Multi-Disciplinary Publishing Institute Pakistan **Vol.** 03, **N0.** 01

Submitted: 03 DEC 2024 Accepted: 08 DEC 2024 Published: 31 DEC 2024

Original Article

Detection of Aflatoxins M1 Contamination in Fresh Milk Sold at Different Outlets in Lahore, Pakistan

Muhammad Mohsin
Department of Epidemiology & Public Health, University of Veterinary & Animal Sciences, Lahore, Pakistan
Email: drmohsin.uvas@gmail.com

Ayaz Qadir, Mukhtiar Ahmed, Muhammad Zahir, Fazal Ur Rehman[4]
Faculty Veterinary & Animal Sciences, Lasbela University of Agriculture, Water & Marine Sciences, Uthal, Pakistan
Email[4]: fazal.vas@luawms.edu.pk

Muhammad Shakeel,
Department of Pharmacology &Toxicology, University of Veterinary & Animal Sciences, Lahore, Pakistan
Email: shakeeldewan34@gmail.com

Fawad Khalil Pitafi,
Department of Veterinary Surgery, University of Veterinary &Animal Sciences, Lahore, Pakistan
Email: fawadkhalil02@gmail.com

Zabeeh Ullah
Department of Molecular Biology & Biotechnology, University of Veterinary & Animal Sciences, Lahore, Pakistan
Email: zabeeh06644@gmail.com

Lubna Kandhro
Department of Livestock Management, Sindh Agriculture University, Tando Jam, Pakistan
Email: lubnakandhro3@gmail.com

Arfa Tehreem
Department of Pathobiology, University of Veterinary & Animal Sciences, Lahore, (Sub-campus, CVAS, Jhang), Pakistan
Email: arfatehreem020@gmail.com

Abstract

The aflatoxin study was conducted to detect aflatoxin M1 in a variety of milk samples obtained from various milk shops in Lahore, Pakistan. A total of 190 samples were collected from different zones of Lahore. All samples were processed through ELISA to detect aflatoxins. A questionnaire containing 14 open-ended questions, and 27 closed-ended questions was designed. The data was analyzed using the chi-square method with mean ± standard deviation (SD) to identify the association between risk factors and positive results for aflatoxin M1 concentrations in the fresh milk samples. The level of significance was set at $P \leq 0.05$. All milk samples were found to be positive for aflatoxins. About 90% of the samples were found to exceed the permissible limit of 50 ng/kg for aflatoxins. Using the questionnaire, an association between risk factors such as education, preventive measures in milk collection, cleaning areas, disinfection, commercial feed, toxin binders, and different seasons was evaluated. The results were non-significant for all factors except the use of toxin binders. It was observed that the use of toxin binders can reduce the aflatoxin levels in milk. It is concluded that all milk samples contain significant amounts of aflatoxins in Lahore, Pakistan. There is a strong need to establish strict rules and regulations to control the levels of aflatoxin B1 in animal feed. Milk producers, the dairy industry, and milk shop owners must be aware of the health risks and preventive measures associated with aflatoxins.

Keywords: Aflatoxins M1, Milk, ELISA, Milk Samples, Lahore.

WEBSITE: www.mdpip.com
ISSN: Print: 2959-619X
ISSN: Online: 2959-6203
PUBLISHER: MDPIP

OAPH&HAR Open Access Public Health & Health Administration Review

Mohsin, M., Shakeel, M., Ahmad, M., Zahir, M., Qadir, A., Pitafi, F.K., Ullah, Z., Kandhro, L., Tahreem, L., & Rehman, F.Ur. (2024), 48-59

Introduction

Aspergillus parasiticus and Aspergillus flavus produce aflatoxins, which are secondary metabolites of molds (Hussain, 2009; Asghat *et al.*, 2018; Iqbal *et al.*, 2015). Aflatoxins are toxin-producing molds of the genus Aspergillus that produce chemically similar compounds (Ahmaed, 2019). Molds produce mycotoxins for a variety of reasons, including the avoidance of plant parasite colonization, external environmental stress, and plant defense from ultraviolet sun radiation (Cary & Ehrlich, 2006). Aflatoxin M1 (AFM1), a liver carcinogen, is secreted in milk by lactating animals fed Aspergillus-contaminated diets (Nidhina *et al*., 2017). A total of 20 varieties of aflatoxins with a chemical structure of dihydro or tetrahydrofuran groups connected to a coumarin ring have been identified in these molds (Imtiaz & Yunus, 2019).

There are many types of aflatoxins. The most significant metabolite of AFB1 is aflatoxin M1 (AFM1) (Hussein & Brasil, 2008). During the formation of hepatocellular cancer, Aspergillusflavus, Aspergillus pseudotamari, Aspergillus bombycis, Aspergillus parasiticus, Aspergillus sochraceoroseus, and Aspergillus nomius have constituted the main etiological factors of aflatoxins (Campagnollo *et al*., 2016). AFBI is the most dangerous of the nearly 18 aflatoxins that have been identified. AFBI contaminates dairy cow feed by contaminating peanut cake and maize (Xiong *et al.*, 2018). There is a strong correlation between mold-infested cattle feed and the safety of milk, which is the most common route for aflatoxins to enter human bodies. AFM1 is created in the liver by cytochrome P450 enzymes in animals or humans who eat an AFB1-contaminated diet. These metabolites are eventually released in both human and dairy animal urine and milk (Prandini *et al*., 2009).

Many countries' governments and organizations have established worldwide acceptable severe residue level limits ranging from 0 to 1000 (Egypt and Romania, Nigeria) (Iqbal *et al*., 2015). China and the United States have set a severe residual level of 500 ng/liter for milk Aflatoxin M1, which is ten times higher than the European standard of 50 ng/L (Guo *et al*., 2019).

Aflatoxin M1 (AFM1) pollution is currently causing worry among experts all over the world. AFM1 safe limits in milk and dairy products have been established globally, ranging from 50 ng/kg in Europe to 500 ng/kg in North America (Reverberi *et al*., 2010).

