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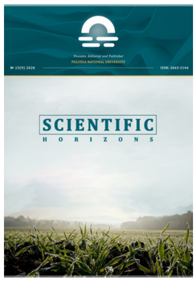

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Evaluation of aflatoxin M1 and nutritional content in Kosovo's market cheese samples

Arieta Camaj Ibrahimi
PhD in Food Science and Technology, Assistant Professor
University Haxhi Zeka
30000, "UÇK" Str., Pejë, Republic of Kosovo
https://orcid.org/0000-0002-3560-3704

Bajram Berisha
PhD in Agricultural Sciences, Full Professor, Doctor Habilitatus
University of Prishtina
10000, 31 George Bush Str., Prishtine, Republic of Kosovo
https://orcid.org/0000-0001-9112-054X

Hata Dibrani Sopjani
Master of Science, Chemist
Food and Veterinary Agency
10000, "Nëna Terezë" Sq., Prishtinë, Republic of Kosovo
https://orcid.org/0009-0008-6245-9024

Aferdita Camaj Isa
PhD in Chemistry, Quality Control Manager
Kosova Medicines Agency
10000, Lagjia e Spitalit, Prishtinë, Republic of Kosovo
https://orcid.org/0000-0001-7044-8810

Shyhrete Muriqi*
PhD in Agroeconomics, Assistant Professor
University Haxhi Zeka
30000, "UÇK" Str., Pejë, Republic of Kosovo
https://orcid.org/0000-0002-3389-0594

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Abstract. The ubiquitous presence and potent carcinogenic effects of aflatoxins, a group of mycotoxins, pose a significant threat to global food safety, especially within the dairy sector. This research aimed to evaluate the prevalence and distribution of Aflatoxin M1 in diverse cheese varieties within Kosovo's markets in 2022 and discern potential correlations with cheese types, processing conditions, and nutritional parameters to enhance understanding and ensure the safety of dairy products. In the latter half of 2022, a total of 93 different types of manufactured cheese samples from 15 different

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**Corresponding author*

countries were analysed for Aflatoxin M1 toxin content using the enzyme-linked immunosorbent assay technique. The results of the research indicate that several countries have exceeded the concentration of 0.05 ug/kg for Aflatoxin M1, set as the maximum tolerable limit for milk and milk products in many countries. Notable, cheese samples from Albania exhibited substantially (p=0.000) higher levels of Aflatoxin M1 compared to other nations. The results showed that the Aflatoxin M1 concentration did not exhibit statistically significant variations based on the regulated total fat content (p=0.902), carbohydrate content (p=0.741), protein concentration (p=0.298), or salt content (p=0.122) within the cheese. The results demonstrated that Aflatoxin M1 levels were not significantly (p=0.926) affected by cheese consistency. While different starter cultures were associated with varying Aflatoxin M1 concentrations, these differences were not statistically significant (p=0.472). Similarly, ageing time did not exert a significant (p=0.691) impact on AFM1 levels, as concentrations remained relatively consistent across different ageing periods. These findings provide valuable insights into the variations in Aflatoxin M1 levels in cheese samples among different countries. They highlight the importance of regulatory measures and continuous monitoring to ensure food safety and adherence to maximum allowable limits

Keywords: cheese types; ELISA; Aflatoxin M1 contamination; dairy product safety

INTRODUCTION

Aflatoxins, toxic by-products created predominantly by the fungi Aspergillus flavus and Aspergillus parasiticus, are a type of mycotoxin. These toxins frequently contaminate food and are especially prevalent in the basic foods of numerous developing nations. Aflatoxins are generated through fungal activity at various stages, including production, harvesting, storage, and processing of food. The US Food and Drug Administration (FDA) (2022) regards these toxins as unavoidable food contaminants. Of all the various aflatoxins, Aflatoxin B1 (AFB1), poses a distinct risk to the dairy industry. When ruminants consume feed contaminated with AFB1, their bodies metabolize it, leading to the secretion of aflatoxin M1 (AFM1) in their milk. Aflatoxins are known to cause liver toxicity, cancerous effects, and suppression of the immune system (Ferrari *et al.,* 2023). As noted by the World Health Organization (2018) also, AFM1 is associated with liver and kidney cancers and may potentially compromise the immune system, weakening its disease-fighting capacity.

