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Prevalence of aflatoxins in dairy products and the biocontrol potential of *Lactobacillus acidophilus* for detoxification and fungal inhibition

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ABSTRACT

This study aimed to evaluate the preponderance of aflatoxin M1 (AFM1) and aflatoxin M2 (AFM2) in different cheese varieties (Ras, processed, and soft cheese) and raw milk and assess their compliance with Egyptian safety standards. This study investigated the potential detoxification effect of *Lactobacillus acidophilus* against AFM1 and its inhibitory activity on *Aspergillus flavus*. One hundred dairy samples were collected from various Menoufia Governorate, Egypt markets. AFM1 and AFM2 levels were confined using high-performance liquid chromatography (HPLC). The probiotic detoxification study was conducted by inoculating milk samples with *Aspergillus flavus* spores and treating them with *L. acidophilus* at 1%, 2%, and 3%, monitoring fungal growth and toxin levels over 15 days. The results showed that AFM1 exceeded the permissible limit of 0.05 ppb in 28% of Ras cheese, 16% of processed cheese, 20% of soft cheese, and 8% of raw milk samples, while AFM2 contamination was lower across all categories. *Lactobacillus acidophilus* exhibited a dose-dependent inhibitory effect on *Aspergillus flavus*, achieving complete fungal inhibition at 3% concentration by day 6. Moreover, AFM1 levels were significantly reduced, with complete detoxification observed at 2% and 3% *L. acidophilus* concentrations. These findings highlight the widespread occurrence of AFM1 in the dairy products sector and underscore the potential of probiotic interventions as a natural mitigation strategy for aflatoxin contamination.

Introduction

Milk and dairy products are principal components of the human diet because they contain appreciable portions of macro- and micronutrients, specifically for infants and older adults (González-Montaña*et al*., 2019).

Fungal spoilage is a perilous problem in the dairy products industry. Raw milk and milk products are generally considered ideal growth mediums for many fungal species, as they provide all the crucial nutrients for their growth (Gulbe and Valdovska, 2014).

Aflatoxins are secondary metabolites assembled by different kinds of fungi, mostly *Aspergillus flavus* and *Aspergillus parasiticus*, which induce various health intricacies, including carcinogenesis, malformations, and immunosuppression, especially in children; they also contribute to significant economic losses (Admasu *et al*., 2021). There are four types of aflatoxin B1, B2, G1, and G2, and the most contamination of food used by humans and animals is with aflatoxin B1(AFB1) (Bhardwaj *et al*., 2023). In the liver, AFB1 transforms into an intermediate reactive epoxide metabolite. Then it is hydroxylated and form aflatoxin M1 (AFM1). After hydroxylation in lactating animals, it is secreted into milk (Stella *et al*., 2024) AFB1 appears very quickly, and after 15 min, in the form of its metabolite, aflatoxin M1 (AFM1), in the blood. Six hours after feeding a diet contaminated with AFB1, AFM1 appears in milk (Rodrigues *et al*., 2019). Aflatoxin B2 (AFB2), like aflatoxin M1 (AFM1), undergoes metabolic conversion into aflatoxin M2 (AFM2), which can be detected in milk and cheese (Fedele *et al*., 2007). Furthermore, Bräse *et al*. (2013) proposed that AFM2 might transform into AFM1 via an alternative metabolic pathway, given that all aflatoxins originate from a common precursor.

AFM1 is considered one of the most significant contaminants in milk due to its thermal stability (Admasu *et al*., 2021). This mycotoxin remains intact even after sterilization and pasteurization processes (Liu *et al*., 2023). Beyond milk, AFM1 has been identified in several dairy products, including yogurt, infant formula, cream, and cheese (Xiong *et al*., 2018). According to the U.S. Food and Drug Administration, the maximum allowable AFM1 concentration in milk is 0.5 µg/kg, while aflatoxin B1 (AFB1) in animal feed must not exceed 20 µg/kg (Jiang *et al*., 2018). Infants and young children are particularly vulnerable to AFM1 exposure, and its limit in infant food is set at 0.025 µg/kg (Admasu *et al*., 2021). In Egypt, the Ministry of Health has established a maximum threshold of 0.05 µg/kg for AFM1 in raw milk, processed milk, and milk used for dairy product manufacturing (European Commission, 2006; Egyptian Standard, 2010).

