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DETECTION AND REMOVAL OF SEASONAL AFM1 OCCURRENCE IN VARIOUS MILK SAMPLES COLLECTED FROM DIFFERENT SHOPS OF LAHORE

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Abstract

Aflatoxin shows carcinogenic results in hepatocellular carcinoma, mutations and primary immunodeficiency disorders. Aflatoxin M1 is found in milk of animals fed on aflatoxin B1 contaminated food. AFM1 detection is being done by different techniques like thin layer chromatography, high-performance liquid chromatography and enzyme-linked immunosorbent assay, among these ELISA shows more efficacy regarding its diagnosis because it takes short time, small volume of sample is required, and also its budget friendly. The European Union has set range of AFM1 limit as 0.05 ppb in milk while 0.5 ppb limit by the Food and Drug Administration. In this study a total of 100 milk samples were tested, 50 during summer (25 branded and 25 non-branded) and total 50 during winter (25 branded and 25 non-branded), and the results were compared by using IBM SPSS Statistics 28.0 for windows. Results showed that 6% branded milk samples having Mean ± SD of 1.38±0.255 and 16% non-branded milk samples having Mean ± SD of 1.33±0.462 were found beyond the permissible levels of AFM1 as set by FDA. Brand names were kept confidential due to ethical values while ensuring the integrity and generalizability of the research findings. Seasonal variations showed more contamination in winter than summer, both in branded (6% and 0.0%) and non-branded milk samples (20% and 12%). Four lactic acid bacteria isolated from local yoghurt namely; *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus*, *Lactobacillus plantarum*, and *Streptococcus thermophilus* showed percentage bound aflatoxin M1 of 78.70%, 54.40%, 50.50%, and 37.10% with Mean ± SD of 55.18±0.173 after 24h incubation period. It is essential to test AFB1 contaminated stored feed daily and apply steps and modern methods to remove aflatoxins from food to ensure health fitness without deteriorating the quality and texture of food.

Keywords: **Aflatoxin B1, aflatoxin M1, enzyme-linked immunosorbent assay, lactic acid bacteria, yoghurt, percentage decontamination.**

Introduction

Aflatoxins are group of mycotoxins which are released by certain genus of filamentous fungi specifically *Penicillium*, *Fusarium* and *Aspergillus* (Anjum et al., 2011). The name aflatoxin is derived from the fact that the toxins which are produced by *Aspergillus flavus* (a= *Aspergillus*, fla= *flavus*) but other *Aspergillus* species are also included namely; *A. nomius*, *A. parasiticus* and *A. fumigatus* which are responsible for aflatoxins production (Jakšić et al., 2021). Aflatoxins are categorized as Aflatoxin B1 (AFB1), Aflatoxin B2 (AFB2), Aflatoxin M1 (AFM1), Aflatoxin M2 (AFM2), Aflatoxin G1 (AFG1), Aflatoxin G2 (AFG2), aflatoxicol and aflatoxin Q1 (Sun & Zhao, 2018). B and G are abbreviated with the fact of blue and green fluorescence nature of aflatoxin below Ultraviolet light respectively while 1 and 2 are positions of aflatoxins on Thin Layer Chromatography (TLC) (Zhao et al., 2019). Aflatoxins are the mycotoxins which are posing an alarming and intoxicating challenges to human health. Some of the aflatoxins are inhaled while others are ingested through various food commodities like meat, egg, milk, dairy products, coffee, cereals, beer, wine, nuts, corn, wheat, gluten, cotton seeds and many others (Hedpara et al., 2022). AFM1 and AFM2 are the metabolites (4-hydroxy derivatives) of AFB1 and AFB2, are found in milk of mammals made by fungi namely; A. flavus and A. parasiticus (Puga-Torres et al., 2020). Milk and milk products contain aflatoxin M1 (AFM_1), a hydroxylated form found in the mammary glands of lactating animals after taking aflatoxin B1 (AFB_1) contaminated (Pandey et al., 2021). Aflatoxin B1, being the most toxic form of aflatoxins is declared as human carcinogenic in Group 1 by the International Agency for Research on Cancer (IARC) (Ferrari et al., 2023). About 0.3-6.2 percentage of Aflatoxin B1 is changed into aflatoxin M1, depending on various variables such as the DNA, seasons, the milking process and habitat features of the lactating animals (Unusan, 2006). AFM1 is a probable human carcinogen and is categorized in Group 2B (possibly carcinogenic to humans) by the IARC (Jafari et al., 2021). The United States Food and Drug Administration (USFDA) and China have prescribed AFM1 range in milk at 500 ng/L or 0.5 parts per billion (ppb) which translates to 500 parts per trillion (ppt), but European Union (EU) has imposed AFM1 levels at 50 ng/L in milk or 0.05 parts per billion (ppb) corresponding to 50 parts per trillion (ppt) (Ferrari et al., 2023). While the Punjab Pure Food Regulations imposed by the Punjab Food Authority (PFA) in Pakistan has set the level of aflatoxin M1 contamination as 0.5 ppb, as set by the USFDA (Ahmad et al., 2019). Contamination level of AFM1 is ten times less than that of the parent compound. After intaking the contaminated feed, aflatoxins are transferred to liver which is the first organ to be affected after their absorption, distribution, metabolism and excretion (Antonissen et al., 2017). In lactating animals, AFB_1 is metabolized into toxic and contaminated by-product aflatoxin M1 (4-hydroxy aflatoxin B1), by a Cytochrome P450 (CYPs) monooxygenase enzyme present in liver (Lewis et al., 2005). Milk being a nutritious liquid is the only source of food for children and is rich in micro and macro-nutrients having rich-quality proteins, lipids, different minerals, and vitamins (riboflavin, vitamin B12, pantothenic acid)

