in the United Kingdom. The liquid chromatographic system comprises the HPLC pump, auto-injector, column oven, and fluorescence detector (1260 Infinity Agilent Technologies, USA). The following were the HPLC parameters for the AFM1 analysis: The Hypersil 5AA-ODS 200 x 2.1mm column (Agilent Technologies, USA) has a temperature of 25°C. The mobile phase consists of water, acetonitrile, and methanol (60:30:10). The flow rate is 0.7 ml/min, the retention time is 2 m, the injection volume is 5 µl, and the detector is a fluorescence spectrophotometer with an excitation of 360 nm and an emission of 440 nm.

Extraction and purification of aflatoxin M1

The extraction process was carried out in accordance with Ruangwises and Ruangwises' earlier description (2013). To reach a temperature of 4ºC, 100 ml of raw cow milk sample was measured into a 100 ml glass beaker and stored in the freezer. Pipetting the sample into a 50 ml polypropylene centrifuge tube was done. Centrifugation was done for 10 minutes at 4,000 rpm to defat the milk samples. After separating and filtering the fatty layer with a Whattman filter size 4, the skimmed milk was put into a 50 ml plastic syringe fitted with a Luer tip and connected to an immunoaffinity column. Due to gravity, the skimmed milk entered the column at a flow rate of around 2 milliliters per minute. Following the skim milk's passage through, 20 ml of Phosphate Buffered Saline (PBS) was used to wash the column at a flow rate of 5ml/min. Air was passed through the column to remove residual liquid.

AFM_1 was eluted from the column at a flow rate of 1 drop/second with 1.25 ml of acetonitrile: methanol (60:40v/v) and 1.25 ml of HPLC water, giving a total volume of 2.5 ml. One hundred (100) µl was injected into the HPLC system, and AFM_1 in the final solution was measured using HPLC analysis. Equations for the amount of aflatoxin are made according to the following;

$$Wm = Wa \times \left(\frac{Vf}{Vi}\right) \times \left(\frac{1}{Vs}\right)$$

Where: Wm = Amount of aflatoxin M1 in the test sample in µg/ L
Wa = Absorbance corresponding to area of aflatoxin M1 peak of the test extract (ng)
Vf = Final volume of re-dissolved eluate (µL)
Vi = Volume of injected eluate (µL)
Vs = Volume of test portion (milk) passing through the column (mL) (Yohannes *et al.*, 2018)

Statistical Analysis

Duncan's Multiple Range Test was used to distinguish the mean AFM1 and analyze the quantified amounts of AFM1 in the samples using ANOVA single factor.

Determination of aflatoxin M1 binding potential of *E. faecium* using a surface binding assay

Preparation of bacterial inoculum

The isolate was grown 48 hours at 37 °C in 100 milliliters of MRS broth. Using a U.V. visible spectrophotometer (Agilent Technologies) at a wavelength of 600 nm and an absorbance range of 0.08-0.1 and 0.225-0.257 for 0.5 and 1.0 McFarland, respectively, the bacterial inoculum was adjusted and maintained at these values, which are equivalent to $1.5x10^8$ and $3.0x10^8$cfu/mL.

Surface binding assay

Eight (8) milliliters of aflatoxin M1-contaminated milk were inoculated with two milliliters of the bacterial inoculum, which was equivalent to 1.5 McFarland ($1.5x10^8$ cells) and 1.0 McFarland ($3.0x10^8$ cells) separately. The inoculated milk was then incubated at 4 and 37 degrees Celsius for 30 and 60 minutes, respectively. After 30 minutes, 5 milliliters of the inoculated milk were removed and centrifuged at 3500g for 10 minutes and the same for 60 minutes. The amount of unbound AFM1 in the supernatant was measured using HPLC analysis. A positive and a negative control were included in every experiment run in triplicate.

Fluorescence was used for detection with excitation and emission wavelengths of 360 and 440 nm, respectively. Using an extremely sensitive hypersil, the retention period was 1 to 2 minutes.

Following the equation, the amount of AFM1 eliminated was determined.

$$\left[1 - \frac{Peak\ area\ of\ AFM1\ in\ test\ sample}{Peak\ area\ of\ AFM1\ in\ positive\ control}\ X\ 100\right]$$

(Carolyn *et al.*, 2001).

RESULTS

Table 1 displays the results of various morphological, microscopic, physiological, and biochemical tests for identifying *E. faecium* isolated from locally fermented cow milk. All isolates were gram-positive, non-spore-forming rods or cocci in pairs, chains, and clusters; they were non-motile, catalase, indole, and citrate-negative. Some isolates could grow at 45 °C and in MRS broth containing 6.5% NaCl.