Several approaches have been developed to mitigate AFM1 contamination in milk, categorized into three primary strategies: biological, physical, and chemical methods (Nilkaram *et al*., 2023).

Biological decontamination procedures are being used vastly as an encouraging alternative to chemical methods because of their efficiency, low cost, and nature-friendly properties. LAB eliminates mycotoxins without leaving toxic residues (Shetty and Jespersen, 2006). Probiotic bacteria can detoxify aflatoxin M1 in contaminated milk positively. This finding is achieved within two years of study (2015–2017), according to Abdelmotilib *et al*. (2018).

Lactobacillus acidophilus is the most common probiotic bacteria among other Lactobacillus species. The FDA has categorized it as GRAS (Generally Regarded as Safe) (Parvez *et al*., 2006). According to (Elsanhoty *et al*., 2014), *Lactobacillus acidophilus* strains lead to the most considerable reduction in AFM1 quantities. Furthermore, Sarlak *et al*. (2017) have shown that *Lactobacillus acidophilus* has the best binding capacity of AFM1 among all the probiotic strains.

This study aimed to assess the prevalence of aflatoxin M1 (AFM1) and aflatoxin M2 (AFM2) in raw milk and various cheese types, comparing the findings with Egyptian regulatory standards. Additionally, it investigates the potential detoxification of AFM1 using *Lactobacillus acidophilus*.

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Materials and methods

Sample Collection

A total of 100 dairy samples, including Ras cheese, processed cheese, soft cheese, and raw milk, were obtained from various supermarkets and grocery stores in Menoufia Governorate, Egypt. Each sample set comprised 25 units. The collected samples were transported under controlled conditions to the Central Laboratory, Faculty of Veterinary Medicine, Benha University, for the determination of AFM1 and AFM2 concentrations.

Chemicals and Supplies

Standards

Standard and blank aflatoxin M1 (AFM1) and aflatoxin M2 (AFM2) were sourced from Sigma Aldrich, Steinheim, Germany, for use in this study.

Solvents

Analytical-grade acetone, acetonitrile, methanol, and HPLC-grade solvents were procured from Merck, Darmstadt, Germany. Deionized water, along with all other reagents and chemicals, met at least analytical-grade specifications.

Apparatus and equipment

Aflatoxin determination was conducted using high-performance liquid chromatography (HPLC) with an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany). This system was equipped with a quaternary pump (Model G 1311A) and a UV detector (Model G 1314A) set at a wavelength of 254 nm. Additionally, an autosampler (Model G1329A VP-ODS) and a Shim-pack column (150× 4.6 mm) (Shimadzu, Kyoto, Japan) were utilized. Data acquisition and integration were performed using Chemstation Software. Ultra-high purity (99%) argon gas and liquid nitrogen were employed where necessary. The study also utilized Easi-Extract Aflatoxin immunoaffinity columns for sample purification.

Standard Aflatoxin Solutions (AFM1 & AFM2)

Stock standard solutions of AFM1, AFM2, and ochratoxin A were prepared by dissolving the solid standards in a benzene: acetonitrile mixture (98:2, v/v). The precise concentration of each solution was measured using a Shimadzu UV-1601 PC spectrophotometer (Shimadzu Scientific Instruments, Japan) following AOAC (2005) guidelines.

HPLC Analysis of AFM1 and AFM2

AFM1 extraction and purification followed the method described by Fernandes *et al*. (2012), with minor modifications recommended by the immunoaffinity column manufacturer. The identification and quantification of AFM1 residues were carried out by injecting 20 µL of purified sample extracts into an HPLC system. A calibration curve was established using AFM1 standard solutions (Sigma, St. Louis, MO, USA) at concentrations of 3.125, 6.25, 12.5, 25, and 50 ng/mL. The analytical method had a detection limit of 0.01 ng/mL. HPLC analysis was conducted using an Agilent 1260 series system with a C18 column (4.6 mm × 250 mm, 5 µm particle size). The mobile phase consisted of water, isopropanol, and acetonitrile (80:12:8), operating at a flow rate of 1 mL/min. The fluorescence detector was set to an emission wavelength of 435 nm and an excitation wavelength of 365 nm. Each sample injection volume was 10 µL, and the column temperature was maintained at 35°C.

