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Article

Aflatoxin M1 Contamination in Dairy Milk in Kathmandu, Nepal

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Abstract: Aflatoxins (AF), secondary metabolites produced by fungi, pose significant health risks, especially to children and adults. In developing countries like Nepal, the tropical climate promotes fungal growth, leading to high levels of AF in animal feed and milk. In this study, we investigated the incidence of aflatoxin M1 (AFM1) contamination in 84 milk samples collected from the Kathmandu district using the competitive enzyme-linked immunosorbent assay (c-ELISA) technique. We also interviewed farmers to gather information on feeding and storage practices. All the collected milk samples were contaminated with AFM1, with 97.6% of the samples exceeding the European Union (EU) maximum permissible limit of 50 ppt. The majority (98.5%) of the farms included paddy straw, and all farms (100%) included concentrate in their feed regimen. Only half (52%) of the farms had proper storage facilities. Straw was mostly stored in sacks outdoors or left open in a shed, while concentrates were stored in a closed room or shed. The study reports very high contamination levels of AFM1 in the milk samples, presenting a serious public health issue, and recommends comprehensive surveillance and further investigation across the country, especially given the limited research and literature available.

Keywords: Aflatoxin M1; mycotoxin; contamination; milk; food safety; Nepal; Kathmandu

Key Contribution: This study demonstrate 100% of the milk samples collected from Kathmandu, Nepal were contaminated with aflatoxin M1, with 97.6% exceeding the EU's maximum permissible limit of 50 ppt, posing a serious health risk among milk consumers.

1. Introduction

The Mycotoxins are naturally occurring, low molecular weight toxic chemicals produced as secondary metabolites by certain filamentous fungi [1]. Among over 300 mycotoxins detected and reported, aflatoxins are among the most commonly found, highly toxic and presents a significant global food safety concern [2]. Aflatoxins are primarily produced by *Aspergillus flavus*, *Aspergillus parasiticus* and infrequently *Aspergillus nomius*, which grow on various of food commodities such as cereals, peanuts, groundnuts, cottonseeds [3]. Among the dozens of identified aflatoxins, aflatoxin B1, B2, G1, G2, M1 are considered important due to their toxicity and associated health risks to both the domesticated animals and the humans [2]. Aflatoxin B and G are usually found in dairy animal feeds of dairy animal, which is a major source of exposure [4].

Aflatoxin M1(AFM1) is metabolite form of aflatoxin B1, excreted through the milk, thereby posing a public health risk to milk consumers [5,6]. AFM1 is a stable compound that is not removed by common domestic heating methods like microwaving, baking, or boiling. However, its stability during pasteurization is debated. Some studies suggest that pasteurization does not affect AFM1 levels [7–9], while others report a 16% reduction, possibly due to the breakdown of casein during

heat treatment [10]. Agricultural residues such as rice straw, wheat straw, maize stovers, and crop hulls, along with feed components like maize grain, rice bran, and wheat bran, are important animal feeds but are susceptible to aflatoxin contamination, particularly under poor storage conditions [11,12]. When dairy animals consume moldy feeds contaminated with aflatoxin, they ingest AFB1, which is partially converted in the rumen into aflatoxicol (Error! Reference source not found.). The remaining AFB1 is absorbed through the digestive tract, hydroxylated in the liver by cytochrome P450 enzymes into AFM1, and then excreted in milk [13].