Table 1: Colony morphology and Biochemical tests for identification of *E. faecium*

Isolate	Colony morphology	Gram reaction and morphology	Spore staining	Temperature survivability test 15°	30°	45°	Salt tolerance test	Catalase test	Indole	Citrate	motility
LAB A	Big creamy-colored colonies with smooth edges	Gram +ve rods in chains	-	-	+	+	+	-	-	-	-
LAB B	Small, rough, flat colonies	Gram +ve rods in chains	-	+	+	-	+	-	-	-	-
LAB C	Big whitish colonies with rough edges	Gram +ve cocci in clusters	-	-	+	-	+	-	-	-	-
LAB D	Small, flat opaque colonies with smooth edges	Gram +ve coccabacilli in chains	-	-	-	-	-	-	-	-	-
LAB E	Spherical, cream colored colonies	Gram +ve rods in pairs	-	-	-	+	+	-	-	-	-

Among the five (5) isolates identified LAB A-E, LAB E is the only isolate identified as *E. faecium* strain OZC108 74 (MK333711.1) with a percentage similarity of 92.14% (Table 2).

Table 2: Molecular Characterization of Lactic Acid Bacteria

S/N	Isolates	Source	Representative species (Accession number)	Percentage Similarity
1.	LAB. E		*Enterococcus faecium* strain OZC108 74 (MK333711.1)	92.14%

Table 3 shows the result for screened milk samples for aflatoxin M1 contamination, all of which were contaminated with aflatoxin M1 after testing with a rapid test kit.

Table 3: **Screened Milk Samples for AFM1 Contamination**

S/N	Location	No. of samples tested	Samples 1	2	3	Number positive (%)
I	Dan-magaji	3	+	+	+	3(100)
ii	Gabari	3	+	+	+	3(100)
iii	Kufena	3	+	+	+	3(100)
Total		9	3	3	3	9(100)

Key: + Aflatoxin M1 detected, 1, 2 and 3 = samples tested

Table 4 shows results for the quantity of AFM1 in all the samples collected using High-Performance Liquid Chromatography.

Table 4: Quantified AFM1 in fresh raw cow milk samples using HPLC analysis

S/N	Location	No. of samples tested	AFM1 in each sample(µg/L)	MeanAFM1 concentrations/location(µg/L)	No. below EU limit(<0.05µg/L)	No. above EU limit(>0.05µg/L)
1.	Dan-magaji	3	118.99 102.84 80.22	101.02[a]	0	3
2.	Gabari	3	71.15 58.60 78.22	60.71[b]	0	3
3.	Kufena	3	79.28 62.34 48.26	62.96[b]	0	3

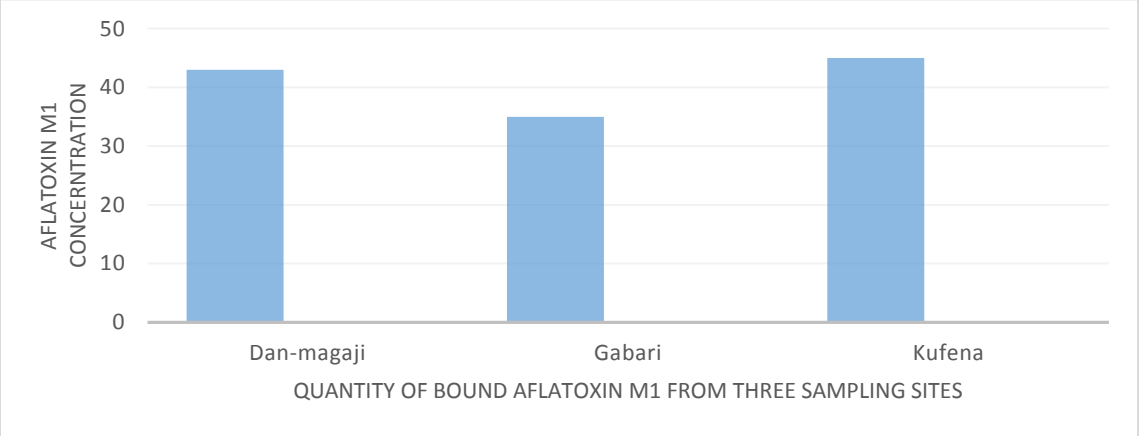

Figure 1. Quantity of bound AFM1 (µg/L) by *E. faecium* from three sampling sites at a cell density of $3.0x10^8$CFU/mL, temperature of 37°c, for 60 minutes

DISCUSSION

Table 1 shows the results of various morphological, microscopic, physiological, and biochemical tests for identifying *E. faecium* isolated from locally fermented cow milk.

