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ASSESSMENT OF AFLATOXIN M1 (AFM1) IN MILK FROM CHAKWAL CITY USING ELISA TECHNIQUE

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ABSTRACT

Aflatoxins are a group of mycotoxins produced primarily by fungi *Aspergillus flavus* and *Aspergillus parasiticus* that commonly contaminate crops. When livestock such as cattle eat feed contaminated with aflatoxin B1 (AFB1), the compound is metabolized in the animal's liver to aflatoxin M1 (AFM1). AFM1 is excreted in milk following contamination, representing a potential health hazard to humans who consume milk or dairy products containing elevated levels of this mycotoxin. The presence of AFM1 in milk is of concern due to toxicological effects, including various adverse health consequences. The purpose of this study was to assess the prevalence of aflatoxin M1 (AFM1) in raw milk samples collected from Chakwal city. 100 raw milk samples were collected from the main milk production areas, which are the main source of milk in the city. The evaluation of AFM1 in the milk samples is performed by using Enzyme-Linked Immunosorbent Assay (ELISA) kit. All 100 raw milk samples were found to be contaminated with AFM1, whereas 99% of the samples showed above the limits of the European Commission (EC), while 1% were below the limits. According to US regulations, 1% of samples exceeded the AFM1 acceptable limit, though the US limit is ten times higher than the EU limit. Maximum permissible limits prescribed by the Pakistan Standards and Quality Control Authority (PSQCA) were met by all samples. AFM1 concentrations ranged between a minimum of 050ng/a maximum of 750ng/L/L. All raw milk samples contained AFM1 contamination. While the concentration in all samples was below the Pakistan Standards and Quality Control Authority (PSQCA) limit of 10,000ng/L (10µg/L), 99% of the samples exceeded the European maximum allowable limit. These findings highlight the critical need to implement stricter measures to mitigate AFM1 contamination in milk produced in the city of Chakwal, as high levels of this mycotoxin can cause significant public health risks.

***Keywords*:** AFM1, Aflatoxin, Mycotoxin, Raw Milk, ELISA

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1. INTRODUCTION

More than 300 mycotoxins have been identified so far, with aflatoxins being the most significant in terms of human health and agricultural impact. These climate-sensitive toxins are produced by various Aspergillus species and pose severe health risks as well as substantial economic losses (El-Sayed et al. 2022). Moreover, there are approximately 20 derivatives of aflatoxin (AF). AFM1 is the hydroxylated form of aflatoxin B1 (AFB1), found in milk and other foods. Eating food contaminated with AFM1 can be harmful to your health. It has been linked to cancer, genetic mutations and birth defects. Furthermore, it weakens the immune system and causes DNA damage (Muaz et al. 2022).

Aflatoxin has been linked to liver cancer, genetic mutations and immune diseases. AFM1 is present in the milk of animals consuming feed contaminated with AFB1. There are several methods for detecting AFM1, including TLC, HPLC, and ELISA. ELISA is preferred due to rapid results and minimal sample requirements. This is also a cost-effective method for aflatoxin detection (Luqman et al. 2024). Dairy cows fed aflatoxin-free feed typically require approximately 72 hours to produce aflatoxin-free milk. However, the cancer risk associated with AFM1 is only about 2-10% of that of AFB1 (Creppy 2002). Monitoring of AFM1 in dairy products and milk products is critical. Ensuring food safety helps protect public health from its harmful effects (Naghshbandi et al. 2023). Food safety is a major risk for agribusiness, which has a social responsibility to ensure product safety. They must follow guidelines set by food safety authorities and go beyond basic regulatory requirements. Implementing additional security measures helps protect consumers and maintain industry standards (Nganje et al. 2021). Contamination of

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milk by mycotoxins affects food safety, public health, and the economy, affecting agriculture and global trade. Because of these risks, mycotoxins are among the most regulated natural toxins worldwide (Meneely et al. 2023).

Milk is a vital source of protein for all ages and plays a crucial role in the dairy industry. It is rich in essential nutrients such as calcium, magnesium, vitamin B12, and energy to support overall health. Various dairy products, including cream, cheese, yogurt, ice cream, milk powder and ghee, contribute significantly to the daily nutritional requirements (Mahfuza et al. 2024).