Aflatoxins are the toxic secondary metabolites of various *Aspergillus* spp. that commonly contaminate food and feed ingredients (Akbar, 2020). The aflatoxins encountered in agricultural commodities include aflatoxin B_1, B_2, G_1 and G_2 (Younus, Abbas, Rafique *et al*., 2013). In contaminated foodstuffs, the percentage of aflatoxin

B_1 (AFB_1) in total aflatoxins is over 90%. Once ingested by animals, AFB_1 is also carried to milk in the form of the toxic metabolite aflatoxin M_1 (AFM_1).

All these toxins are known to exert potent hepatotoxic, immunotoxin and carcinogenic effects in animals and humans consuming the contaminated food. Due to high carcinogenicity, aflatoxins are the only group of mycotoxins for which legislation and control protocols are in place, even in many developing countries. The toxicity of aflatoxins is known to be higher in younger age groups (infants, children and young animals). Monitoring the levels of AFM_1 in milk and baby foods is therefore more critical. Consequently, the levels of the mycotoxin allowed in milk are lower than the levels allowed in other foodstuffs. The EU further restricts the levels allowed in infant milk formula to half of the levels allowed in milk.

The maximum tolerable limit of AFM_1 in liquid milk is 500 ng/L in the USA and in the Codex standards, while only 50 ng/L in the EU. In Pakistan, the maximum tolerable limit of AFM_1 is 10 µg/kg in milk powder while no legislation has been made for liquid milk. This is even though specific monsoon conditions in the country favor mycotoxin development in food and feedstuffs, pushing Pakistan into a high-risk area. The studies conducted in Pakistan also show that 25 to 90% of milk samples could be contaminated with AFM_1.

There have been notable differences in the AFM_1 levels in milk reported by different authors from Pakistan. In this regard, Ismail *et al*. (2016, 2017) and Tahira *et al*. (2019) reported 17,380 ng/L as the mean AFM_1 level in milk sampled from Lahore in the year 2007, with 81% samples exceeding the 500 ng AFM_1/L limit.

Contrary to this, Iqbal *et al*. (2011a, 2011b, 2014) reported 64 ng/L mean AFM_1 level in milk sampled in the year 2011 in the urban areas of Punjab province, with 15% samples exceeding the 500 ng AFM_1/L limit. These differences in the AFM_1 contamination level reported by various authors could be due to different seasons, different feeds used by farmers in different areas, and different methods of AFM_1 quantification. Overall, such differences make it impractical to infer risk of exposure for the consumers of milk in other cities. The present study was therefore conducted as a longitudinal one-year study to determine the AFM_1 levels in various types of milk, primarily processed, available in Islamabad the capital city of Pakistan.

To the best of our knowledge, there is no previous longitudinal study on AFM_1 contamination in processed milk in Pakistan. Also, AFM_1 contamination of milk has not been previously investigated in Islamabad city. Data on processed milk from one city are however applicable to milk consumers in other cities because processing companies collect milk from farmers located in different areas and distribute it to consumers in all cities of Pakistan.

There are different categories of Aflatoxins for example, (AFB1- aflatoxin B1), (AFB2- aflatoxin B2), (AFG1- aflatoxin G1) and (AFG2- aflatoxin G2). Because of the higher level of toxicity, teratogenicity, photomutagenicity and mutagenicity, the International Agency for Research on Cancer categorized aflatoxins included AFB1 under "Group I" (Ostry *et al*., 2017).

The animals consuming fungal-infested feed can also consume Aflatoxin B1 with it, which is then hydroxylated to Aflatoxin M1 by the enzyme Cytochrome P450 present in the cattle' liver, though very limited studies have been conducted on this issue therefore very information could be explored on this important public health issue (Jawaid, Talpur, & Afridi, 2015). This is because it is very hard to isolate a large quantity of Aflatoxin M1 in pure form to conduct extensive toxicological research for this compound (Eaton & Groopman, 2013). However, studies have verified and confirmed that Aflatoxin M1 is comparatively less toxic than Aflatoxin B1 according to the following order AFB1+AFM1 > AFB1 > AFM1 (Li *et al*., 2018).

The toxicological effects of Aflatoxin M1 that have been reported to date include carcinogenic effects (Cullen *et al.* 1987), oxidative stress on Kidney (Li *et al*., 2018), and several immunosuppressive effects (Luongo *et al*., 2014). The combined effect of AFM1 and AFB1 working synergistically with Hepatitis B virus and causing a 12-fold rise in liver cancer risk have been reported as well (Sun *et al*., 2013). There are various regulatory limits for Aflatoxin M1 contamination in liquid raw milk throughout the world depending upon the economic conditions and availability of resources in the region (Stoloff *et al*. 1991; Van Egmond, 1989).

As per the standards of the European Union, the maximum limit allowed for AFM1 contamination in liquid raw milk is 50ng/kg (European Commission, 2010). The limit of 500ng/kg has been allowed by US Food and Drug Administration, and Punjab Pure Food Regulations, Pakistan (US Food and Drug Administration, 2000; Punjab Pure Food Regulations, 2018) Hence, there are varying differences in the maximum permissible limit of AFM1 in liquid raw milk among different countries and regions of the world (Egmond, 1989).

Data from existing studies on Aflatoxin M1 Milk contamination showed varying contamination levels in different regions of Pakistan (Punjab Pure Food Regulations, 2018). A previous study from year 2011 conducted in various regions of Punjab province reported 64ng/kg as the mean AFM1 contamination level in milk from urban areas, with over 42% and 15% samples exceeding the European Union (i.e., 50ng/kg) and USFDA (i.e., 500ng/kg) limit, respectively (Iqbal *et al*., 2014). The same study showed 40ng/kg as the mean AFM1 contamination level in milk from rural farmhouses with over 27% and 8% samples exceeding the European Union and USFDA limit, respectively (Iqbal *et al*., 2014).

On the contrary, a study from Lahore in 2007 reported 17.380ng/kg as the mean AFM1 contamination level in milk, with over 81% of samples exceeding the European Union limit (Muhammad *et al*., 2010). Another longitudinal one year-long study from 2018 reported a concentration of 1535ng/kg as the mean AFM1 contamination level in raw milk of Islamabad, with over 91.9% of samples exceeding the European Union limit (Yunus *et al*., 2019).

These varying differences in the results could be due to varying temperature conditions, different seasons, feed, and storage conditions used by the farmers in different areas and differences in the approach for quantification of AFM1 by the researchers. Such studies can therefore help to determine the factors responsible for varying levels of AFM1 contamination in different regions which allows taking region-specific measures to control further contamination in the future. The present 4 months' study was also designed to assess AFM1 contamination levels in raw milk from different regions of Lahore throughout summer in compliance with the International and National regulatory limits using ELISA technique.