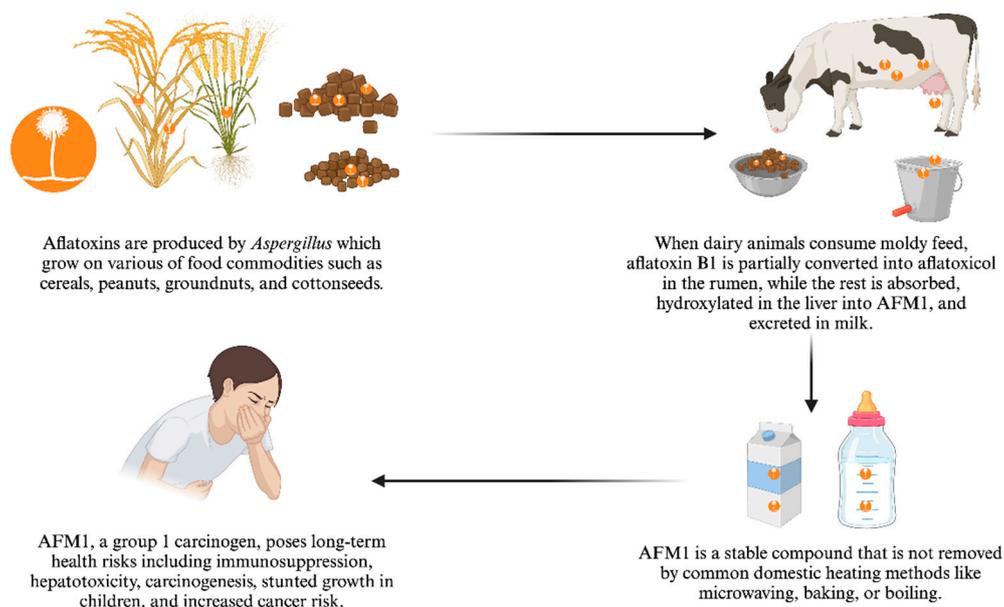


Figure 1. Aflatoxin contamination in milk from feed.

Although AFM1 is less toxic than AFB1 it still poses serious long-term health risks, especially to children and the elderly, as it is a potent immunosuppressive agent, hepatotoxin, and carcinogen [14]. AFM1, along with AFB1, AFB2, AFG1 and AFG2, is categorized as group 1 carcinogen by the International Agency for the Research on Cancer (IARC) [15]. Exposure to AFM1 in early life and childhood is associated with reduced Height-for-Age Z (HAZ) scores in children [16], reduced birth weight [17], reduced height at birth [5] and stunted growth [18]. The cumulative effects of the toxin are also correlated to the higher risk of cancer, immune suppression, hepatocellular carcinoma, Reye's syndrome, cirrhosis and Kwashiorkor [1,19]. A study in Egypt showed a correlation between increased AFM1 levels in blood and high hepatitis C virus titer in patients with chronic liver disease [20].

Aflatoxin contamination in human food and dairy products is a global issue, affecting approximately 25% of food products annually, with particularly severe impacts in developing and underdeveloped countries due to poor husbandry practices and socioeconomic conditions [21]. The European Commission has set the maximum permissible level (MPL) for AFM1 as 0.025 $\mu\text{g}/\text{kg}$ for infant formulae and 0.05 $\mu\text{g}/\text{kg}$ for raw milk [22]. This MPL is found violated by a marketed milk in many African, Middle East and South Asian countries. In some studies from these regions, the prevalence of AFM1 in milk exceeding the EU limit has been found to be 100%, highlighting a concerning food safety situation [23].

Nearly all Nepalese, from children to the elderly, consume milk; however, there is a significant lack of information about AFM1 contamination in milk, and the public remains largely unaware of the potential health risks associated with consuming AFM1-contaminated milk [24,25]. This issue is particularly severe in the Kathmandu district, where milk consumption is high, yet milk is rarely screened for adulteration and contamination, leaving its safety for human consumption uncertain [26]. Similarly, livestock management on peri-urban farms near large cities like Kathmandu faces challenges, particularly in feeding, housing, and storage space, due to land and feed scarcity, which has been found to be significantly associated with higher levels of aflatoxicosis in milk [26]. A study

by Pokharel et al. (2021) found AFM1 in 94% (i.e. 1355/1439) of breast milk samples, highlighting the significant risk of toxin exposure to humans in Nepal [25]. Similarly, research in neighboring countries like India, Pakistan, China has reported AFM1 levels in milk exceeding permissible limits (≥ 50 ppt.) [27–30], reflecting a broader regional concern.