Activation of Lactobacillus acidophilus

The *Lactobacillus acidophilus* DSMZ 20079 strain was obtained from Cairo MIRCEN (Microbiological Resource Center), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. The strain was activated in sterile 9 mL De Man, Rogosa, and Sharpe (MRS) broth (Biolife, Italy) and incubated at 37°C for 24 hours. Three successive subcultures were performed to ensure full activation, achieving a final concentration of 10^9 CFU/mL. The activated strain was stored under refrigeration and used within 24 hours, following the protocol of Ogunbanow *et al*. (2003).

Preparation of Aspergillus flavus Suspension

A food-origin *Aspergillus flavus* strain was cultivated at 30°C for five days on Sabouraud's dextrose agar (Difco Laboratories, Detroit, MI, USA) until sporulation occurred (approximately seven days). The spores were collected by adding 10 mL of a sterile 0.05% (v/v) aqueous Tween-80 solution (Merck, Germany) to the culture surface and gently scraping the conidiophores with a sterile inoculation loop. The resulting spore suspension was filtered through four layers of sterile cheesecloth to remove mycelial debris. The spore concentration, estimated to be 10^6-10^7 spores/mL, was determined using the spread plate technique on Potato Dextrose Agar (Difco Laboratories, Detroit, MI, USA), following the method described by Mellout *et al*. (2014).

Experiment design of Lactobacillus acidophilus effect on A. flavus and their aflatoxin

Milk samples free from mold and aflatoxins were grouped into four groups. The 1st group (control) was inoculated with One ml spore suspension of *Aspergillus flavus*. However, the 2nd, third, and fourth groups were inoculated with One ml spore suspension of *Aspergillus flavus* and 1, 2, and 3 % pure *Lactobacillus acidophilus* (109) cultures, respectively. All treatments were incubated for (3, 6, 9, 12, and 15 days.) at 30ºC with triplicates. Therefore, the *Aspergillus flavus* count and the concentrations of Aflatoxin M1 were determined, and the reduction % was calculated and recorded according to Pierides *et al*. (2000).

Statistical analysis

The results are expressed as Mean ±Standard error mean (SEM). Data was analyzed using the SPSS program (2008) (Statistical Package for Social Science, version 16).

Results

Prevalence of Aflatoxin M1 and M2 in Cheese and Raw Milk

The occurrence of aflatoxins M1 and M2 was assessed in different cheese varieties (Ras, processed, and soft cheese) and raw milk samples (Tables 1 and 2). AFM1 was detected in 28% of Ras cheese, 16% of processed cheese, 20% of soft cheese, and 8% of raw milk samples, with mean concentrations of 0.814±0.11 ppb, 0.358±0.14 ppb, 0.529±0.08 ppb, and 0.147±0.02 ppb, respectively. Similarly, AFM2 was found in 16% of Ras cheese, 8% of processed cheese, 8% of soft cheese, and 4% of raw milk, with mean concentrations of 0.246±0.05 ppb, 0.069±0.02 ppb, 0.087±0.11 ppb, and 0.012±0.01 ppb, respectively.

Comparison with Egyptian Standards

According to Egyptian standards (2010), the permissible limit for AFM1 in dairy products is 0.05 ppb Table 3. The data show that 28% of

Ras cheese, 16% of processed cheese, 20% of soft cheese, and 8% of raw milk samples exceeded this threshold, raising potential health concerns regarding their safety.

Table 1. Aflatoxin M1 levels (ppb) in examined cheese varieties and raw milk.

Samples	No. of Examined samples	Positive samples		Mean±SEM
		No.	%	
Ras cheese	25	7	28	0.814±0.11
Processed cheese	25	4	16	0.358 ±0.14
Soft cheese	25	5	20	0.529±0.08
Raw milk	25	2	8	0.147±0.02

Table 2. Aflatoxin M2 levels (ppb) in examined cheese varieties and raw milk.