Several investigations on the prevalence, anticipation, and transmission of milk AFM1 have been undertaken around the world (Bodbodak *et al*., 2018; Younas, 2019). The level of aflatoxin in milk differs depending on the style of handling/milk processing (Aziz, Noor-ul-Ain, Majeed *et al.*, 2012; Guo *et al*., 2019; Zahra, 2020). It has been found that an initial amount of aflatoxin B1 in animal feed (1–3%) resulted in AFM1 secretion in milk. The type of animal and the number of milking have an impact on AFM1 secretion in milk. Pasteurization or processing of contaminated milk into cheese did not affect AFM1 (Baruki *et al*., 2018). Pakistan is regarded to be an excellent environment for fungi to thrive and create mycotoxins in general (Sadia, Jabbar, Deng, Hussain *et al*., 2012; Barham, Khaskheli, Soomro, & Nizamani, 2014). The goal of this research is to look for Aflatoxin M1 in a variety of milk samples obtained from various shops in Lahore, Pakistan.

Materials and Methods

The study was designed in raw milk retailer shops, including commercial, semi-commercial, and rural shops selected from different towns, societies, and union councils of Lahore, Punjab, Pakistan.

A cross-sectional study was conducted to estimate the prevalence of Aflatoxin M1 in fresh raw milk in the Lahore district. Risk factors associated with this toxin were also investigated using a questionnaire in data form. This study was carried out from December 2019 to November 2020.

For sample selection, random and convenient sampling methods were employed based on a list frame of aflatoxin M1 in milk in the Lahore District. A total of 190 samples were collected from different zones of Lahore. Samples were collected in Falcon tubes. After collection, samples were centrifuged and filtered to remove their protein content. Then, the samples were taken for further processing through ELISA.

Investigation of Risk Factors

A face-to-face interview was conducted using a pre-structured questionnaire with the consent of the milk outlet owners. The questionnaire contained 14 open-ended questions and 27 closed questions. It was designed in English but presented in the local Punjabi language of the area. Samples were collected with the assistance of the Nutrition Lab at Copper Road Livestock Complex. The detailed questionnaire is provided in the appendix.

Sample Collection and Criteria

A total of 200 milk samples were collected from different milk outlets situated in various areas of Lahore on different days. Approximately 250 mL of fresh milk was collected and transported to the lab in an icebox. After collection, samples were divided to provide 50 mL sub-samples, which were stored at 4°C until processed in the Provisional Diagnostic Laboratory of the Livestock and Dairy Development Department at Copper Road, Lahore. Fresh milk samples were collected from large milk outlets only. Processed milk and milk products were not included in this present study.

Centrifugation

Following the centrifugation technique at 3500 rpm at a temperature of 10 °C for a total time of 10 minutes, fresh milk samples were thawed at room temperature. When centrifugation was completed, using a Pasteur pipette, the defatted supernatant (bottom layer) was aspirated, and then the skimmed milk was directly tested (100 uL per well) for aflatoxin M1 using the analytical method in the NEOGEN® AFM1 test kit (Veratox, K-blue y Veratox son Marcos's commercials, North America), with a microplate reader at 650 nm.

Preparation for ELISA Procedure

Kit components

1. Purified Aflatoxin M1 coated microplates (12×8 well strips)
2. Conjugate
3. Dilution Buffer
4. Substrate solution
5. Stop solution
6. Standard solution (0 ppt, 5, 15, 30, 60, 100)

Test Procedure

1. Allow all the reagents to warm to room temperature (8-30 °C) before starting.
2. All standards were processed in duplicate wells. This was achieved by transferring standards and samples from the red-marked mixing wells to the antibody-coated microwells.
3. Remove the red-marked mixing well for each sample tested (12 in total).
4. Remove 12 antibody-coated wells that were used.
5. Mix each reagent.
6. Use a new pipette for each transfer.
7. Transfer 250 µL of standard to the red-marked mixing well.
8. Using a 12-channel pipette, transfer 100 µL from the mixing well to the two antibody-coated wells.
9. Place the microwells on a plate shaker at 600 rpm for twenty minutes, then discard the red-marked mixing wells.
10. Fill the wells with diluted washing buffer and dump them out, repeat this 5 times, and then dry the wells with tissue.
11. Pour conjugate from the blue bottle into the trough, then add 100 µL.
12. Place the wells on a plate shaker at 600 rpm for ten minutes.
13. Shake out the content from the antibody wells, fill them with diluted washing buffer, and repeat this washing 5 times.
14. Pour 100 µL of substrate from the green bottle.
15. Place the microwells on the shaker for fifteen minutes at 600 rpm.

16. Discard the remaining substrate and rinse the reagent boat with water.
17. Pour the red stop solution, then wipe the bottom of the wells with tissue, and within 20 minutes read values in the ELISA reader unit.
18. Read and calculate the results using Neogen's Vertex for Windows software.
19. For making regression bends between optical density and aflatoxin M1 (AFM1) concentration, we used commercially available test kits comprised of standard solutions consisting of 0, 5, 15, 30, 60, and 100 ng/liter. When the milk aflatoxin M1 (AFM1) was detected above 100 ng/L, then the sample was diluted in dilution buffer, and re-detection of aflatoxin M1 (AFM1) was done. For the calculation of aflatoxin M1 (AFM1) content, NEOGEN'S VERATOX® WINDOW software (Veratox, K-blue y Veratox son Marcos's commercials, North America) was used. To confirm the presentation of work, the same lot of ELISA kits underwent initial trials, which were tracked by the analysis of aflatoxin M1 in samples of fresh milk. I followed the results of the initial trials: 5 ng/L will be the detection limit, and a range of 94.7–96.1% was the recovery of aflatoxin M1 (AFM1), with a variation coefficient of 3.7–6.3%.

Statistics Based Analysis

The median, minimum, and maximum concentrations, mean ± standard deviation (SD) of aflatoxin M1, were used to elaborate and express all the results of my study. SPSS version 20 was applied for the analysis of the frequencies of data from the questionnaire by applying the chi-square method to identify the association between risk factors and positive results of aflatoxin M1 concentrations in samples of fresh milk from different areas of the Lahore region. ($P \leq 0.05$) was the level of significance, and ($p \leq 0.10$ and $p \geq 0.05$) were set as standard values (Daniel, 2010).