playing as a vital food commodity for human body growth, development, boost immune system, and enzymes production (Al-Bedrani *et al.,*2023). AFM1 in milk and dairy products, therefore, has adverse effects on public health, especially growing children (Ferrari *et al.,*2022). AFM1 is concerned with Hepatocellular Carcinoma (HCC) formation, teratogenicity; affecting the placental development in animals, errors in meiosis, DNA mutations and toxicity in cell which are fatal and threatening the human health worldwide (Tadesse *et al.,*2020). Being hepatocarcinogenic in nature, AFM1 is responsible for 5 – 30 % of liver cancer with more rising in Africa, Asia and China where people come across intoxication of the toxin (Angelopoulou *et al.,*2023). In the United States, economic crisis is high as USD 2.6 billion per annum due to AFM1 (Kachapulula *et al.,*2017).

Seasonal variations of AFM1 toxicity:

The Aspergillus fungus survival capacity, ranges from 10 °C to 50 °C temperatures, wide range of pH (1.7–9.3), ideal relative humidity of 85%-95%, boosting the production in darkness and in less CO_2 concentration and a greater O_2 concentration can cause intoxication to humans and animals (Chang *et al.,*2002; Mahbobinejhad *et al.,*2019). Temperature and moisture contents also affect the presence of aflatoxin B1 in feeds. A. flavus and A. parasiticus can easily grow in feeds having moisture between 13% and 18% and environmental moisture between 50% and 60%, furthermore, they can produce toxins. Another reason of low AFM1 level in summer may be attributed to out-pasturing of milking cattle (Hussain & Anwar, 2008). Higher concentration of AFM1 in winter seasons have been seen high as compared to summer seasons, the reason being in winters mostly milking animals are fed with compound feeds and thus concentration of aflatoxin B1 increases which in turn enhances AFM1 concentration in milk (Akbar *et al.,*2020).

Methods to detect AFM1:

Various analytical methods for detecting AFs in food samples have been mostly used including high performance liquid chromatography (HPLC), Thin-layer chromatography (TLC), Gas chromatography–mass spectrometry (GC–MS), fluorescence, tandem mass spectroscopy, and Direct Analysis in Realtime mass spectrometry (DART-MS) due to low detection limits, the ability to give structural information of the analytes, and to cover a wide range of analytes differing in their polarities (Aramendía *et al.,*2010). These methods show some limitations, such as accurate determination of aflatoxins concentrations is difficult, less accuracy, very expensive equipment, extensive sample pretreatment, slowness, and the need of advanced human skills (Rosi *et al.,*2007).

Enzyme-linked immunosorbent assay (ELISA) based methods have been used widely to measure aflatoxins as ELISA is cost effective, the quickest and simplest method with good sensitivity, requires only a small sample volume for analysis, high precision and optimal recovery (Bilandžić *et al.,*2016). ELISA offers many advantages including shorter analysis time, absence of complicated sample preparation steps, ELISA kits with different detection limits and simplicity of the analytical procedure in comparison to time-consuming and expensive chromatographic techniques (Kos *et al.,*2016). Direct competitive chemiluminescent ELISA (CL-ELISA) has been developed for improved sensitivity, a detection limit of 0.001 ng/mL of AFM1 has been achieved

(Vdovenko *et al.,*2014). ELISA kit is intended for the analysis of raw milk, skimmed milk powder, milk powder, cheese and butter (Veratox® Aflatoxin M1, Product: 8019) AFM1 residues (Jakšić *et al.,*2021).

Methods to reduce AFM1 toxicity:

The methods are divided into biological, chemical and physical methods for the decontamination of aflatoxins. These must be cost-effective to keep the fair market price (Cao *et al.,*2021).

Chemical Methods:

Chemical methods are based on chemical reactants that inactivate and degrade aflatoxins either by oxidation and hydrolysis of the lactone ring from the polyketide backbone of aflatoxins, or by oxidation of the double bond of the terminal furan ring. In spite of that, these agents are linked to the problems with their residues (Maggira *et al.,*2022). Ozone is found to destroy furan rings with the formation of primary ozonide's while ammonia and different bases such as potassium hydroxide and sodium hydroxide have been used in seed treatment to decontaminate AFB1 (Ji & Xie, 2020).