This study aimed to determine the prevalence of AFM1 in various types of milk (farm raw, dairy retailers', and packet) in the Kathmandu district and to compare the contamination level to international standard to assess whether milk was hazardous for human consumption. Additionally, the study sought to evaluate the different farm management factors that might increase the risk for aflatoxin contamination in milk and to guide the relevant authorities in developing strategic measures to mitigate this contamination.

2. Results

2.1. Aflatoxin Contamination

Milk samples collected from eight municipalities in the Kathmandu district were analyzed for aflatoxin M1 using competitive enzyme-linked immunosorbent assay (c-ELISA). All 84 tested milk samples had aflatoxin levels above the lower limit of detection (5 ppt), indicating that 100% of the milk samples were contaminated (Error! Reference source not found.). Of these, 82 samples (97.6%) exceeded the EU's maximum permissible limit of 50 ppt. The two samples that did not exceed the EU limit were from pooled milk collected from farms.

Table 1. Comparison of AFM1 in milk by region and sample categories collected from Kathmandu district, Nepal.

Sample	Sample Size	Samples Exceeding EU Limit-AFM1 (%)
Municipalities		
Budanilkantha	12	100
Kirtipur	15	100
Tokha	13	77.8
Tarkeswor	6	100
Gokernswor	7	100
Kageswori-manohara	6	100
Chandragiri	7	100
Nagarjun	7	100
Sample categories		
Farms' raw milk	48	95.8
Dairy retailers' milk	25	100
Packet milk	11	100

2.2. Farm Characteristics

Forty-eight cattle farms from the Kathmandu district were enrolled in the study. Among the farmers who responded to the questionnaire, the average age was 39 years, and 70.8% were male. Only two respondents had ever heard of aflatoxin contamination in milk. Most of the respondents were small-scale herders, with an average herd size of 15.8 animals per farm (SD=17.3), ranging from 4 to 86 animals (Q1=6, Q2=8, Q3=20). Daily milk yield varied from 9 liters to 400 liters, with an average of 88.8 liters. Of these farms, 87.5% were managed intensively, while the remaining 12.5% were managed semi-intensively with mixed grazing practices. The two farms with AFM1 levels below 50 ppt both had smaller herd sizes and were located Pre-Urban areas (Error! Reference source not found.).

Table 2. Comparison of AFM1 contamination levels with farm characteristics in dairy farms in Kathmandu district, Nepal.

Farms Characteristics	Number (%)	Samples Exceeding EU Limit-AFM1 (%)
A. Location		
Urban	33 (68.8%)	100%
Pre-Urban	15 (31.3%)	86.7%
B. Rearing Practice		
Intensive	42 (87.5%)	97.6%
Semi-intensive	6 (12.5%)	83.3%
C. Herd Size		
0-10	29 (60.4%)	93.1%
11-20	10 (20.8%)	100%
Above 20	9 (18.8%)	100%

2.3. Feeding Practice

All farms (n=48), except for two, included cut-and-carry forage in their livestock feeding practices. The exceptions relied on other feed sources, such as concentrates and straw. Only three farms incorporated silage into their feeding regimen. On 98.5% of the farms, the feedstuffs consisted of paddy straw, with all but one sourced from the Terai, the southern region of the country. All farms used concentrates, either as "homemade ready-made feed" or "commercial compound pellet feed". Eleven farms exclusively used commercial feed, two relied solely on homemade feed, and the remaining 35 farms used a combination of both in varying ratios. Twenty-three farms also included unconventional feedstuffs, such as leftovers and brewers' dry yeast. Crushed maize and paddy husk were the primary ingredients in the "homemade ready-made" feed. The inclusion percentages of the various ingredients in "homemade ready-made feed" and the different unusual feedstuffs are provided in supplementary file (Table S1) and (Table S2). None of the farms used antitoxins in their regimen. Data on the inclusion of these ingredients in animal feed and the percentage of samples exceeding the EU limit are presented in (Error! Reference source not found.).

Table 3. Comparison of AFM1 contamination levels with inclusion percentages of various feedstuffs in animal rations in dairy farms in Kathmandu district, Nepal.