Ethical Approval and Consent to Participate

The study protocol and consent procedure were approved by the University of Veterinary and Animal Sciences, Lahore, Pakistan, and the Provisional Animal Nutrition Laboratory at the Livestock Complex, 16 Copper Road, Lahore. The consent form was translated into the local language, Punjabi. All participants and their attendants were briefed on the purpose of the research, the interview questions, voluntary participation, and other aspects of the study. Verbal consent was obtained from the individual milk shopkeepers.

Results and Analysis

Variation in Aflatoxin M1 Levels During Different Seasons of the Year

Aflatoxin M1 was measured in fresh milk samples during all four seasons of the year: autumn, winter, spring, and summer. The quantity of aflatoxin M1 analyzed in fresh milk samples, which numbered 190, ranged from low to high levels. Ninety percent of the tested samples were found to exceed the permissible limit of aflatoxin (50 ng/kg) according to the European Community and Codex Alimentarius standards. However, the USA has a different limit, which is 500 ng/kg.

All tested samples met the US criteria for aflatoxin limits. The mean AFM1 level was highest in winter, while the minimum level was recorded in the summer season. The sequence order of AFM1 levels in the four seasons of the year is autumn > spring > winter > summer. According to the permissible EC levels in the different four seasons, they were found to be 94%, 90%, 89%, and 88%, respectively. The results of this study show an increasing trend of aflatoxin M1 levels from April, reaching the maximum limit in September, and decreasing to the minimum level of AFM1 in July. This indicates poor animal feeding practices and a lack of awareness about testing and even about AFM1.

Concentration of Aflatoxin M1 in Different Milk Samples

All 190 samples were found to be contaminated with aflatoxin M1, but the quantity of aflatoxin varied according to the European Community (EC) and US limit ranges. Out of the 190 samples, 90% crossed the EC limit of aflatoxin, while only 5% of samples were unacceptable according to the US range. Various concentrations of aflatoxin M1 are shown in Figure 1 and the calibration curve of standard samples is shown in Figure 2.

Figure 1

Concentrations of Aflatoxin M1 in Different Milk Samples

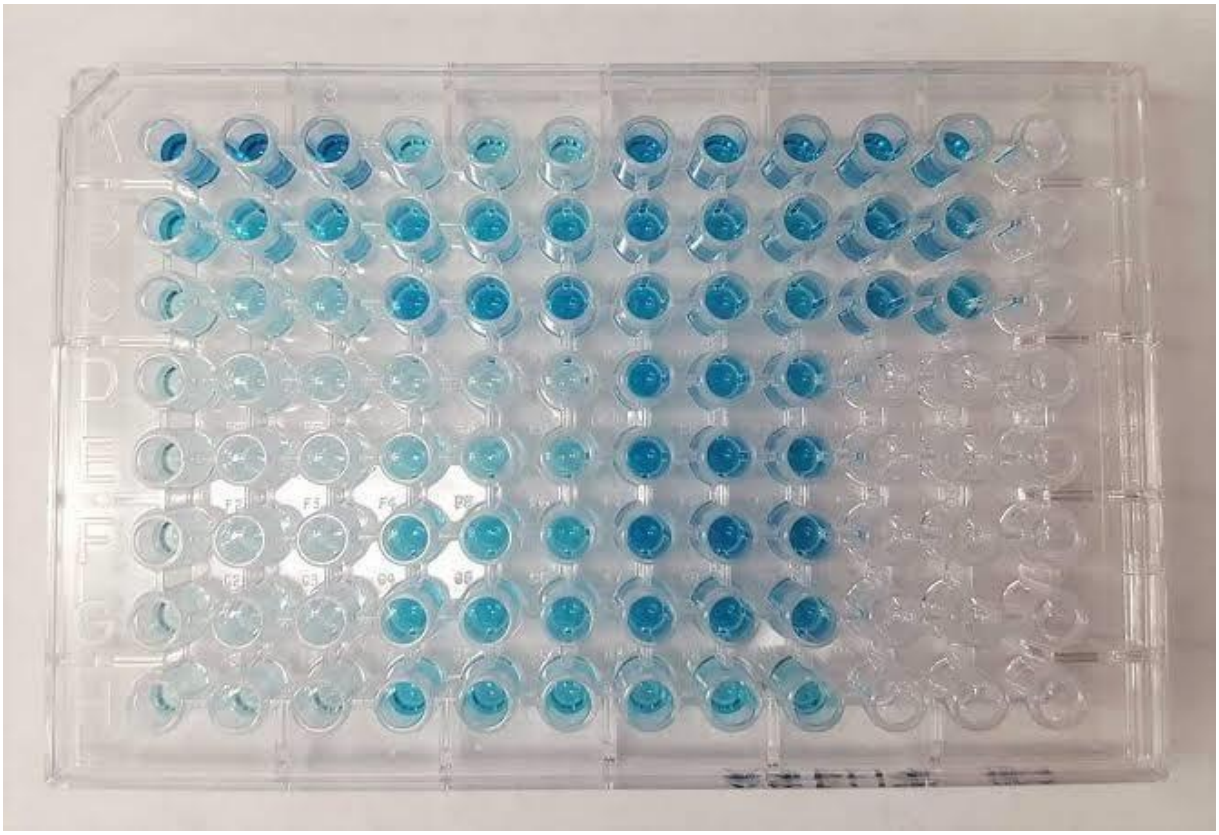
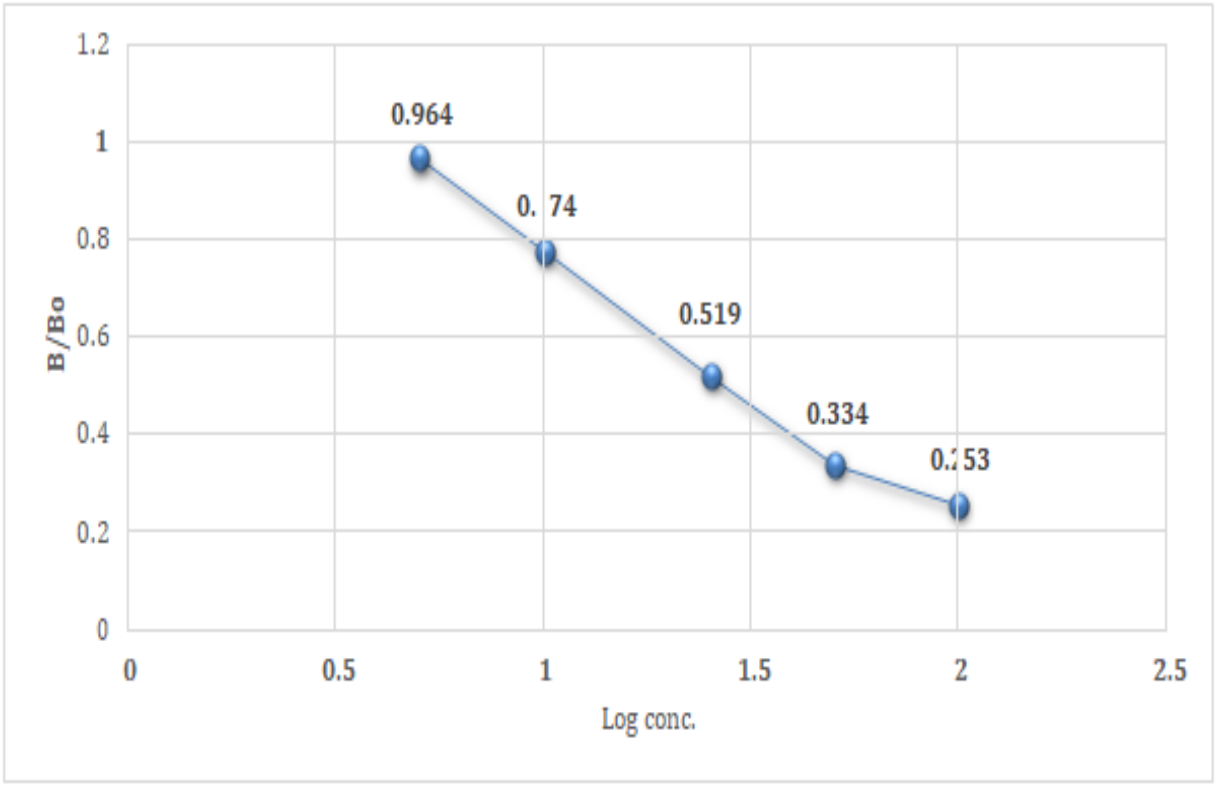