Pakistan is the third-largest producer of milk in the world, with an annual production of 57 million tons. Most of this (approximately 80%) comes from small farms in rural areas, with 15% in suburban areas and the remaining 5% in urban areas. Despite its significant nutritional value, about 95-97% of milk consumed in the country is unpackaged as shown in Fig. 1, raising hygiene concerns (Khalid et al. 2024).

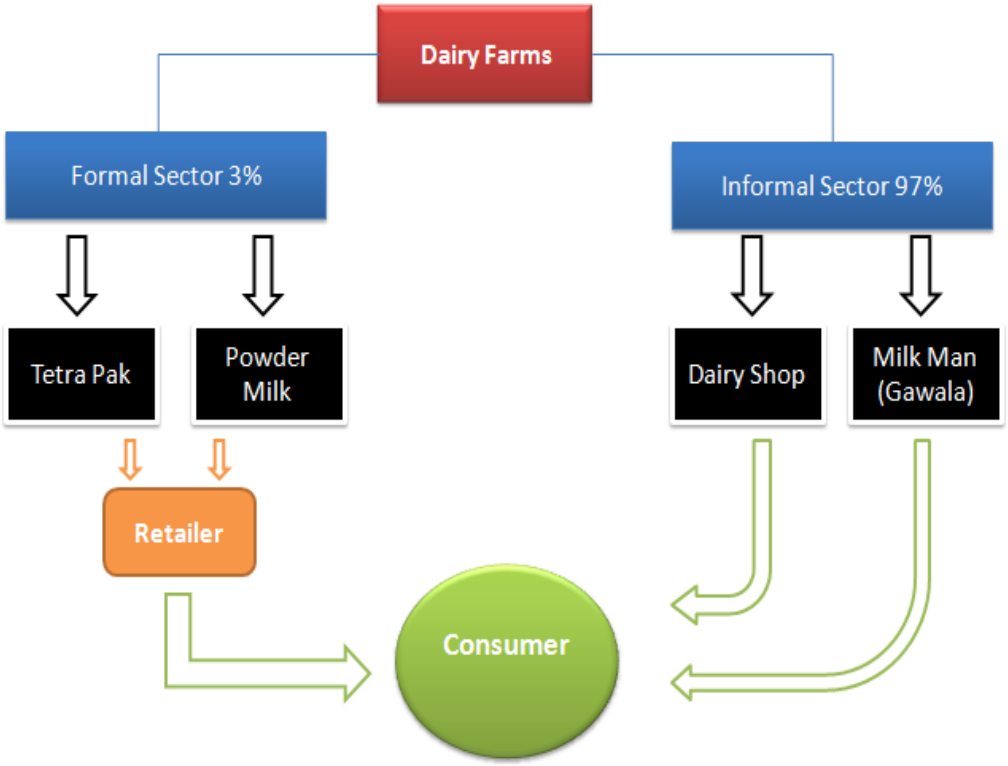

Fig. 1: Milk supply chain distribution in various sectors of Pakistan.

The maximum permitted levels of AFM1 in raw milk vary from country to country based on risk assessment and economic factors. For example, in the EU, the limit for liquid milk is 50ng/L. In comparison, the United States and many Asian countries have a higher threshold of 500ng/L, while Pakistani regulations allow an upper limit of 10µg/L, which is significantly higher than the EU's allowable value (Anukul et al. 2013; Van de Perre et al. 2015; Ashiq 2015).

One of the most effective methods of monitoring AFM1 levels in milk is through an enzyme-linked immunosorbent assay (ELISA). This method is fast, simple, has hypersensitivity, high degree of accuracy, and reliable recuperation (Cai et al. 2023).

The presence of toxins in milk can cause serious health problems for consumers. This concern prompted us to assess AFM1 levels in milk from various dairy farms in Chakwal city, on which the city is heavily dependent for its milk supply.