Inclusion in animal feed diet	Opinion	Number (%)	Samples Exceeding EU Limit-AFM1 (%)
Cut and carry Forage	Yes	46 (95.8%)	95.7
	No	2 (4.2%)	100
Silage	Yes	3 (6.3%)	100
	No	45 (93.8%)	95.6
Concentrates	Yes	48 (100%)	95.8%
	No	0 (0%)	
Inclusion of unusual feedstuffs in animal diet	Yes	23 (47.9%)	100%
	No	25 (52.1%)	92.0%
Types of Concentrate in animal diet	Only commercial pellet	11 (22.9%)	100%
	Only homemade concentrate	2 (4.2%)	50%
	Mixtures of both	35 (72.9%)	97.1%

2.4. Storage Practice

Although farmers were aware of mold infestation in stored feedstuffs, they had few options other than to store them for future use, particularly dry fodder (straw) sourced from outside the valley, especially the Terai region of the country. The duration of straw storage (Mean = 35.76 days, SD = 34 days, IQR = 19-30 days) was longer than that of concentrates (Mean = 22.40 days, SD = 10.7

days, IQR =15-30 days). Farmers stored straw for up to 6 months and concentrates for up to 2 months. Only 52% of farms had dedicated storage facilities, most of which were simply open spaces allocated for storage next to the farms. Farmers used different storage methods for storing straw and concentrates, including sacks, sheds (open or with compartments), and rooms (with open or closed doors), illustrated in **Figure 2**, and adopted various storage practices (*Error! Reference source not found.*).



Figure 2. Different storage methods for storing straw and concentrates in dairy farms of Kathmandu district, Nepal.

Table 4. Comparison of AFM1 contamination levels with various storage practice of animal feed in dairy farms in Kathmandu district, Nepal.

Storage Practice of animal feed		Frequency (%)	Samples Exceeding EU Limit-AFM1 (%)
Storage of dry fodder (straw)	Sack (outdoor)	18 (40%)	100%
	Shed (open)	16 (35.6%)	93.8%
	Shed (separate compartment)	3 (6.7%)	100%
	Room	6 (13.3%)	100%
	Others	2 (4.4%)	100%
Storage of concentrates	Room (closed)	24 (50%)	91.7%
	Room (Open)	7 (14.6%)	100%
	Shed (open and/or in compartment)	15 (31.3%)	100%
	others	2 (4.2%)	100%
	Raised	24 (50%)	100%

Floor state of storage facilities?	Floored	24 (50%)	91.7%
Regular monitoring of temperature, humidity and moisture in storage facilities?	Yes	0 (0%)	
	No	48 (100%)	95.8%
Have you seen mold infestation in storage facilities?	Yes	13 (27.1%)	100%
	No	35 (72.9%)	94.3%
Have you seen rodent and insect infestation in storage facilities?	Yes	33 (68.8%)	100%
	No	15 (31.3%)	86.7%

3. Discussion

The study was conducted to assess AFM1 contamination in milk samples from Kathmandu district. Given the limited data and scarce research on AFM1 in milk in Nepal, this study aimed to provide insights into the current situation and guide future surveillance in the region and other part of the country. The results were alarmingly concerning. Of the 84 milk samples analyzed by competitive ELISA for AFM1, all samples exhibited some level of contamination, indicating a 100% prevalence of AFM1 in the milk. Even more troubling, 97.6% of the samples had AFM1 levels exceeding the EU's permissible limit of 50 ppt, which is considered for human consumption. This presents a serious food safety issue for consumers in Kathmandu district. Since the same milk is used to produce dairy products such as yogurt, cheese, and sweets, these products may also exhibit similar contamination trends, as observed in India and Pakistan [29,31].