Figure 2

Calibration Curve of Standard Samples



Association of Prevalence of Aflatoxin M1 with Various Factors

The chi-square test is a test of independence. It tells us if the association is significant. The association between risk factors, such as education level, milk chiller usage, cleaning area, washing and disinfection practices, commercial feed usage, toxin binders, and different seasons, was examined. Results were non-significant with all samples except for the use of toxin binders. It was observed that the use of toxin binders can reduce aflatoxin levels in milk. All results are shown in Tables 1, 2, 3, 4, and 5.

Table 1
Frequency Analysis of Various Factors Asked in Data Capture Form

Variables	Responses	Frequency	Percentage%
Sample	**Milk**	**190**	**100**
Education	Intermediate	12	6.3
	Elementary	119	62.6
	No schooling	59	31.1
Type of Outlet	Rural	80	42.1
	Semi-commercial	101	53.2
	Commercial	9	4.7
Type of Milk	Cattle	5	2.63
	Buffalo	6	3.15
	Both	179	94.22
Other Products sold at	Cheese	5	2.6

Outlet	Butter	63	33.2
	Yogurt	122	64.2
Any separate Grouping of	Yes	152	80
Milk Quality	No	38	20
Do you have any biosafety at	Yes	33	17.4
outlet	No	157	82.6
Does Punjab food authority	Yes	172	90.5
give any awareness about	No	18	9.5
safety of milk			
Does food department visit	Yes	61	32.1
your shop on regular basis	No	129	67.9
When does food department	If customer complain	76	40
visits your shop	Regular visit	113	59.5
	Other reason	1	0.5
Is the boundary wall around	Yes	33	17.4
the farm present where the	No	157	82.6
milk is collected			
What feeding method at farm	Stallfeeding	161	84.7
from where milk is collected	Grazing	29	15.3
Food department check your	Yes	33	17.4
milk quality	No	157	82.6
When was the previous toxin	Yes	152	80
level tested	No	38	20
From where you collect milk	Corporate dairy	1	0.5
for your shop	Commercial dairy	131	68.9
	Any other	58	30.5
Do you know about aflatoxins	Yes	25	13.2
M1	No	165	86.8

Do you know aflatoxins M1 cause cancer?			
	Yes	22	11.6
	No	168	88.4
Do you know aflatoxins come from feed source B1 toxin	Yes	31	16.3
	No	159	83.7
Is there any othersource of milk collection	Yes	160	84.2
	No	30	15.8
Is there any other source of milk collection	Yes	170	89.5
	No	20	10.5
Any precautionary measures do you adopt during milk collection	Yes	161	84.7
	No	29	15.3
What do you do with daily spoiled milk	Waste	118	62.1
	Mix in milk	1	0.5
	Any other	71	37.4
Did you clean the area of your outlet	Yes	166	87.4
	No	24	12.6
Do you regularly wash your shop with disinfectant	Yes	119	62.6
	No	71	37.4
Did you purchase milk from source other than farm	Yes	168	88.4
	No	22	11.6
Do you use toxin binder at your farm where milk is collected	Yes	21	11.1
	No	169	88.9
	Yes	158	83.2

Was animal feed commercial feed from where milk is collected	No	32	16.8
Do you regularly check aflatoxins M1 level at your outlet	Yes	14	7.4
	No	176	92.6
Do you know about toxin in feed and milk	Yes	22	11.6
	No	168	88.4
Do you know what is permissible level of aflatoxins M1 in milk	Yes	23	12.1
	No	167	87.9
Which method did youuse to diagnose aflatoxin M1	Elisa	190	100
	PCR	0	0
	No idea	0	0
ELISA result	Positive	170	89.4
	Negative	20	10.6