To assess AFM1 contamination, multiple analytical techniques, including high-performance liquid chromatography (HPLC), capillary electrophoresis, and ultra-performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) can be used. However, in this study, we chose to use an ELISA method to detect AFM1 in milk samples. This choice was made because ELISA is readily available in our laboratory and provides a faster, more reliable, and cost-effective method for measuring these toxic compounds.

2. MATERIALS AND METHODS

This research was done at the Food Microbiology laboratory and the Hi-Tech Laboratory of the Chemistry Department at the University of Engineering and Technology, Lahore. Milk samples were collected from nearby dairy farms of Chakwal city for the assessment of AFM1.

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2.1. Collection of Milk Samples

For this research study, we chose Chakwal city, the capital of Chakwal region of Punjab province, Pakistan. It is the 66th most populous city in the country and is recognized as one of the largest milk-producing city in Pakistan.

Usually, in Winter, the probability of milk contamination with Aflatoxin M1 increases. Therefore, in the months of January and February, when it's Winter season in Pakistan, we collected 100 raw milk samples from 20 different dairy farms around Chakwal, which are the main milk suppliers to the city (Table 1). To determine the amount of Aflatoxin M1 in the samples, five samples were collected from each farm using a simple random sampling technique. Samples were collected in sterile bottles to prevent contamination during collection. Sterility is crucial in ensuring that the sample accurately represents the original condition of the material being studied, without interference from extraneous microorganisms or substances. To maintain integrity and slow down any potential biochemical processes, such as bacterial growth or chemical degradation, samples were transported in a refrigerated environment.

In the laboratory, the samples were stored at −20°C to preserve their components, particularly in cases where further analysis is to be conducted at a later time. At −20°C, enzymatic activity is significantly slowed, and microbial activity ceases, preventing any degradation or alteration of the sample's composition. This storage method is crucial in ensuring that the analysis results are representative and reliable, thereby minimizing the risk of degradation or contamination during the period between sample collection and analysis.

2.2. Assessment of Aflatoxin M1

The ELISA method was used for quantitative analysis of AFM1 using a competitive assay with an AFM1 kit (RIDASCREEN; R-Biopharm AG, Darmstadt, Germany). The kit is characterized by several key features, including a detection limit of 5 ng/L, 95% recovery, and 100% cross-reactivity for AFM1, as well as 30% cross-reactivity for AFM2. The standard solutions used for calibration included concentrations of 0, 5, 10, 20, 40, and 80ng/L.

This test is based on the principle of antigen-antibody reaction. The wells of a microtiter strip dedicated to AFM1 were coated, and 100µL of the prepared sample or standard solution was added to each well. After gently shaking the plate, it was incubated for 30 minutes at room temperature in the dark, allowing the antibodies to bind. Subsequently, the wells were washed with 250µL of wash buffer, and the washing procedure was repeated twice to remove any excess liquid.

Following the washing steps, 100µL of peroxidase-conjugated AFM1 was added to the wells, where it was bound to any free antibodies. Any unbound conjugated AFM1 was removed by washing. Next, 100µL of substrate and chromogenic solution was added, gently mixed, and incubated for an additional 15min at room temperature in the dark. During this incubation, the colorless chromogen turned blue due to the bound enzyme conjugate. To complete the reaction, 100µL of 1N H_2SO_4 was added, resulting in a color change from blue to yellow (Assem et al. 2011).

Absorption was measured at 450nm using an ELISA plate reader (BioTek, Winooski, VT, USA). The absorption intensity was inversely correlated with the AFM1 concentration in the sample. To analyze the data, dedicated software (RIDA SOFT Win; R-Biopharm AG) was used. The sample was considered positive for AFM1 if its concentration was 5ng/L or greater.

2.3. Statistical Analysis

All statistical analyses were performed using SPSS for Windows 16.0.0 (SPSS Inc., 2007, Chicago, USA). A descriptive analysis of the data was conducted initially.

3. RESULTS AND DISCUSSION

3.1. Occurrence of AFM1

AFM1 was found in all 100 samples we tested, giving a 100% contamination rate. The content of AFM1 varied between 50 and 750ng/L (Table 1), with an average concentration of 287.8ng/L. According to the European Commission, the maximum residue limit (MRL) for AFM1 in milk is 50ng/L, while in the United States, the MRL for milk and dairy products is significantly higher at 500ng/L (Anukul et al. 2013; Van de Perre et al. 2015).