Physical Methods:

Physical methods include adsorption, heating, boiling, baking, cooking, roasting, and radiation but due to the limited solubility of aflatoxins, these processes become impractical and economically ineffective (Müller *et al.,*2018). Adsorption includes the binding of a toxic compound, to the adsorbent compound, during digestion in the gastrointestinal tract of farm animals. Adsorbent compounds include active carbon, kieselguhr, alumina clay, alumina bentonite, montmorillonite, zeolite, cellulose, glucomannans, peptidoglycans, polyvinyl pyrrolidone, and its derivatives (Khadem *et al.,*2012). Controlled conditions of storage, such as proper air humidity, ventilation, temperature control, and packaging techniques, reduce fungal growth and mycotoxin accumulation (Huang *et al.,*2020). Drying of farmed feed is a productive measure against fungal growth and aflatoxin production soon after the harvest (Kamala *et al.,*2018). Cold plasma uses a low-temperature plasma (non-thermal technology), produced by the electrical discharge in gases or reduced pressures (sub atmospheric pressures) effectively removed AFB1 by 66% after 10 min (Wielogorska *et al.,*2019).

Biological Methods:

The available physical and chemical AF removal and detoxification methods have effects on nutritional status, food safety and change traditional organoleptic properties of the fermented product, forming the toxic metabolites and by-products. Biological decontamination can be done by food fermentation, employing modified strains of Aspergillus to remove aflatoxins by competitive inhibition, or by applying genetically modified plants e.g., in Africa, Central America and Asia, transgenic maize (Bt corn) is used for removal of mycotoxin contamination (Hamza *et al.,*2019). Food supplement (NovaSil clay) can be used to absorb aflatoxins in the gastrointestinal tract for reduction of aflatoxins. Antioxidant compounds in animal feed, for example chlorophyll and its derivatives, selenium, waste from wine production, medicinal herbs and plant extracts can

be used to eliminate the toxicity of aflatoxins (Juglal *et al.,*2002). Feeds containing polyphenolic compounds specifically of the flavonoid group, can weaken the aflatoxin-induced inflammatory process by regulating the activities of NF-κB and Nrf2 signaling pathway (Muhammad *et al.,*2018). Fermentation for the removal of aflatoxins has been proven to be effective but biological control may not be appropriate to some foods and feeds (Zhang *et al.,*2019). The use of microbial laccase enzymes, catalase, oxidase enzymes, manganese peroxide and *Aspergillus* enzymes like chitinases and β-1,3-glucanases can be effective for AFB1 reduction (Meerpoel *et al.,*2018). The use of microorganisms to degrade mycotoxins in feed and food has been widely employed, including bacteria such as lactic acid bacteria (LAB) isolated from yoghurt or milk, and other species of bacteria, such as *Flavobacterium aurantiacum*, *Micrococcus luteus* and *Bacillus subtilis* and fungi such as *Saccharomyces cerevisiae* etc (Xia *et al.,*2017). Polysaccharides and peptidoglycans present in the cell walls of bacteria were involved to cause the aflatoxins binding with the help of microorganisms with the production of microorganisms with the production of little or no toxic intermediates and end products (Umesha *et al.,*2007).

Emerging and green strategies

Other processes include High-pressure processing (100 to >1000 MPa of pressure) (HPP), pulsed electric field (80 kV/cm to 100 V/cm) (PEF), and also ultrasound have been proven as emerging and green strategies effective in controlling fungi and aflatoxins in field and post-harvested crops (Mirza Alizadeh *et al.,*2021). Nanoparticles (NPs) such as chitosan-coated NPs of Fe_3O_4, silver NPs, and magnetic carbon nanocomposites have been applied to remove AFB1 (Tarazona *et al.,*2019).

The main objective of the present study was to detect and quantify the percentage contamination of aflatoxin M1 (AFM1) in various branded and local milk samples, and to compare these results in terms of summer & winter seasons and contamination in branded versus non-branded milk samples using IBM SPSS Statistics 28.0 for windows. Biological method was also applied to decontaminate the AFM1 spiked milk samples by using the Lactic acid bacteria (LAB), isolated from local yoghurt sample.

Materials and Methods:

Branded and non-branded raw milk (n=100), purchased from supermarkets and shops of Lahore, Pakistan. Of these, 50 during summer (25 branded and 25 non-branded) and total 50 during winter (25 branded and 25 non-branded) were collected in year 2022. All samples were homogenized, subsampled and stored at 2-8 °C (35-46 °F) until AFM1 analysis was done.

Aflatoxin M1 detection by ELISA

ELISA kit used for AFM1 detection was refrigerated at 2-8 ˚C and all the materials provided with kit and the materials not included with kit were arranged before performing Veratox® for AFM1.

Sample preparation and extraction

Liquid milk sample extraction was done by centrifugation of milk samples to separate fat for 10 minutes at 3500 rpm at 10°C. Atter centrifugation, defatted supernatant (bottom layer) was collected as upper fatty layer can adversely impact the outcome of assay by adding an additional matrix to sample. The skimmed milk was used as sample for further testing procedure.