Samples	No. of Examined samples	Positive samples		Mean±SEM
		No.	%	
Ras cheese	25	4	16	0.246 ±0.05
Processed cheese	25	2	8	0.069 ±0.02
Soft cheese	25	2	8	0.087±0.11
Raw milk	25	1	4	0.012±0.01

Effect of Lactobacillus acidophilus on Aspergillus flavus Growth

The inhibitory effect of *Lactobacillus acidophilus* on *Aspergillus flavus* was monitored over 15 days at different probiotic concentrations (1%, 2%, and 3%) (Table 4). In the control group (milk inoculated only with *Aspergillus flavus*), fungal counts increased from 1.5×10^4 CFU/mL to 2.1×10^6 CFU/mL by day 15. In contrast, *Lactobacillus acidophilus* exhibited significant antifungal activity, with higher concentrations yielding more potent effects. At 1% *Lactobacillus acidophilus*, fungal growth was reduced by 46.7% on day 3 and 79.3% by day 9, with complete inhibition (ND: undetected) after day 12. At 2% *Lactobacillus acidophilus*, growth reduction reached 63.3% by day 3, with total fungal inhibition by day 9. The highest reduction was observed at 3% *L. acidophilus* (82.7% by day 3), leading to complete fungal elimination on day 6.

Effect of L. acidophilus on Aflatoxin M1 Production

AFM1 production by *Aspergillus flavus* was also evaluated in the presence of *Lactobacillus acidophilus* (Table 5). In the control group, AFM1 concentration steadily increased to 0.209 µg/L by day 15. However, the probiotic-treated samples exhibited significantly lower toxin levels. At 1% *Lactobacillus acidophilus* (T1), AFM1 remained at 0.013 µg/L from day 6 onward, marking a 94% reduction compared to the control. At 2% *L. acidophilus* (T2), AFM1 production was fully inhibited from day 3 onward. At 3% *L. acidophilus* (T3), no detectable AFM1 was observed throughout the study, confirming complete suppression of toxin formation.

Effect of Lactobacillus acidophilus on Aspergillus flavus Growth

The growth of *Aspergillus flavus* in milk samples was monitored over 15 days with and without *Lactobacillus acidophilus* treatment at different concentrations (1%, 2%, and 3%), as presented in Table 4. In the control group (milk inoculated only with *Aspergillus flavus*), the fungal count showed a progressive increase from an initial count of 1.5×10^4 CFU/mL to 2.1×10^6 CFU/mL by the 15th day. In contrast, adding *Lactobacillus acidophilus* significantly suppressed fungal proliferation in a dose-dependent manner. At 1% *Lactobacillus acidophilus*, fungal growth was reduced by 46.7% on the third day, with a 79.3% reduction by the ninth day and complete inhibition (ND: undetected) from day 12 onward. At 2% *Lactobacillus acidophilus*, a more potent antifungal effect was observed, with a 63.3% reduction in fungal growth by the third day and complete inhibition by day 9. The highest suppression was recorded at 3% *Lactobacillus acidophilus*, with an 82.7% reduction by the third day and total fungal elimination from day 6 onwards.

Effect of L. acidophilus on Aflatoxin M1 Production

The production of aflatoxin M1 (AFM1) by *Aspergillus flavus* was also assessed in treated and untreated milk samples (Table 5). The control group (milk inoculated with *Aspergillus flavus* only) continuously increased AFM1 concentration, reaching 0.209 µg/L by the 15th day. However, the presence of *Lactobacillus acidophilus* significantly mitigated toxin production. At 1% *Lactobacillus acidophilus* (T1), AFM1 levels were maintained at 0.013 µg/L from the sixth day onward, representing a 94% reduction compared to the control. At 2% *Lactobacillus acidophilus* (T2), AFM1 was completely inhibited from day 3 onward. At 3% *Lactobacillus acidophilus* (T3), no detectable AFM1 was recorded throughout the experimental period, indicating total suppression of toxin formation.