Of the 100 raw milk samples tested, all were found to be contaminated with AFM1. Of these, 99 samples (99%) exceeded the limits set by the European Commission, while only 1 sample (1%) fell within the acceptable range. As far as US regulations are concerned, 1 sample (1%) is above the permitted limit, while the other 99 samples (99%) are below the permitted limit. Although the maximum residue limits in the United States are ten times higher than those in the EU, this sample still exceeded the permitted limits. On the other hand, all 100 samples were below the maximum permissible limits prescribed by the Pakistan Standards and Quality Control Authority (PSQCA). Test results showed that each sample was contaminated with AFM1, with concentrations ranging from a minimum of 50ng/L to a maximum of 750ng/L, as shown in Table 2.

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Table 1: AFM1 level (ng/L) value of raw milk samples from different dairy farms.

Dairy Farm Code	Mean±SE (ng/L)	Range (ng/L)		No. of Samples Exceed limit (%)*
		Minimum	Maximum	
F1	253.0±37	054	404	5
F2	176.2±39	080	261	5
F3	282.2±29	056	431	5
F4	274.6±53	220	401	5
F5	323.6±32	177	458	5
F6	292.4±46	212	430	5
F7	259.6±33	124	480	5
F8	341.4±29	068	460	5
F9	326.6±51	139	427	5
F10	330.2±32	180	750	5
F11	313.8±53	098	411	5
F12	330.4±33	222	420	5
F13	282.8±37	062	425	5
F14	244.4±46	050	492	4
F15	313.4±52	230	382	5
F16	216.6±41	060	380	5
F17	322.8±35	180	425	5
F18	228.0±43	098	357	5
F19	321.0±39	151	430	5
F20	324.2±33	151	496	5

*European Union Limit 50ng/L; Five raw milk samples were collected from each dairy farm and AFM1 contamination 100% was detected in every sample.

Table 2: Summarized AFM1 level (ng/L) value of raw milk samples from Chakwal

Parameters	Results Obtained
Total No. of Samples	100
Total No. of Farms	20
Milk Samples	Raw Milk
AFM1 Contaminated Samples (%)	100
Mean±SEM* (ng/L)	287.8±39.0
Range (ng/L)	050-750
No. of Samples Exceed limit (%)[a]	99
No. of Samples Exceed limit (%)[b]	1
No. of Samples Exceed limit (%)[c]	0

[a]European Union Limit 50ng/L; [b]US Regulation Limit 500ng/L; [c]Pakistan Standard and Quality Control Authority (PSQCA) Limit 10000ng/L (10µg/L); *Standard Error of the Mean.

AFM1 is a global problem, and numerous studies have been conducted around the world using various methods to assess its content in milk. Table 3 summarizes the results of some studies that measured AFM1 using ELISA. The average AFM1 values found in our study were similar to the levels reported in Serbia (Tomašević et al. 2015) but lower than those observed in the Bangladeshi study (Tarannum et al. 2020).

Moreover, the mean AFM1 in raw milk samples of Brazil (Picinin et al. 2013) were detected as 19.50ng/L, Croatia (Eastern Part) (Bilandžić et al. 2014) as 20.60ng/L, in Indonesia (Yogyakarta) (Nuryono et al. 2009) as 8.53ng/L, in Iran (Hamadan) (Ghiasian et al. 2007) as 43.40ng/L, in Iran (Ilam) (Vagef and Mahmoudi 2013) as 43.98ng/L, in Iran (Sanandaj) (Mohamadi Sani et al. 2012) as 12.65ng/L, also Iran (Southern Iran) (Hashemi 2016) as 18.26ng/L, in People's Republic of China (Central- South China) (Xiong et al. 2020) as 15.9ng/L, in Spain (Leon) (Rodríguez Velasco et al. 2003) as 20.50ng/L, in Turkey (Kaysen) (Ertas et al. 2011) as 8.73ng/L. These results were under the tolerance limit of EU Commission (Van de Perre et al. 2015) as summarized in Table 3. Furthermore, the following studies were exceed the tolerance limit of EU Commission (Van de Perre et al. 2015), in Bangladesh (Tarannum et al. 2020) the mean AFM1 in raw milk samples were observed as 699.07ng/L, in Iran (Ahvaz) (Rahimi et al. 2010) as 60.10ng/L, in Iran (Qazvin) (Mohamadi Sani et al. 2012) as 90.00ng/L, in Lebanon (Assem et al. 2011) as 60.40ng/L, in Serbia (Tomašević et al. 2015) it was observed as 282.00ng/L, in Syria (Ghanem and Orfi 2009) as 143.00ng/L, and in Turkey (Kaysen) (Buldu et al. 2011) as 59.9ng/L.