Test procedure

After mixing, each solution was transferred to a different antibody-coated wells and 100 µL of each skimmed milk samples were added to respective wells and kept at room temperature for 30 minutes. After shaking for 10 minutes at room temperature (or 600 rpm for 20 mins), each well was washed for three times and discarded with AFM1 washing buffer which was made as 1:3 in distilled water. Bluer color indicates less AFM1. Antibody wells were dried with help of paper towel and 100 µL AFM1 substrate was added to well and kept for 15 minutes with continuous shaking. Then 100 µL AFM1 red stop solution was added to each well. After 15 minutes all wells containing the samples and controls were put in ELISA reader (Model 680 Microplate Reader S/N 17007) and AFM1 concentration was checked at 650 nm. Optical density values obtained from the reader were put in the calibration curve of controls in computer software to get preliminary result which was divided by 1000 to get AFM1 values in parts per billion (ppb). The controls /standard AFM1 solutions of 0, 5, 15, 30, 60 and 100 ppt, in the test kit were simultaneously used for comparison to assure the validity of results (Maggira *et al.,*2021).

Synthesis of LB Agar and yoghurt preparation

Every instrument and the media were autoclaved at 120°C for 2 hours prior to use for the isolation of LAB. Lysogeny Broth/Luria Broth (LB) agar was made by mixing 1g of yeast extract, 2g peptone/tryptone, 2g of sodium chloride, and 4g of agar by making a volume of 200 ml with distilled water in 250 ml flask. Flask containing LB agar was autoclaved after labelling and covering up with cotton and then aluminum foil.

Local yoghurt sample was purchased from local market of Lahore. Sterile saline solution (0.85%, pH:7.0) was autoclaved to make the four serial dilutions. For this purpose, four test tubes each containing 9 ml autoclaved saline were taken (named test tube 1,2,3,4), 1 ml of yoghurt sample was put in test tube 1 and mixed thoroughly. After that 1 ml of yoghurt sample was taken from test tube 1 and then was put in test tube 2 and so on in such a way that four serial dilutions were made in all test tubes (İspirli & Dertli, 2018).

Lactic acid bacteria isolation

LB agar was put in four glass petri-dishes for bacterial growth carefully in the chamber to avoid contamination. After 5 minutes, 100 µL of four yoghurt serial dilutions from test tubes was spreaded on four petri-dishes containing LB agar with spreader, respectively. After spreading, bacterial plates were sealed with parafilm tape to avoid contamination and kept at 37 °C for 1 day. Further dilutions of LAB were done from original LAB containing plates to obtain the pure colony

from each glass plate by streak plate method. After obtaining pure colony, liquid culture was obtained by putting single colony into LB broth (without agar) and incubated at 37 °C for 1 day.

Glycerol stock of lactic acid bacteria

Glycerol stock solution of four LAB was made by mixing 800 µL of LAB liquid culture (LB broth culture, without agar) in Eppendorf tube containing 200 µL 80% glycerol. Four Eppendorf tubes of four LAB cultures were obtained and store at -20°C.

Gram's staining of lactic acid bacteria

Smear suspension was prepared of all LAB strains on clean glass slide with help of sterilized platinum loop and was allowed to air dry. Heat fixation was done by moving slide to and fro on blue flame. The counterstain safranin was poured for about 1 minute and then was washed with water. Observations were done to check the color change and type of bacteria present after air drying the slide.

Application of LAB on Milk

Four lactic acid bacteria namely; *L. rhamnosus, L. bulgaricus, L. plantarum* and *S. thermophilus* were allowed to make complex with aflatoxin in milk by spiking in presence of Phosphate Buffered Saline buffer (PBS) (1X, pH 7.2). First of all, bacterial pellet was obtained by centrifuging 1ml fresh bacterial culture at 5000 x g for 10 minutes for each bacterial strain. Skimmed milk was obtained by centrifuging raw milk at 3500 x g for 10 minutes. In Eppendorf tube containing bacterial pellet, 1ml skimmed milk spiked with 10 µL of 50 ppb standard AFM1 and 50 µL of PBS buffer were added for each strain of LAB. Eppendorf tube was incubated aerobically at 37 °C to study the binding efficiency of LAB strains after 24h.

Results:

Detection of AFM1 by ELISA

Measuring the optical densities of each sample at 650 nm, optical densities' values were compared with calibration curve of AFM1 controls 0 ppt, 5 ppt, 15 pp, 30 ppt, 60 ppt, and 100 ppt to get preliminary result.

Preliminary result values obtained of AFM1 controls, were divided by 1000 factor to convert into parts per billion (ppb). AFM1 values (ppb), after comparing with controls and dividing preliminary values by 1000, total 50 during summer (25 branded and 25 non-branded) and total 50 during winter (25 branded and 25 non-branded) detected through ELISA. AFM1 controls (ppt) with their optical densities and preliminary results is shown in Table 1.