These substances can be present in numerous crucial agricultural and food items, predominantly influenced by factors such as product moisture content, water activity, relative air humidity, temperature, pH value, composition of the food matrix, the extent of physical damage, and the existence of mould spores (Pleadin *et al.,* 2019). The resilience of AFM1 across various temperatures and pH levels, which enables it to remain stable through different phases of dairy processing, is very concerning. Consequently, conventional heat methods like pasteurization and sterilization are unable to eliminate it effectively. Existing and developing physical techniques, including pulsed electric fields and various nonthermal processes, along with the use of chemical substances like acids, enzymes, gases, and absorbents in livestock management, have proven to be successful in diminishing the levels of mycotoxins in both feed and food products (Sipos *et al.,* 2021). Although AFM1 is less toxic than its precursor AFB1, it is still considered a potential human liver carcinogen and is categorized as Group 2B by the International Agency for Research on Cancer (IARC, 2002).

The European Commission (2006), enforces strict regulations on AFM1 levels, given its ubiquitous presence and serious health consequences. Continuous monitoring and effective control measures are crucial for managing AFM1 contamination in dairy products, ensuring food safety and public health, as AFM1 could be passed from milk into milk products. N. Salari *et al.* (2020) conducted a systematic review and meta-analysis to assess the global occurrence of AFM1 in milk. They examined 122 articles, encompassing a total of 18,921 samples from January 1988 to February 2020, and found that 79.1% of the milk samples contained AFM1, indicating a widespread presence of this toxin in milk around the world. According to a study done by J. Kos *et al.* (2023), as the climate conditions in Europe continue to provide increasingly favourable conditions for the proliferation of Aspergillus flavus, there is an escalating likelihood of cereals being exposed to elevated risks of contamination by highly hazardous aflatoxins in the future.

The transfer of AFM1 from milk to cheese is unavoidable, considering that this toxin is not removed through conventional treatment methods. Nevertheless, the concentration of AFM1 in various types of cheese and other dairy products has been found to vary. Kosovo, a Balkan region with substantial dairy production and consumption, is no exception to this challenge. Yet, comprehensive, and systematic data on AFM1 contamination in the region is scant. This issue becomes even more critical considering the local population's dietary habits, with milk and dairy products playing a key role (FAO, 2013). As per a study done by A. Camaj *et al.* (2018), high percentage of milk samples which were non-compliant with AFM1 maximum levels. However, a subsequent study by A. Camaj *et al.* (2023) concluded that the prevalence of AFM1 contamination of raw milk in Peja, Kosovo, was lower than previously reported. All samples complied with European Union regulations for

AFM1, indicating increased farmer awareness regarding feed storage and regular AFM1 monitoring. However, the study emphasized the need for regular institutional monitoring of imported UHT milk samples. Yet, published data on AFM1 levels in cheese consumed in Kosovo, particularly those imported, is lacking. This study is crucial as a variety of foreign cheese types are available in Kosovo's markets.

Accordingly, this study aimed to assess the presence and distribution of AFM1 in various cheese types available in Kosovo's markets during 2022, including both locally produced and imported cheeses, and to assess if there is a correlation between cheese types, processing conditions, and nutritional parameters, to the aflatoxin M1 level in cheese. This study is the first article of this context done in Kosovo.