Several studies from South Asian countries have reported a very high prevalence of AFM1 in milk and milk products, and our study is no exception. The prevalence rate in Nepal is comparable to findings from the Middle East, Africa, and South America also. For instance, Iran has documented a significant number of research articles on AFM1, highlighting it as a serious issue, with multiple reports indicating a 100% prevalence rate [32]. Similar findings have been reported in Ethiopia, where Tadesse et al. (2017) and Gizachew et al. (2016) found AFM1 contamination in 100% of milk samples [33,34]. In Kenya and South Africa also, studies reported occurrence levels above 85% [35,36]. In Brazil, AFM1 was found in 100% of pasteurized and Ultra Heat Treated (UHT) milk samples [37]. Prevalence levels above 90% have also been reported in Thailand [38], Taiwan [39] and Indonesia [40]. This widespread aflatoxin contamination in milk and dairy products in tropical countries is largely attributed to the common contamination of crops, crop residues, and animal feedstuffs [41]. The environmental conditions in tropical areas are conducive to fungal growth and mycotoxin production [42]. The problem is particularly severe in low- and middle-income countries, where poor harvesting, storage, and transportation practices are prevalent, and where policies or the implementation of regulations and mitigation strategies are often lacking [43].

Aflatoxin M1 contamination in milk is associated with various risk factors, with the primary source being the feedstuffs consumed by the animals [44]. Therefore, the feed regimen and storage practices are critical factors associated with AFM1 contamination in milk. All farms in the study relied on concentrates, with most using a mixture of homemade ready-made feed and commercial pellet feed, both of which have been frequently recognized as potential risk factors for AFM1 contamination in milk [45,46]. Although evaluating aflatoxin levels in cattle feed in Nepal is crucial –given the sparse literature on the subject–this aspect was not addressed in our study. However, a study on poultry feed by Aryal and Karki, (2009) revealed a high prevalence (75.4%) of aflatoxin B1 and B2, with levels as high as 366 µg/kg [47].

The primary ingredients in both commercially produced and homemade concentrates in our study –crushed maize, paddy husk, mustard oil cake, and wheat bran–are particularly susceptible to mold infestation and mycotoxin contamination, especially during storage [28]. Joshi et al. (2022) found that 78% of maize samples were contaminated with aflatoxin, with a mean concentration of 23.04 ppb, exceeding the permissible limit (20 µg/kg) established by the government of Nepal [48].

Other studies have also reported the contamination of maize with aflatoxin in Nepal [48–50]. In our study, all farms relying on commercial pellet feed had AFM1 levels above EU limits and exhibited higher contamination levels. Studies by Kang'ethe and Lang'a, (2009) and Akbar et al. (2020) found a positive correlation between higher aflatoxin contamination in commercial concentrate feed and increased AFM1 concentrations in milk [11,51]. The possible reasons for this include the poor quality of raw materials, suboptimal harvesting practices, and inadequate storage and shipping procedures for both raw materials and the final product.

Additionally, 53% of farms supplemented animal diets with unconventional feedstuffs, mainly leftover grains, vegetables, and fruits, likely due to the scarcity of fodder and roughages and the high cost of concentrates. Patyal et al. (2021) found that the inclusion of leftover grains was significantly associated with higher AFM1 levels in milk [45].

Storage facilities and the duration of feed storage, particularly for concentrates, have been found to be significantly associated with aflatoxin contamination in animal feed and milk [45,46]. Half of the farms in the study lacked proper storage facilities; roughages and concentrates were stored in open areas, either in sheds or outdoors. Observations during farm visits revealed that farms in urban areas had limited space to extend sheds for additional storage, leading to the shared use of sheds for storing straw and concentrates in sacks. In contrast, farms on the outskirts, being more spacious, often had designated storage areas, although these were not always equipped with proper facilities. Among all the samples tested, only two did not exceed the EU MPL, both of which were from peri-urban farms. The lower level of AFM1 in peri-urban farms, compared to urban farms, could be due to the presence of spacious and designated storage areas that help prevent mold growth. Paddy straw, a byproduct of seasonal rice cultivation, constituted a major component of the animal diet and was usually procured in bulk mainly from the southern part of the country. Straw was stored for an average of 35 days, with some farms storing it for up to 6 months. Inadequate transportation and improper storage practices promote fungal growth and mycotoxin production in these roughages [52].