Table 1 AFM1 controls (ppt) with their optical densities and preliminary results

AFM1 controls (ppt)	Optical density	Preliminary result

0	1.491	0
5	1.424	5.32
15	1.322	11.71
30	0.965	36.79
60	0.706	64.59
100	0.548	91.17

Aflatoxin M1 detection during summer

AFM1 detection during summer season, total 50 milk samples (25 branded and 25 non-branded) detected through ELISA, are given in Table 4.2.

Table 2 AFM1 concentration (ppb) in total 50 during summer (25 branded and 25 non-branded)

** = AFM1 values beyond permissible levels according to USFDA, and ND* = Not Detected

Branded milk samples (Summer)	**AFM1 concentration (ppb)**	**Non-branded milk (Summer)**	**AFM1 concentration (ppb)**
1	0.0331	**26**	0.048
2	0.0455	**27**	0.0652
3	0.0422	**28**	0.0258
4	0.0199	**29**	0.0465
5	0.0281	**30**	0.0819
6	0.0315	**31**	0.0492
7	0.0463	**32**	0.0294
8	0.0265	**33**	0.0188
9	0.0173	**34**	0.0344
10	0.0475	**35**	1.41**

11	0.0397	**36**	0.0148
12	0.0189	**37**	0.0241
13	ND*	**38**	0.0295
14	0.046	**39**	0.0394
15	0.0158	**40**	0.0261
16	0.045	**41**	0.0258
17	0.0374	**42**	0.04784
18	0.0421	**43**	0.0241
19	0.0335	**44**	1.251**
20	0.0378	**45**	0.0753
21	0.0263	**46**	1.971**
22	0.0351	**47**	0.0498
23	0.0107	**48**	0.0258
24	0.0199	**49**	0.0239
25	0.0289	**50**	0.0147

Aflatoxin M1 detection during winter

AFM1 detection during winter season, total 50 milk samples (25 branded and 25 non-branded) detected through ELISA, are given in Table 4.2.

Table 3 AFM1 concentration (ppb) in total 50 during winter (25 branded and 25 non-branded)

** = AFM1 values beyond permissible levels according to USFDA, and ND* = Not Detected

Branded milk samples (Winter)	**AFM1 concentration (ppb)**	**Non-branded milk (Winter)**	**AFM1 concentration (ppb)**
1	0.0342	**26**	0.0286

2	0.0323	**27**	0.891**
3	0.0327	**28**	0.0176
4	0.0292	**29**	1.084**
5	0.0321	**30**	0.0413
6	0.0346	**31**	0.021
7	0.0286	**32**	0.0367
8	0.0236	**33**	0.0281
9	0.0227	**34**	0.0234
10	0.0128	**35**	0.0129
11	0.0618	**36**	0.0184
12	ND*	**37**	0.038
13	1.299**	**38**	1.398**
14	0.0443	**39**	0.0861
15	0.0295	**40**	0.0294
16	1.221**	**41**	0.0167
17	0.0492	**42**	0.0132
18	1.645**	**43**	1.987**
19	0.0386	**44**	0.0474
20	0.0315	**45**	0.0241
21	0.0426	**46**	0.0422
22	0.0306	**47**	0.0489
23	0.0248	**48**	0.0149
24	ND*	**49**	0.709**

25	0.0481	**50**	0.0174

Branded milk samples number, exceeding the USFDA prescribed limits are 3 out of 50, while non-branded milk samples are 9 out of 50 and it can be stated that the field crops or stored feed have high amount of AFB1 which is hydroxylated into AFM1 in liver of lactating animals as elaborated in Figure 4.2.