Table 2

Association of Prevalence of Aflatoxins M1 with Education, Milk Chiller, Washing Area, Toxin Binders, and Commercial Feeds

		Results			**P-value**
Education		**Positive**	**Negative**	**Total**	
	Intermediate	2	10	12	0.247
	Elementary	197	100	119	
	Noschooling		52	59	
Do you use milk precautionary measure while milk collection	Yes	145	15	160	0.59
	No	23	7	30	
	Total	168	22	190	
	Yes	120	46	166	0.40
	No	15	9	24	

Do you clean the area of outlet, wash the shop with disinfectant	Total	135	55	190	
Do you use toxin binder at your farm from where milk is collected	Yes	14	7	21	0.026
	No	140	29	169	
	Total	154	36	190	
Was the animal fed on commercial feed	Yes	130	28	158	0.265
	No	23	9	32	
	Total	153	37	190	

Table 3

Mycotoxins Level with Six Standards Using ELISA Kit

Standard level	**Absolute Value**	**Absolute Mean**	**Log concentration.**	**B/Bo (Mean Absorbance Value)**
0	1.688	1.688		1.00
5	1.627	1.627	0.699	0.964
10	1.307	1.307	1.000	0.774
25	0.876	0.876	1.398	0.519
50	0.563	0.563	1.699	0.334
100	0.427	0.427	2.000	0.253

Table 4

Prevalence of Aflatoxins M1 in Fresh Milk During Four Seasons of the Year (2019-2020)

Month	n	Range Aflatoxins M1 (ppt)	Mean Aflatoxins M1 (ppt) ± SD
Autumn			
September	21	34.177-219.60	162.680±0.51
October	14	120.75-186.22	153.48±0.45
Total	35		
Winter			
November	20	41.33-88.04	64.68±0.48
December	21	37.91-85.39	61.65±0.53
January	14	42.92-137.16	90.04±0.58
Total	55		
Spring			
February	32	68.93-175.17	122.05±0.49
March	19	49.67-211.52	130.59±0.50
Total	51		
Summer			
April	15	135.08-239.04	187.06±0.45
May	8	142.87-224.86	183.86±0.6
June	16	34.55-216.98	125.76±0.37
July	9	175.17-206.20	190.68±0.57
August	11	192.98-256.71	224.84±0.67
Total	59		

Table 5

Aflatoxins M1 Concentrations (%) in Different Seasons

Season	No.	Positive	Negative	Percentage
Autumn	35	33	2	94%
Winter	55	49	6	89%
Spring	51	46	5	90%
Summer	59	52	7	88%

Discussion and Conclusion

Aflatoxin M1 is a hydroxylated metabolite of Aflatoxin B1 and is discovered in milk or milk products obtained from animals that ingested contaminated feed (Sadeghi *et al.,* 2010). Therefore, the presence of M1 is highly concerning. In Pakistan, the hot weather runs from April through September, with a 7- to 9-week rainy period in August and July. This situation significantly impacts the production of animal feed. Temperatures range from 30 to 42 degrees Celsius, with maximum temperatures reaching 45.3 degrees Celsius (Pakistan Meteorological Department, 2016).

Climate change has been reported to have a significant impact on temperature, which encourages mycotoxin contamination (Paterson & Lima, 2010). Furthermore, during the summer, humidity levels range from 45 to 82 percent, which is extremely high (Pakistan Meteorological Department, 2016). Variation in seasons results in the highest growth rate of fungi and mold, leading to higher toxin levels in more humid environments than in less humid seasons. These hot and humid climatic conditions are conducive to fungal growth and food/feed spoilage (Probst *et al*., 2007). AFM1 was detected in 200 milk samples. The average concentration of AFB1 was found to be 0.62 in 32 samples, 0.53 in 37 samples, 0.48 in 29 samples, 0.56 in 35 samples, 0.39 in 31 samples, and 0.45 in 36 samples. AFM1 contamination was found in milk samples in several instances.

Previous investigations in Pakistan revealed high occurrences of AFM1. All milk samples (168) were found to contain toxin M1, with a mean concentration of 37 ng/L and a range of 10 to 700 ng per liter (Duarte et al., 2013). Approximately 167 samples, or 99.4%, exceeded European and American standards. Another investigation in our country found AFM1 in all 468 raw milk samples, with a mean content of 2600 ng/L, and 423 samples (87.2%) exceeded US limits (Aslam et al., 2016). The high percentage of AFM1-infected samples in the current investigation may be due to sample manufacturing using affected batches of milk powder or contaminated raw milk. Many

researchers have discovered that liquid milk had a higher rate of AFM1 contamination (89%) than solid milk, with 7.4% exceeding European regulatory levels (Redouane-Salah *et al*., 2015). AFM1 levels were calculated in 520 milk samples using an ELISA kit during the four different seasons: autumn, spring, winter, and summer in south Punjab. Almost 93% of samples were found to be positive, with 53% exceeding the EC standard.

In this study, the M1 level in milk differed across the four seasons: autumn, winter, spring, and summer. Aflatoxin levels and percentages varied according to the season because in Lahore, Pakistan, and Punjab province, the climate became humid in July, August, and September. In these seasons, fungal growth reaches its maximum level in silage and concentrates feed ingredients. Milk samples collected during this season had the highest levels of aflatoxin M1 due to fungal growth, while toxin levels remained high in winter and rainy seasons. Out of 200 samples, almost 180 samples tested positive according to European standards (Asghar *et al*., 2014). Potential relationships and various factors associated with seroprevalence were checked. The levels of Aflatoxin M1 in milk samples from the summer and winter seasons in the recent study were lower compared to a previous study (Iqbal & Asi, 2013). The results showed that 49 out of 55 samples were positive in winter and 52 out of 59 samples were positive in the summer season, exceeding the EU permissible level of Aflatoxin M1. However, toxin levels were found to be higher in the autumn and spring seasons, with 33 out of 35 samples contaminated in autumn and 46 out of 51 samples found to be positive (Chavaria *et al*., 2015).

In the present study, AFM1 levels in milk were found in 90% of milk samples. Also, they were to be significantly higher in autumn and spring, with significant amounts (94%) and (90%) respectively, contaminated. These levels exceeded those of the European Union. It was also recorded that the use of toxin binders in animal feed helped to reduce alfatoxins in milk. The findings of our study emphasize the need to establish strict rules and regulations to control the level of AFB1 in animal feed.