This study focused exclusively on milk produced within the Kathmandu district, selecting only farms and dairy retailers sourcing their milk from this area. However, the milk produced by these farms significantly falls short of meeting the demand in Kathmandu, the country's most populous city and capital. To address this shortfall, milk is imported from neighboring districts, particularly Kavre district, which also needs to be assessed to determine the AFM1 contamination level in Kathmandu. The AFM1 concentration could vary in milk from this region due to different feeding and storage practices on farms situated in areas with abundant forages and ample storage space, unlike in Kathmandu.

4. Conclusions

This study has revealed an alarming 100% prevalence of AFM1 contamination in milk samples from the Kathmandu district, with 97.6% of the samples exceeding the EU's maximum permissible level. The findings underscore the urgent need for comprehensive national investigations to establish contamination trends, identify risk factors, and assess the health and economic impacts of aflatoxicosis. Such efforts will be crucial in supporting the implementation of effective measures and regulations to mitigate the risk of aflatoxin contamination in food, animals, and humans. Furthermore, it is essential to educate the public—especially dairy farmers, retailers, processing companies, and consumers—about the risks and economic impact of aflatoxin in milk.

5. Materials and Methods

5.1. Study Design and Study Area

The cross-sectional study was conducted from October to November 2022 in the Kathmandu district, located in the Bagmati province of central Nepal. The study focused on major milk-producing and distributing areas within the district. Out of the 11 municipalities, eight were selected: Kageswori-Manohara, Kirtipur, Gokarneshwor, Chandragiri, Tokha, Tarkeshwor, Nagarjun, and

Budanilakantha (Error! Reference source not found.). Kathmandu (latitude 27°41'38.76" North, longitude 85°22'39.00" East), the nation's capital, is densely populated and has one of the highest rates of milk and dairy product consumption. The areas within the district were classified as urban and peri-urban; 'peri-urban' refers to areas transitioning towards urbanization, while "urban" describes areas that have fully transitioned and exhibit the characteristics of a developed city or town.