These differences may be traced to changes in AFB1 levels in the feed consumed by dairy cows. Local weather conditions before and during harvest, as well as poor storage practices, can seriously affect feed quality. AFB1 is produced by certain molds that thrive in feed with a moisture content of 13 to 18% and in an environment with a humidity of about 50 to 60% (Unusan 2006). To lower the AFM1 matter in raw milk, dairy farmers should specify good administrative operations, and investigator should promote adequate bio-detoxifying procedures, (Carraro et al. 2014) have published that elected bentonites can safely and effectively purify AFM1 in milk and dairy products. In addition, the bacterium *Pseudomonas putida* has been disclosed to transmute AFB1 into a much less toxic compound (Samuel et al. 2014).

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Table 3: Results of AFM1 contamination in raw milk detected by ELISA from different countries.

Country	Samples tested	Contaminated Samples (%)	Mean (ng/L)	Range (ng/L)	No. of samples exceed limit (%)^	References
Bangladesh	50	35 (70)	699.07	22.79−1489.28	34 (97)	Tarannum et al. (2020)
Brazil (MG)	129	129 (100)	19.50	0.2-106	18 (13.95)	Picinin et al. (2013)
Croatia (Eastern part	194	47 (24.23)	20.60	3.7-162.3	13 (27.66)	Bilandžić et al. (2014)
Indonesia (Yogyakarta)	113	65 (57.52)	8.53	NR	0 (0.00)	Nuryono et al. (2009)
Iran (Ahvaz)	75	59 (78.67)	60.10	NR	27 (45.76)	Rahimi et al. (2010)
Iran (Hamedan)	186	119 (63.98)	43.40	10-410	14 (11.76)	Ghiasian et al. (2007)
Iran (Gilan)	90	56 (62.22)	NR	2.1-131	28 (50.00)	Rokhi et al. (2013)
Iran (Ilam)	54	34 (62.22)	43.98	10.03-85.24	31 (57.40)	Vagef and Mahmoudi (2013)
Iran (Qazvin)	288	163 (56.60)	90.00	10-250	113 (69.33)	Mohamadi et al. (2012)
Iran (Sanandaj)	240	226 (94.17)	12.65	0.01-115.9	10 (4.42)	Mohamadi Sani et al. (2012)
Iran (Southern Iran	135	64 (47.41)	18.26	0.00-99.92	20 (31.25)	Hashemi (2016)
Kosovo	96	43 (44.8)	NR	4.00-109	7 (7.3)	Camaj et al. (2019)
Lebanon	38	28 (73.68)	60.40	2.63-126	17 (60.71)	Assem et al. (2011)
China	133	100 (75.2)	15.9	5.3-36.2	0 (0.00)	Xiong et al. (2020)
Spain (Leon)	92	5 (5.43)	20.50	14-24.9	0 (0.00)	Rodríguez Velasco et al. (2003)
Serbia	678	540 (79.65)	282.00	NR	382 (70.74)	Tomašević et al. (2015)
Syria	74	70 (94.59)	143.00	20-690	41 (58.57)	Ghanem and Orfi (2009)
Turkey (Kayseri)	50	43 (86.00)	8.73	1-30	0 (0.00)	Ertas et al. (2011)
Turkey (Kayseri)	90	90 (100.00)	59.9	5-80	63 (70.00)	Buldu et al. (2011)

[a] European Union Limit 50ng/L. NR=Not Reported.