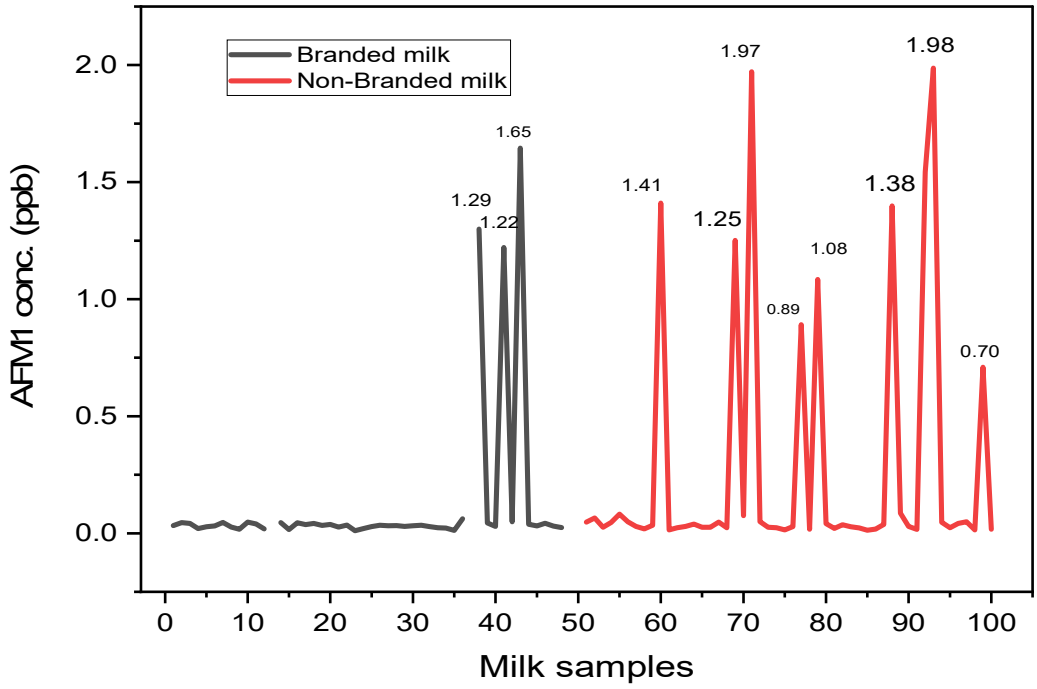

Figure 4.2: AFM1 conc. (ppb) exceeding the USFDA limits

AFM1 contamination in branded milk samples indicates that mostly samples have passed the both the EU MRL (European Union Maximal Residual Limit) and USFDA (United States Food and Drug Administration) MRL, showing only 8% and 6% samples exceeding MRL respectively. As mostly countries including Pakistan are following USFDA limits therefore we consider the levels as set by FDA. Statistical analysis of the difference in contamination between branded and non-branded milk samples was done by IBM SPSS Statistics 28.0 for windows. Maximum AFM1 concentration (ppb) was found to be 1.645 and a mean of 1.388 with standard deviation ±0.255. Local milk samples in contrast, showed more AFM1 contamination of 26% and 18% samples exceeding EU MRL and USFDA MRL respectively. Maximum AFM1 concentration in non-branded milk samples was 1.971 and mean of 1.360 with SD ±0.438 as described in Table 4.3.

Table 4 Branded and non-branded milk samples (n=100) with AFM1 positive samples according to USFDA with MRL in accordance to EU and USFDA and AFM1 contamination value (ppb) in terms of maximum value, median and Mean±SD

Milk type	Total samples	Positives/ (%)	Exceeding EU MRL (>0.05 ppb) no (%)	Exceeding USFDA MRL (>0.5 ppb) no (%)	AFM1 concentration in positive samples (ppb)		
					Maximum	Median	Mean ±SD
Branded milk	50	3/6.0%	4/8.0%	3/6.0%	1.645		1.299 1.38±0.255
Non-branded milk	50	8/16%	13/26%	8/16%	1.971		1.324 1.33±0.462

Aflatoxin M1 detection during summer and winter:

AFM1in branded and non-branded milk samples during summer:

Branded milk samples tested during summer season show no AFM1 contamination exceeding the USFDA prescribed limits while non-branded milk samples (3) are exceeding the AFM1 range as set by the USFDA. First 25 are the branded milk samples in black shade while the remaining are non-branded milk samples shaded in red as shown in Figure 4.3:

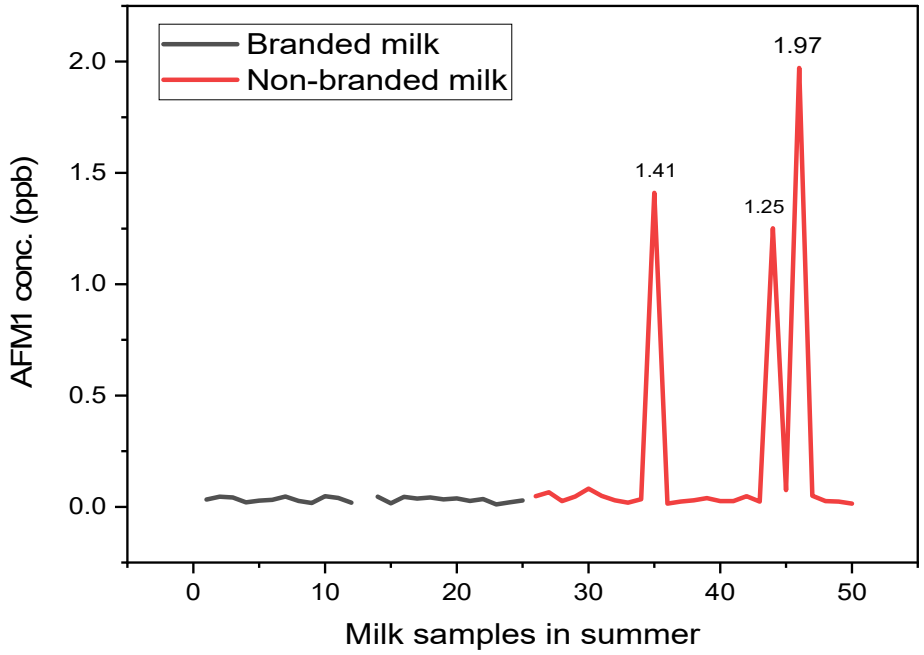

Figure 4.3: AFM1 concentration (ppb) during summer season

AFM1in milk samples during winter

Branded and non-branded milk samples collected during winter season have AFM1 contamination exceeding the USFDA limits. Three branded milk samples and six non-branded milk samples are showing AFM1 concentration greater than 0.5 ppb which indicates that during winter seasons mycotoxins attack stored grains and compound feed on which milking animals depend as there is no fresh grass and pastures during harsh winter season as shown in Figure 4.4.