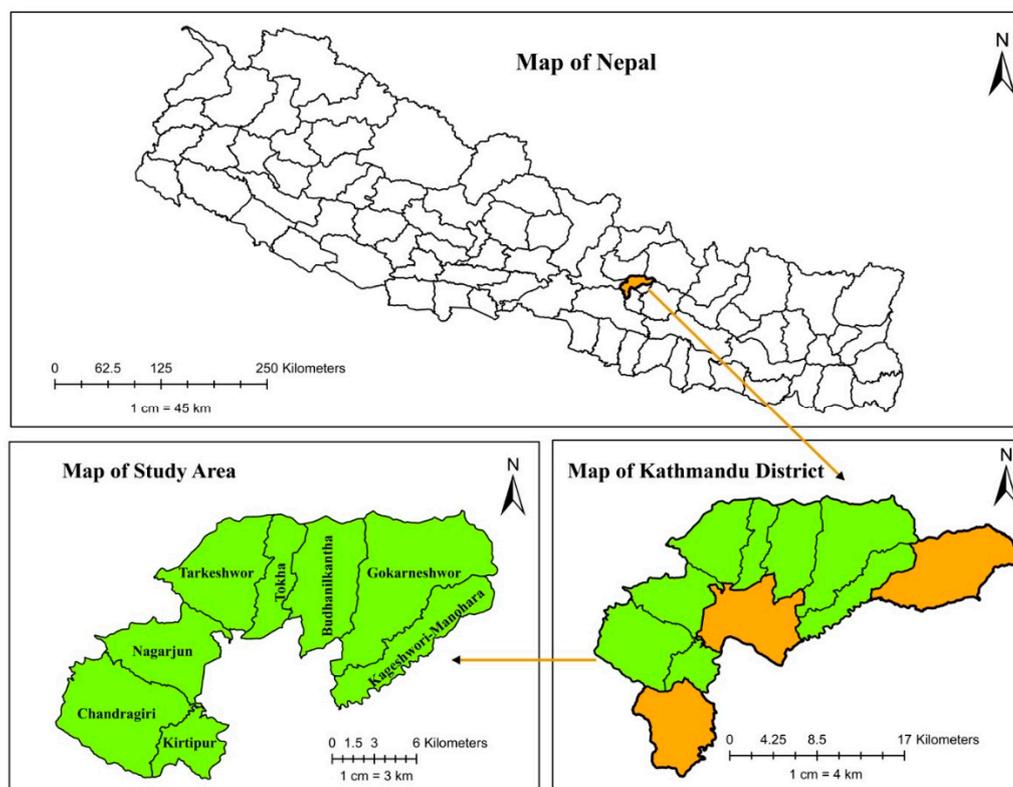


Figure 3. Map of Nepal and Kathmandu district showing study area.

5.2. Sample Collection

A total of 84 samples were collected for the study, categorized into three groups: farm raw milk (48 samples), dairy retail shop milk (25 samples), and packet milk (11 samples). Forty-eight commercially registered farms with varying herd sizes were randomly selected from eight municipalities within the Kathmandu district. Fresh raw milk samples were collected in sterile, screw-capped 100 ml centrifuge tubes from the bulk milk tanks after proper mixing during early morning visits. Similarly, milk samples were obtained from the bulk tanks of 25 dairy retail shops in these municipalities. These retail shops, which pool milk from various nearby farms, are major distributors of milk in Kathmandu, alongside packet milk. Eleven milk packets from different brands were purchased from supermarkets for the study. After collection, all milk samples were placed in an insulated cold box and transported to the Central Veterinary Laboratory, Kathmandu where they were stored in a refrigerator at -20°C . All samples were analyzed for AFM1 within two days of collection. During the farm visits for sample collection, a pretested questionnaire was administered via Kobo Toolbox [53] to farmers, owners, or managers to collect data on farm characteristics, as well as feeding and storage practices.

5.3. Laboratory Analysis

The milk samples were thawed and then centrifuged at 1000 rpm for 5 minutes. The upper layer of fat was removed, and AFM1 was screened in the defatted milk samples using a competitive ELISA (CUSABIO Technology LLC), following the manufacturer's instructions provided with the ELISA kit. The kit included a precoated ELISA plate, standards (0, 5, 15, 45, and 135 ppt), HRP-conjugate,

substrates, wash buffer, and stop solution. Briefly, 100 microliters of standards and milk samples were added to the precoated ELISA plate and incubated for 30 minutes at 25°C. The plate was then washed five times, and 100 microliters of HRP conjugate were added to each well, followed by a 15-minute incubation at 25°C. After another five washes, 50 microliters of substrate A and substrate B were added, mixed, and incubated for 15 minutes at 25°C in the dark. Finally, 50 microliters of stop solution were added, and absorbance was measured at 450 nm using an ELISA reader (Multiskan™ FC Microplate Photometer). The calibration curve was constructed from the standard solutions and their absorbance, and the regression equation ($R^2 = 0.98$) was used to quantify aflatoxin levels.

5.4. Statistical Analysis

All data from the questionnaire and laboratory analysis were compiled into an Excel file (Microsoft Excel 2019) and subsequently imported into SPSS version 25 for a descriptive assessment of the farms and husbandry practices. Contamination levels of AFM1 in milk were categorized based on the EU's maximum permissible limit (MPL) of 50 ppt, above which milk is considered unsafe for human consumption. Consequently, contamination levels were reported as either below or above 50 ppt, indicating whether the milk complied with or exceeding the EU limit.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: Inclusion percentage of various ingredients in 'homemade ready-made feed' in dairy farms in Kathmandu district, Nepal. ; Table S2: Inclusion percentage of different unusual feedstuffs in dairy farms in Kathmandu district, Nepal.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are included in the article and supplementary files. Further inquiries or data requests can be directed to the authors.

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Conflicts of Interest: The authors declare no conflicts of interest.

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