Additionally, milk stakeholders, the dairy industry, milk shop owners, and the public should be aware of the health risks and preventive measures associated with these aflatoxins in milk. It is evident from the findings of this study that raw milk being sold at retail shops in Lahore is unhealthy for human consumption. Lahore is one of the largest cities with an estimated population of 11,119,985 (Pakistan Bureau of Statistics, 2017). Most of the participants expressed that it poses a higher degree of contamination among its inhabitants with AFM1, this means that citizens of Lahore are at greater risk of being exposed to AFM1 in more humid months as compared to dry months. Therefore, the need of the time is to take steps, especially by the government functionaries from the grass-roots level to ensure the supply of quality milk being sold particularly during the humid months of the season.

Limitations

This study, though conducted on a limited scale, identifies raw milk as less suitable for human consumption in Punjab's capital city Lahore due to high aflatoxin M1 levels. Processed liquid milk was found to be not a safer option for consumers. In all types of milk, the levels of aflatoxin M1 levels were higher during the humid months. However, due to small scale study the results could not be generalized.

Future Research

A study for the detection of aflatoxins M1 in Lahore and other major cities of Pakistan with a larger sample size should be considered. It will help to rule out health hazards caused by aflatoxins M1 present in unhygienic milk consumed by the city population of Pakistan.

Funding

Not applicable.

Conflict of Interest

There is no conflict of interest.

Availability of Data and Materials

All data analyzed during the study are available in the manuscript.

Ethics Approval

Approved by the Ethical Review Committee of the concerned department.

Informed Consent

Approved by all authors.

References

Ahmad, M., Awais, M., Ali, S.W., Ali, Khan H.A., Riaz, M., Sultan, A., Bashir, M.S., & Ishtiaq Chaudhry, A. (2019). Occurrence of aflatoxin M1 in raw and processed milk and assessment of daily intake in Lahore, Multan cities of Pakistan. *Food Additives and Contaminants*, Part B, 12, 18-23. https://doi.org/ 10.1080/ 19393210. 2018.1509899

Akbar, N., Nasir, M., Naeem, N., Ahmad, M.U.D., Saeed, F., Anjum, F.M., Iqbal, S., Imran, M., Tufail, T., Shah, F. U., & Atif, M. (2020). Assessment of Aflatoxin in milk and feed samples and impact of seasonal variations in the Punjab, Pakistan. *Food Science and Nutrition* 8, 2699-2709. https://doi.org/10.1002/fsn3.1557

Asghar, M.A., Ahmed, A., & Asghar, M.A. (2018), Aflatoxin M1 in fresh milk collected from local markets of Karachi. *Pakistan. Food Additives & Contaminant*, Part B 11, 167-174. https://doi.org/10.1080 /19393210.2018. 1446459

Asghar, M.A., Iqbal, J., Ahmed, A., & Khan, M.A. (2014). Occurrence of afflation contamination in brown rice from Pakistan. *Iranian J Public Health*, 43(3), 291-295.

Aslam, N., Tipu, M.Y., Ishaq, M., Cowling, A., McGill, D., Warriach, H.M., & Wynn, P. (2016). Higher levels of aflatoxin M1 contamination and poorer composition of milk supplied by informal milk marketing chains in Pakistan. *Toxins*, 8(12), 347.

Aziz, S., Noor-ul-Ain, W., Majeed, R., Khan, M.A., Qayum, I., Ahmed, I., & Hosain, K. (2012). Growth centile charts (anthropometric measurement) of Pakistani pediatric population. *J. Pakistan Med. Assoc*., 62, 367–377.

Barham, G.S., Khaskheli, M., Soomro, A.H., & Nizamani, A.A. (2014). Screening of adulterants and extent of added water I market milk at central zone of Sindh, Pakistan. *IOSR J. Environ. Sci. Toxicol. Food Technol*, 8, 77–83. doi: 10.9790/2402-08727783.

Baruki, I., Bilandzic, N., Markov, K., Jakopovic, K.L., & Bozanic, R. (2018). Reduction in aflatoxin M1 concentration during production and storage of selected fermented milk. *International Journal of Dairy Technology*, 71(3), 734-740.

Bodbodak, S., Hesari, J., Peighambardoust, S.H., & Mahkam, M. (2018). Selective decontamination of aflatoxin M1 in milk by molecularly imprinted polymer coated on the surface of stainless-steel plate. *International Journal of Dairy Technology*, 71(4), 868-878.

Campagnollo, F.B., Ganev, K.C., Khaneghah, A.M., Portela, J.B. *et al.* (2016). The occurrence and effect of unit operations for dairy products processing on the fate of aflatoxin M1: A review. *Food Control*, 68: 310-329.

Cary, J.W., & Ehrlich, K.C. (2006). Aflatoxigenicity in Aspergillus: Molecular genetics, phylogenetic relationships and evolutionary implications. M*ycopathologia*, 162(3), 167-77.

Chavaria, G., Granados, F., & Molina, A. (2015). Detection of aflatoxins M1 in milk, cheese, and sour cream samples from Costa Rica using enzyme-assisted extraction and HPLC. *Food Add Cont*., 8(2), 128- 135.

Daniel, W.W. (2010). *Biostatistics: Basic concepts and methodology for the health sciences*. John Wiley & Sons, New York. 9th Ed., pp 346-353.

Duarte, S., Almeida, A., Teixeira, A., Pereira, A., *et al*. (2013). Aflatoxin M1 in marketed milk in Portugal: Assessment of human and animal exposure. *Food Control*, 30(2), 411-417.

Guo, L., Wang, Y., Fei, P., Liu, J., & Ren, D. (2019). A survey on the aflatoxin M1 occurrence in raw milk and dairy products from water buffalo in South China. *Food Control*, 105, 159-163.

Hussain, I. (2009). *Micro-analysis of Aflatoxin M1 in dairy products at trace levels and its elimination*. Ph.D. Thesis. Institute of Chemistry, University of the Punjab; Punjab, Pakistan.

Hussain, I., & Anwar, J. (2008). A study on contamination of aflatoxin M1 in raw milk in the Punjab province of Pakistan. *Food Control*, 19, 393-395.