Table 3. Comparison of Aflatoxin M1 level in examined cheese and raw milk with Egyptian standards (2010)

Samples	No. of Examined samples	Permissible Limit ES, (2010). (ppb)	Samples above the permissible limit	
			No.	%
Ras cheese	25	0.05	7	28
Processed cheese	25	0.05	4	16
Soft cheese	25	0.05	5	20
Raw milk	25	0.05	2	8

Table 4. Effect of Lb. Acidophilus on *A. flavus* growth experimentally inoculated to milk.

Treatment	Control (+ve *A. flavus*)	*Lb. acidophilus* 1%		*Lb.* a*cidophilus* 2%		*Lb. acidophilus* 3%	
Storage (Day)	Initial injected Count	Count	R (%) *	Count	R (%)*	Count	R (%) *
Zero	$1.5\times10^4\pm 0.1\times10^4$	$1.5\times10^4\pm 0.1\times10^4$	-----	$1.5\times10^4\pm 0.1\times10^4$	----	$1.5\times10^4\pm 0.1\times10^4$	-----
3rd Day	$4.8\times10^4\pm 0.3\times10^{4\,a}$	$8.0\times10^3\pm 0.5\times10^{3\,b}$	46.7	$5.5\times10^3\pm 0.4\times10^{3\,c}$	63.3	$2.6\times10^3\pm 0.1\times10^{3\,d}$	82.7
6th Day	$9.0\times10^4\pm 0.6\times10^{4\,a}$	$6.0\times10^3\pm 0.1\times10^{3\,b}$	60	$2.9\times10^3\pm 0.1\times10^{3\,c}$	80.7	ND	-----
9th Day	$5.0\times10^5\pm 0.3\times10^5$	$3.1\times10^3\pm 0.2\times10^{3\,b}$	79.3	ND	----	ND	-----
12th Day	$7.0\times10^5\pm 0.4\times10^5$	ND	-----	ND	----	ND	-----
15th Day	$2.1\times10^6\pm 0.1\times10^6$	ND	-----	ND	----	ND	-----

R (%) *: Reduction %; ND: Not detected. *Mean values with different superscript letters in the same rows differ significantly (P<0.05).

Table 5. Effect of Lb. Acidophilus on aflatoxin M1 production produced by *A. flavus* experimentally inoculated to milk samples.

Storage (Day)	Treatments			
	C	T1	T2	T3
Zero time	0	0	0	0
3rd Day	0.02	0.01	0	0
6th Day	0.04	0.01	0	0
9th Day	0.09	0.01	0	0
12th Day	0.15	0.01	0	0
15th Day	0.21	0.01	0	0

C: milk with *A. flavus*; T1: milk with *A. flavus* + Lb. acidophilus1%
T2: milk with *A. flavus* + Lb. acidophilus2%; T3: milk with *A. flavus* + Lb. acidophilus3%

Discussion

The widespread detection of AFM1 and AFM2 in cheese and raw milk highlights a significant food safety concern. Several samples exceeded Egyptian regulatory limits, consistent with prior studies reporting global aflatoxin contamination in dairy products (Ashraf *et al*., 2024). Aflatoxins in milk and cheese are primarily attributed to *Aspergillus flavus* and *Aspergillus parasiticus* contamination in animal feed (Casquete *et al*., 2017).

The findings align with previous studies showing that AFM1 levels in cheese are typically 3-5 times higher than those in raw milk due to its strong affinity for casein (Prandini *et al*., 2009). Similarly, Saad (2017) reported AFM1 concentrations in Ras cheese comparable to our results, while higher contamination levels were recorded by El-Seadawy *et al*. (2000). Additionally, Aiad and Abo El-Makarem (2013) detected AFM1 in 46% of soft cheese and 56% of Ras cheese samples.

Regarding AFM2, our results align with those of Saad (2017), who reported lower AFM2 levels in Ras cheese. However, Camarillo *et al*. (2016) detected a higher incidence (20%) in cheese samples, suggesting variability due to regional, seasonal, and analytical differences. The elevated AFM1 concentrations in cheese may be linked to AFB1-contaminated feed, particularly in Egypt's warm and humid climate, which favors fungal proliferation (Govaris *et al*., 2002).