Several authors from Iran (Kamkar 2005; Nemati et al. 2010; Fallah et al. 2011; Vagef and Mahmoudi 2013; Mahmoudi & Norian 2015), Croatia (Bilandžić et al. 2014), Serbia (Tomašević et al. 2015) and Turkey (Golge 2014) noted that AFM1 levels tend to be higher in cooler months compared to hotter months. This increase may be due to dairy cows being fed more stored feeds during the winter, such as hay, corn, concentrates and silage, which are more likely to contain AFB1. As a result, AFM1 levels in milk increased accordingly. These seasonal changes appear to stem from differences in feeding patterns.

This study calculated estimates of daily AFM1 exposure through milk intake based on average AFM1 concentrations detected in milk samples, average adult milk intake data, and average body weight. Although specific human exposure data for AFM1 from milk consumption in Pakistan are lacking, it is noteworthy that the Joint Expert Committee on Food Additives (JECFA) has developed guidelines for AFM1 intake in Latin America (Additives 2002). According to JECFA, the estimated intake of AFM1 is 0.058 ng/kg body weight per day, based on a body weight assumption of 60 kg and AFM1 concentrations detected in milk from several Latin American countries. In comparison, the results of this study showed that daily AFM1 intake increased significantly to 3.1ng/kg body weights by drinking branded or non-branded milk. This value is approximately 53 times higher than the intake calculated for the Latin American population. The apparent differences in AFM1 uptake may be attributed to several factors, including variations in feed quality, feed composition, and the dietary habits of dairy cows. Geographic and seasonal differences also play key roles in AFM1 contamination levels in milk. These changes may be influenced by changes in climatic conditions, which affect both the growth of aflatoxin-producing fungi (such as Aflatoxin) and the storage conditions of food. Furthermore, changes in agricultural practices and dairy cow genetic variation can significantly affect AFM1 levels in milk. For example, farm management practices such as food storage, sanitation, and controlling mold growth in food are critical to minimizing aflatoxin contamination. The presence of aflatoxin B1 (AFB1), the precursor of AFM1, in foods is directly related to environmental conditions during harvest. Hot and humid climates, especially during the rainy season, can create favorable conditions for the growth of aflatoxin-producing fungi, leading to higher levels of pollution. In addition, insufficient or improper food storage increases the risk of contamination, as mold may continue to grow on improperly stored grains and other food sources. Another factor that affects AFM1 levels is the genetic makeup of the dairy cow. Different breeds of cattle may have different sensitivities to aflatoxin contamination, and some animals may metabolize aflatoxin more efficiently than others. This may result in different concentrations of AFM1 in milk from different herds or regions. Additionally, the animal's diet, which may include different types of forages and supplementary feeds, may lead to changes in AFM1 concentrations in milk. For example, if cattle are fed high-risk grains or crops contaminated with aflatoxin-producing mold, there is an increased likelihood that their milk will have elevated levels of AFM1. Understanding these factors is critical to developing strategies to reduce AFM1 contamination in milk. For example, improving food safety by using appropriate drying and storage techniques for

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crops and ensuring animals are fed low-risk feed can help minimize contamination. Regular testing of milk and food for aflatoxin, as well as better regulation of the use of contaminated food, can also play a vital role in controlling human exposure to these toxic compounds.