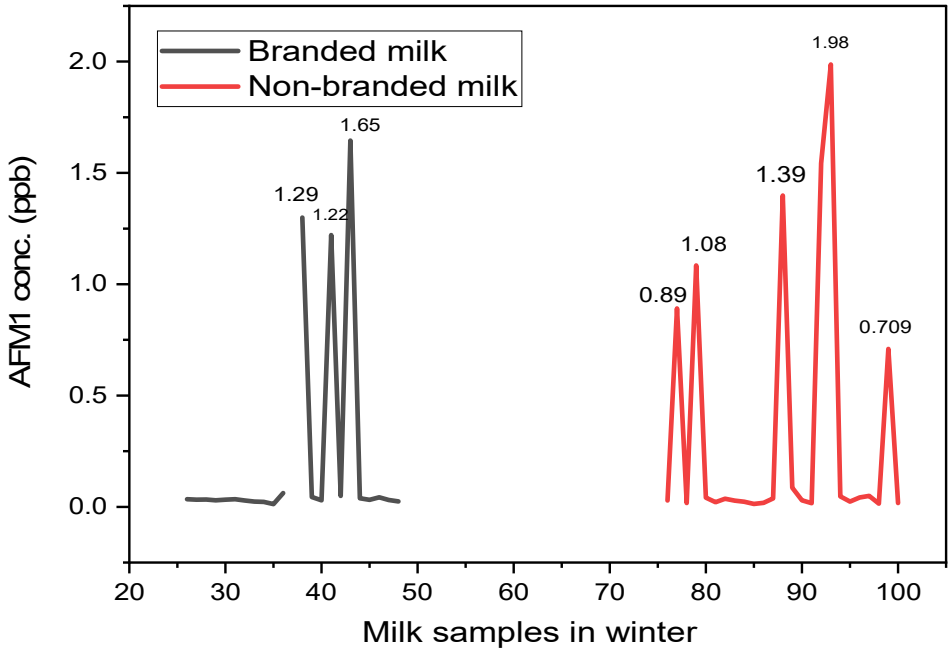

Figure 4.4: AFM1 concentration (ppb) during winter season

According to Table 4.1, it is shown that AFM1 contamination can be seen mostly in milk samples, collected in winter, whether branded or non-branded according to USFDA prescribed limits. Statistical analysis of the difference in contamination between branded & non-branded milk samples during summer & winter was done by IBM SPSS Statistics 28.0 for windows. Data shows 0.0% contamination in summer and 6.0% contamination in winter of branded milk samples while in local milk samples, 12% and 24% contamination are seen during summer and winter seasons respectively. These percentage contaminations of AFM1 during summer and winter seasons of branded and non-branded milk samples according to USFDA limits is shown in Table 4.4 as follows.

Table 5 Percentage contaminations of AFM1 during summer and winter seasons of milk samples according to USFDA

Milk type	Summer season samples	Winter season samples	Positives during summer/ (%)	Positives during winter/ (%)	AFM1 max concentration in milk samples (ppb)	
					Summer	Winter

Branded milk	25	25	0/0.0%	3/6.0%	0.0475 1.645
Non-branded milk	25	25	3/12%	5/20%	1.971 1.987

LAB identification Gram's staining, Morphological tests and Biochemical tests:

Gram staining showed blue/purple color indicating that all strains are Gram-positive. Shapes of mostly bacterial strains were rods (bacilli) and round (cocci). *Streptococcus thermophilus* was identified on morphological bases like being Gram-positive, non-motile, spherical or ovoid cells, varying length of chains or pairs, and no spore forming. Another Gram-positive bacterium with purple color was identified as *Lactobacillus bulgaricus* which appeared long, creamy-grey, non-motile non-spore forming, rod-shaped, and filamentous under microscope. Two Gram-positive bacteria namely *Lactobacillus plantarum* with rod cells including rounded ends as single or in pairs or in short chains and *Lactobacillus rhamnosus* with single rod or in short chains were also identified in the prepared glass plates after performing Gram's staining. Biochemical tests were performed to confirm these four bacteria isolated from yoghurt in plates. Catalase test done by using drops of 3% hydrogen peroxide on bacterial culture with oxygen gas bubbles formation as positive test and was not shown positive by any of these bacteria as being fermentative and anaerobes. Similarly, sulphide indole and motility (SIM) test was also shown negative by these bacteria.