Among the five (5) isolates identified LAB A-E, LAB E is the only isolate identified as *E. faecium* strain OZC108 74 (MK333711.1) with a percentage similarity of 92.14%.

Table 3 shows the result for screened milk samples for aflatoxin M1 contamination, all contaminated with aflatoxin M1 after testing with a rapid test kit specific for milk samples. The kit had a limit of detection of 0.05ppb, equivalent to 0.05ng/ml/g. This result is consistent with the reports of Maureen *et al.* (2019) in Kenya, where the authors collected 96 raw milk samples, all of which were contaminated with aflatoxin M1.

The ANOVA single-factor analysis of the data revealed statistically significant differences between the quantified AFM1 amounts and sampling locations; the calculated p-value is 0.000785, less than 0.05 at a 95% confidence interval. The Duncan's multiple range test was used to separate the means; the mean obtained from location Dan-Magaji is significantly different from other sampling sites, and the samples collected from the location had the highest level of AFM1 contamination (101.02 µg/L), Kufena (62.96 µg/L) and the least is Gabari (60.71 µg/L). The disparity may be explained by differences in the metabolic activity of the suckling animal, the level of aflatoxin B1 contamination in the feed, and the kind of feed the animals are fed with.

Elgerbi *et al.* (2017) revealed that AFM1 was found in 35 (71.4%) of the 49 raw cow milk samples that were collected in various locations of Libya, with values ranging from 0.03 to 3.13ng/L milk, which is similar to the findings of this research.

Pittet (2008) reported that AFM1 concentrations in raw milk are typically less than 0.1 ng/L in Europe but can exceed 1.0 ng/L in other regions. Global surveys have shown varying levels of AFM1 in milk, though other factors may also influence the production of fungal toxins in food and feedstuffs.

Figure 1 showed that *Enterococcus faecium* bound up to 43% of the toxin in samples collected from Danmagaji, 35% in samples from Gabari and 45% of Aflatoxin M1 from Kufena

E. faecium is a member of Lactic Acid Bacteria found naturally in foods and has various applications in processing some fermented dairy products (Giraffa, 2003). As Ali et al. (2010) reported, it can reduce aflatoxins from liquid media. *E. faecium M74* bound approximately 19 to 30% of AFB1 from aqueous solution, which is similar to the findings of this research.

The highest aflatoxin M1 binding by *Enterococcus faecium* occurred at cell density equivalent to 1.0 Mcfarland, at 37ºC for 60 minutes. These findings align with findings by Rayes (2013), who discovered that the optimal temperature for AFM1 removal from milk is 37°C, with the lowest temperature observed at 5°C. Similarly, Diaa *et al.* (2018) reported that *L. acidophilus* binds AFM1 most effectively at 37°C, whereas *L. plantarum* binds AFM1 best at 4°C in whole milk. El-Nezami *et al.* (1998) noted that the bio-detoxification activity of LAB is significantly influenced by the optimal incubation temperature (37°C) and bacterial cell concentration. Mahmood *et al.* (2018) found that the binding process depends on cell concentration, with the number of mycotoxins bound increasing significantly with higher cell concentrations. This research supports these findings, showing the highest binding at a cell density equivalent to 1.0 McFarland ($3.0x10^8$ cfu/mL) rather than 0.5 McFarland ($1.5x10^8$ cfu/mL).

CONCLUSION

E. faecium was isolated from '*Nono*' and identified using colonial morphology, physiologic, biochemical, and molecular techniques. The isolate was identified as *E. faecium* strain OZC108 74 (MK333711.1) with a percentage similarity of 92.14%. All screened samples were contaminated with AFM1 at concentrations exceeding the EU set limit (≤ 0.05µg/L) adopted by NAFDAC in Nigeria. Samples from Dan-magaji had the highest level of AFM1 contamination (101.02µg/L), while samples from Kufena had the least (62.96µg/L). The highest binding of AFM1 was achieved (45%) at 1.0 McFarland, 37ºC, and for 60 minutes. This study has demonstrated that specific strains of *Enterococcus* can reduce AFs from milk.

RECOMMENDATIONS

1. It is recommended that dairy farmers adopt the best pre- and post-harvest agricultural practices for crops used as animal feeds to curtail fungal colonization, toxin accumulation, and subsequent contamination of animal milk in the local setting.
2. Regulatory bodies should use sufficient monitoring to ensure milk and milk products have AFM1 levels below the predetermined limit.
3. Since the USFDA generally considers lactic acid bacteria safe for human consumption, using *E. faecium* could be a good way to lower the amounts of AFM1 in milk and dairy products.

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