The inhibitory action of *Lactobacillus acidophilus* on *Aspergillus flavus* and AFM1 production reinforces its role as a natural biocontrol agent. Probiotics have been shown to counteract aflatoxin contamination via competitive exclusion, acid production, and enzymatic degradation (Shi*et al*., 2024).

Aiad and Abo El-Makarem (2013) previously reported that 100% of examined soft and Ras cheese samples exceeded permissible AFM1 limits, highlighting an urgent need for enhanced regulatory enforcement. Similarly, Khalifa and Shata (2018) found that dairy products frequently exceeded Egyptian and European safety standards, potentially due to inadequate hygiene during processing and storage.

These results underscore the necessity for routine aflatoxin monitoring in dairy products and advocate for integrating probiotic treatments as a preventive measure. Future research should explore the molecular mechanisms behind *Lactobacillus acidophilus*'s antifungal activity to optimize its application in dairy production.

The results indicate that *Lactobacillus acidophilus* potently inhibits *Aspergillus flavus* growth and aflatoxin M1 production in milk. The antifungal activity observed may be attributed to multiple mechanisms, including competitive exclusion, acidification of the medium, and production of antimicrobial metabolites such as bacteriocins. Higher concentrations of *Lactobacillus acidophilus* (≥2%) were particularly effective, inhibiting fungal growth and toxin production within a short time. The complete inhibition observed at 2% and 3% concentrations suggests that *Lactobacillus acidophilus* limits *Aspergillus flavus* growth and interferes with its aflatoxin biosynthetic pathway. The reduction in AFM1 levels aligns with previous studies demonstrating the ability of probiotic strains to bind or degrade aflatoxins. For instance, research by Heshmati and Khoshfetrat (2015) found that *Lactobacillus acidophilus* effectively reduced AFM1 concentrations in reconstituted milk.

Also, Sokoutifar *et al*. (2018) found that *Lactobacillus acidophilus* effectively reduced AFM1 concentrations in fermented milk with higher temperatures and longer storage times, enhancing the binding capacity. Similarly, Mahmood Fashandi *et al*. (2018) reviewed the detoxification capabilities of *Lactobacillus acidophilus* and Bifidobacterium spp., highlighting their potential to bind AFM1 through interactions with cell wall components. The same results were obtained by Møller *et al*., (2021), who reported that lactic acid bacteria, including *Lactobacillus acidophilus*, can degrade aflatoxins through cell wall adsorption and the production of antifungal metabolites. Furthermore, (Rabie *et al*., 2019) demonstrated that *Lactobacillus acidophilus* and Bifidobacterium lactis could significantly reduce AFM1 levels in contaminated milk and yogurt, achieving complete elimination after three days of refrigerated storage.

These findings support the potential application of *Lactobacillus acidophilus* as a natural biocontrol agent in dairy products to mitigate fungal contamination and aflatoxin risks, ensuring food safety and compliance with regulatory standards. Overall, these findings highlight the effectiveness of *Lactobacillus acidophilus* in controlling *Aspergillus flavus* and aflatoxin M1 in dairy products, providing a promising strategy as a natural biocontrol agent to mitigate fungal contamination and aflatoxin risks for improving the microbial safety of milk and cheese and ensuring compliance with regulatory standards.

Conclusion

The study confirmed the presence of AFM1 and AFM2 in cheese and raw milk, with a significant proportion of samples exceeding Egyptian safety standards. The contamination likely originates from AFB1-contaminated feed, exacerbated by Egypt's warm and humid climate, which promotes fungal growth. The application of *Lactobacillus acidophilus* demonstrated substantial antifungal and detoxification properties, effectively inhibiting *Aspergillus flavus* growth and significantly reducing AFM1 levels. The complete detoxification achieved at higher probiotic concentrations (2% and 3%) suggests that *Lactobacillus acidophilus* can be a promising biocontrol agent in dairy processing. These findings advocate enhanced regulatory monitoring of aflatoxins in dairy products and the potential integration of probiotics in food safety management. Further research should focus on elucidating the precise mechanisms of *Lactobacillus acidophilus*'s antifungal activity and its scalability for industrial applications.

Conflict of interest

The authors have no conflict of interest to declare.

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