Additionally, educating farmers about the dangers of aflatoxin and promoting best practices in food management are critical steps in ensuring food safety. Differences in AFM1 levels across regions highlight the complexity of aflatoxin contamination in food systems. Factors such as local climate, agricultural practices, and food quality and storage techniques must be fully understood and managed to mitigate risks associated with exposure to AFM1. Monitoring and controlling the transfer of toxic substances such as AFM1 from the environment into the food supply is critical to maintaining public health. Ultimately, such studies provide data that can provide a solid basis for more effective regulatory, monitoring, and management strategies to protect consumers from harmful levels of aflatoxin in dairy products. Identifying and addressing factors that contribute to AFM1 contamination is critical to reducing potential health risks associated with milk consumption, particularly in areas where exposure levels are above recommended thresholds. This holistic approach to aflatoxin contamination control will contribute to the overall safety and sustainability of the dairy industry, benefiting both producers and consumers (Asi et al. 2012). Zahra (2024) investigated different methods of detoxification of milk during processing. Various techniques have been studied for their effectiveness, including thermal treatments and probiotics. Absorbent materials such as bentonite clay and activated carbon have shown potential to reduce toxins. Chemical methods using ammonia and hydrogen peroxide are also considered, although they carry some risks. Additionally, plant-based detoxification methods using black cumin, garlic, and broccoli are being explored. These plant extracts have antioxidant properties, making them a promising choice. Overall, the study highlights both traditional and innovative approaches to improving milk safety. Studies by (Mukhtar 2024) show that *E. faecium* was isolated from "Nono" and characterized through physiological, biochemical, morphological and molecular techniques. The strain was confirmed to be *E. faecium* OZC108 74 (MK333711.1), with a similarity of 92.14%. AFM1 levels in all tested samples exceeded the EU limit set by NAFDAC in Nigeria (≤0.05µg/L). The Dan-magaji sample had the highest AFM1 concentration (101.02µg/L), while the Kufena sample had the lowest AFM1 concentration (62.96µg/L). Optimal binding of AFM1 (45%) occurred at 1.0 McFarland, 37°C, 60 minutes. This study highlights the potential of specific Enterococcus strains to reduce AF in milk. The study by (Mohsin 2024) at different outlets of Lahore, Pakistan found AFM1 contamination in 90% of the milk samples. Levels were significantly higher in autumn and spring, with 94 and 90% of the sample affected respectively. These concentrations exceed the safety standards set by the European Union. Seasonal changes appear to affect pollution levels. The research by Sirhan et al. (2024) detected AFM1 only in fresh milk, while processed milk and infant milk showed no contamination, highlighting the effectiveness of pasteurization and UHT treatments. The results showed that the type of milk and the processing method can affect the level of contamination. Furthermore, the combined ELISA and PCA assay proved to be a reliable method for the quantification of AFM1. This approach improves the accuracy of contamination monitoring in milk samples. Research by Jyoti and Singh (2024) in terms of aflatoxin M1 contamination, declared on goat's milk was considered safe for human consumption. However, the incidence in cow's milk was 40%, raising safety concerns. This highlights the need for stricter monitoring and control of cow milk quality. It can be concluded by Ibrahimi et al. (2023) that AFM1 contamination in raw milk from Peja, Kosovo, between summer 2021 and spring 2022, was lower than in previous studies. All test samples comply with EU AFM1 safety standards.

4. CONCLUSION

In summary, the findings indicate that raw milk from 20 dairy farms near Chakwal city is safe for consumption by consumers as per the maximum tolerance level of AFM1 prescribed by Pakistan Standards and Quality Control Authority. However, worryingly, 99% of samples exceeded the EU AFM1 limit of 50ng/L. This highlights the need for stronger measures to manage AFM1 levels in milk produced at Chakwal. An effective strategy to control AFM1 is to monitor AFB1 in animal feed. This can be achieved by improving production practices and ensuring appropriate storage conditions. Additionally, increasing awareness among farmers, dairy producers, and consumers of the potential health risks associated with Aflatoxins may help mitigate these risks.

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Author's Contribution: Uzair Ahmad conceptualized the study, laying the foundation for its intellectual framework. The research methodology was meticulously designed by Uzair Ahmad and Shahnshah E Azam, ensuring a robust and systematic approach. Uzair Ahmad and Shahnshah E. Azam collaborated on data collection, while Atiqa Perveez undertook the critical task of data analysis. The initial manuscript was drafted by Uzair Ahmad, with contributions from Shahnshah E. Azam and Atiqa

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Perveez, who provided valuable revisions and refinements. Humayun Ajaz offered supervision and strategic oversight throughout the research process. All authors thoroughly reviewed and approved the final manuscript, affirming their collective contribution and commitment to the study.

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Generative AI statement: The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

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