Decontamination of aflatoxin M1 by lactic acid bacteria

L. rhamnosus showed the highest percentage decontamination of 78.7%. Other Gram-positive bacteria including *L. bulgaricus*, *L. plantarum*, and *S. thermophilus* showed %bound AFM1 efficiency of 54.4%, 50.50%, and 37.10% respectively with mean value of 55.18 and standard deviation of ±0.173.

$$\%\text{age Bound AFM1} = [\,1 - (\text{AFM1 detected}/\text{AFM1 spiked}) \times 100]$$

Table 6 Percentage decontamination shown by different lactic acid bacteria after 24 h incubation time with Mean±SD of 55.18±0.173

Micro-organism	**AFM1 spiked (µg/ml)**	**Time (h)**	**AFM1 detected (µg/ml)**	**Decontamination (%)**
L. rhamnosus	10	24	2.13	78.70

L. bulgaricus	10	24	4.56	54.40
L. plantarum	10	24	4.95	50.50
S. thermophilus	10	24	6.29	37.10

Discussion:

This study was aimed to detect the presence of aflatoxin M1 in various milk samples, finding percentage contamination in milk samples to ensure public health safety, via ELISA kit method. As reported in literature, HPLC-MS is still considered as gold standard and its lower detection limits of aflatoxin M1 confirmation in milk samples (Esam et al., 2022). Heating and ozone gas are the popular physical methods used for decontamination. Furthermore, AFM1 can be removed by several bacteria and other probiotics (Mohammadi et al., 2017). As reported earlier in a study, the immobilized *Saccharomyces boulardii* could remove AFM1 92% in milk within 40 min but *S. boulardii* in combination with the LAB could remove more than 99% of AFM1 (Corassin et al., 2013). In another study, with a lower degree of temperature (4 °C) increased the microorganism ability (100.0±0.58%) to bind AFM1. In this regard, the maximum ability of *L. rhamnosus* to bind AFM1 was observed at a (91.33% and 91.82% at 4°C and 37 °C, respectively) which was more than those of *L. plantarum*, (89.33% and 84.62%). This result could be due to the thick layer of peptidoglycan in the cell wall of *L. rhamnosus* (Khadivi et al., 2020). Another research reports AFM1 binding of *Lactobacillus plantarum* bacteria to reduce AFM1 toxicity in milk which was contaminated artificially. The binding success rate of AFM1 to *Lactobacillus plantarum* cells was good and averaged 80% that was also proven by earlier studies indicating the ability of lactic acid bacteria to reduce the amount of aflatoxin in feed for animals and dairy products (Pavlek et al., 2021). The progress of different results by the microorganisms indicates that various binding sites are linked with different strains and binding sites in each probiotic. Plasma membrane polysaccharide and peptidoglycan are involved for the physical removal of aflatoxins by probiotics (Haskard et al., 2001). The percentage of the complex of AFM1 and *L. rhamnosus* was showed in the PBS medium reached 79.2% and 71.6% after 4 and 8 h of incubation, respectively (Elsanhoty et al., 2014). As reported in literature, major active organisms discovered are lactic acid bacteria (LAB) specifically *Lactobacillus acidophilus, L. bulgaricus*, and *L. plantarum* which can irreversibly metabolize and degrade aflatoxins B1, G1 and M1. Many aflatoxin degradation reactions occur in presence of different enzymes like peroxidase which have been found very effective (Xia et al., 2017). The concentration of AFM1 in milk in winter was higher (196 vs. 39.5 ng/ L) than that in summer. AFM1 concentrations in UHT milk were lower than those in pasteurized milk and ESL milk in winter and summer and the children had the highest risk of exposure to milk AFM1 as compared to the elderly aged people (Xiong *et al*., 2021). In this study lactic acid bacteria strains showed less AFM1 binding capabilities than reported in literature. This might be because of variations in hereditary makeup, environmental conditions, cultural parameters, strain-specific properties, systemic methodologies, cross-reactivity with other

compounds, and post-harvest handling of strains can all contribute to differences in AFM1 binding capabilities observed among different strains of lactic acid bacteria. Further research into understanding these variables is fundamental for optimizing LAB-based techniques for mycotoxin removal.

Conclusion:

Aflatoxin contamination in food chain is a warning to public health safety. Recent advances in technology have revolutionized the field of drugs, medicines and diagnostic tools. Nowadays many techniques and standard procedures are available to check the contamination in food items. Aflatoxin M1 in milk of lactating animals is being detected through ELISA, TLC, HPLC-MS, and HPLC-FLD. Every technique has its advantages over others being sensitive, having lower detection limits, lower sample load, less sample processing, and accurate. ELISA is considered a routine analysis for the detection of aflatoxin M1 in milk and milk related products. Physical, chemical, and biological methods are also available for the removal of AFM1 and reducing its toxicity. All methods can be employed based on retaining the nutritional value of the milk being analyzed for decontamination of AFM1. Additionally, the current study shows that aflatoxin in milk is found more as compared in summer season because during winter there is more chance of fungal contamination in stored feed for cattle. But during summer fresh feed is available for cattle and thus there is lower chance of aflatoxin contamination in milk due to fungus. In future, more advanced methods should be adopted for removal of AFM1 from milk in such a way that nutritional quality of milk remains intact without changing its chemistry. As ELISA is used for routine analysis and has lower detection limits therefore HPLC-FLD is considered a gold standard for detection of very low aflatoxin but is very expensive, time consuming and requires trained personnel.

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