Imtiaz, N., & Yunus, A.W. (2019). Comparison of some ELISA kits for aflatoxin M1 quantification. *J. AOAC Int.*, 01 doi: 10.5740/jaoacint.18-0185.

Iqbal, S.Z., Asi, M.R., & Ariño, A. (2011). Aflatoxin M1 contamination in cow and buffalo milk samples from the Northwest Frontier Province (NWFP) and Punjab provinces of Pakistan. *Food Additives and Contaminant*, Part B 4, 282-288. https://doi.org/ 10.1080/19393210.2011.637237

Iqbal, S.Z., Asi, M.R., & Selamat, J. (2014), Aflatoxin M1 in milk from urban and rural farmhouses of Punjab, Pakistan. *Food Additives and Contaminants*, Part B. 7, 17-20. https://doi.org/10.1080/19393210.2013.828322

Iqbal, S.Z., Jinap, S., Pirouz, A., & Faizal, A.A. (2015). Aflatoxin M1 in milk and dairy products, occurrence and recent challenges: A review. *Trends in Food Science & Technology*, 46(1), 110-119.

Iqbal, S.Z., Paterson, R.R.M., Bhatti, I.A., & Asi, M.R. (2011). Comparing aflatoxin contamination in chilies from Punjab, Pakistan produced in summer and winter. *Mycotoxin Research*, 27, 75-80.

Ismail, A., Riaz, M., Akhtar, S., Yoo, S.H., Park, S., Abid, M., Aziz, M., & Ahmad, Z. (2017). Seasonal variation of aflatoxin B1 content in dairy feed. *Journal of Animal and Feed Sciences*, 26, 33-37.

Ismail, A., Riaz, M., Levin, R.E., Akhtar, S., Gong, Y.Y., & Hameed, A. (2016). Seasonal prevalence level of aflatoxin M1 and its estimated daily intake in Pakistan. *Food Control*, 60, 461-465. https://doi.org/10.1016/

Jawaid, S., Talpur, F.T., & Afridi, H.I. (2015). Contamination profile of aflatoxin M1 residues in the milk supply chain of Sindh Pakistan. *Toxicol. Rep*., 2:1418–1422.

Nidhina, N., Bhavya, M., Bhaskar, N., Muthukumar, S., & Murthy, P.S. (2017). Aflatoxin production by Aspergillus flavus in rumen liquor and its implications. *Food Control*, 71, 26-31.

Pakistan Meteorological Department. 2016. Government of Pakistan. [Internet]. [cited 2017 Sep 05]. Available from: http://www.pmd.gov.pk/.

Paterson, R.R.M., & Lima, N. (2010). How will climate change affect mycotoxins in food? *Food Res*., 43, 1902-1914.

Prandini, A., Sigolo, T.S., Filippi, L., Laporta. M., & Piva, G. (2009). On the occurrence of aflatoxin M1 in milk and dairy products. *Food Chem Toxicol*., 47, 984-991.

Probst, C., Njapau, H., & Cotty, P. J. (2007). Outbreak of an acute aflatoxicosis 369 in Kenya in 2004: identification of the causal agent. *Applied and Environmental Microbiology*, 73, 2762-2764.

Punjab Pure Food Regulations (2018). *Punjab Food Authority, Government of Punjab, Pakistan* (www.pfa. gop.pk/wp-content/uploads/2018/06/Punjab-pure-food -regulations-2018. pdf)

Redouane-Salah, S., Morgavi, D.P., Arhab, R., Messa, A., & Boudra, H. (2015). Presence of aflatoxin in raw, reconstituted, and powdered milk samples collected in Algeria. *Environmental Monitoring and Assessment*, 187(6), 155-163.

Reverberi, M., Ricelli, A., Zjalic, S., Fabbri, A.A., & Fanelli, C. (2010). Natural functions of Mycotoxins and control of their biosynthesis in fungi. *Applied Microbiology and Biotechnology*, 87, 899-911.

Sadeghi, N., Oveisi, M., Jannat, B., Hajimahmoodi, B., *et al*. (2010). Incidence of aflatoxin M1 in human breast milk in Tehran, Iran. *Food Control*, 20, 75-78.

Sadia, A., Jabbar, M.A., Deng, Y., Hussain, E.A., Riffat, S., Naveed, S., & Arif, M. (2012). A survey of aflatoxin M1 in milk and sweets of Punjab, Pakistan. *Food Control*, 26, 235–240.

Tahira, I., Sultana, N., Munir, A., Hasan, S.M., & Hanif, N.Q. (2019). Occurrence of Aflatoxin M1 in raw and processed milk consumed in Pakistan. *Pakistan Journal of Pharmaceutical Sciences*, 32, 1097-1101.

Xiong, J., Xiong, L., Zhou, H., Liu, Y., & Wu, L. (2018). Occurrence of aflatoxin B1 in dairy cow feedstuff and aflatoxin M1 in UHT and pasteurized milk in central China. *Food Control*, 92, 386-390.

Younus, M., Abbas, T., Rafique, K., Sajid M., Aslam, M., & Zafar, M. (2013). Analyses of selected heavy metals and Aflatoxin M1 in milk for human consumption in Jhang City Pakistan. *International Food Policy Research Institute* (IFPRI); Washington DC, USA: 2013. Working Paper 12.

Yunus, A.W., Imtiaz, N., Khan, H., Ibrahim, M.N.M., & Zafar, Y. (2019), Aflatoxin contamination of milk marketed in Pakistan: A longitudinal study. *Toxins*, 11, 110. https://doi.org/10.3390/toxins11020110

Zahra, N., Jamil, N., Ahmad, S.R., Kalim, I., Saeed, M.K., Ijaz, A., & Munir, S. (2020). Assessment of milk quality by analyzing Aflatoxin M1 in Branded and Non-Branded Milk Samples Collected from Different Shops/Areas in Lahore, Pakistan. *Pak Non-Branded of Zoology*, 52, 373.

Note: **Open Access Public Health and Health Administration Review** is recognized by the Higher Education Commission Pakistan in the Y category.

Disclaimer/ Publisher's Note: The statements, opinions, and data contained in all publications in this journal are solely those of the individual author(s) and not of the MDPIP and/ or the editor(s). MDPIP and editor(s) disclaim responsibility for any injury to the people or property resulting from any ideas, methods, instructions, or products referred to in the content.