MATERIALS AND METHODS

Sample Collection. In the latter half of 2022, a total of 93 different types of manufactured cheese samples were procured from the largest food suppliers across Kosovo for the analysis of the AFM1 toxin content. The chemical analysis of the toxin AFM1 was conducted in the laboratories of the Food and Veterinary Agency of Kosovo. Additionally, other parameters like, the cheese's nutritional parameters including fat total (g/100g), carbohydrates total (g/100g), protein (g/100g), and salt (g/100g); parameters such as, consistency (hard, semi-hard, semi-soft, semi-soft creamy, soft, and soft-creamy); starter cultures (bacterial cultures, bacterial-rennet, bacterial-rennet-fungal, rennet, bacterial-rennet-processed, rennet-processed); and ageing time (none, 1-3 months, 4-6 months, 7-12 months, <13 months) are considered for the comparative analysis. These data were gathered from the producers' declarations on labels as well as internet sources. These samples originated from fifteen distinct countries, including: Kosovo (6 samples), North Macedonia (6), Albania (3), Slovenia (3), Greece (5), Italy (27), Germany (7), France (5), Croatia (5), Netherlands (3), Turkey (3), Hungary (5), Lithuania (3), Austria (9), and Denmark (3). To ensure optimal preservation and accurate analysis, these samples were stored in deep freeze conditions at -18°C. They were then brought up to room temperature prior to the execution of the analyses.

Laboratory analyses. The procedure for quantitatively analysing AFM1 in the cheese samples was carried out using the enzyme-linked immunosorbent assay (ELISA) technique. The assessment of AFM1 was conducted utilizing the MEIZHENG Bio-Tech Co. Aflatoxin M1 ELISA Test Kit, strictly adhering to the protocol provided by the manufacturer, briefly outlined below. Each testing reagent Kit offered a certificate with the validation results. All the samples were brought to room temperature before analysis. The dilution factor was 9. For testing, 1 g of each sample and 4 mL of methanol were mixed into a centrifuge tube and put to vortex to mix well. Then the mixture was put into the centrifuge at 4000 rpm for 5 minutes. From the mixture, 1 mL of the supernatant was transferred into a centrifuge tube and blow dry under 50-60°C Termovap Sample Concentrator. In each tube was added 1 mL of Sample Diluting Buffer II and vortexed to mix well. For the assay was taken 50 μL of the sample dilution.

The Aflatoxin M1 ELISA Test Kit operates on the principle of an indirect competitive enzyme-labelled immunoassay. The Aflatoxin M1 antigen is pre-embedded in the wells. Within the sample, the aflatoxin M1 vies for binding with the aflatoxin M1 antibody against the aflatoxin M1 antigen already present on the well. Meanwhile, the M1 antibody associates with the enzyme conjugate. Subsequently, the substrate solution is added to the wells, which prompts a colour change. The colour intensity of the unknown samples is compared to that of the standards, enabling the determination of Aflatoxin M1 concentration. Using a plate reader set to 450 nm, absorbance values were recorded. The AFM1 level was then calculated from a logarithmic standard curve, and the mean value of the duplicates was recorded as results.

Based on the information provided in the manufacturer's certificate, the sensitivity was 0.015 μg/kg, equivalent to the Kit's Limit of Detection (LOD) for raw milk determination. This implies that the Kit might detect aflatoxin M1 at concentrations even lower than the LOD. Typically, sensitivity aligns with the concentration of the second standard (Table 1). The percent recovery was within the range of 100%±30%, and the intra-lab assay precision was denoted with a Coefficient of Variation (CV%) less than 10%. In this study, it's crucial to note that the kit's LOD for cheese determination was 0.06 μg/kg, which is slightly above the maximum permissible limit of aflatoxin M1 in cheese as adapted by regulatory standards in Kosovo. (0.05 μg/kg). The observations included results that fell below the Kit's stated LOD. This suggests that some cheese samples contained extremely low, or potentially even undetectable, levels of aflatoxin M1.

Aflatoxin M1 ELISA Test Kit standard summary. The ELISA test kit validation certificate of analysis by the manufacturer provides reference points for comparing the results obtained from samples tested with the Aflatoxin M1 ELISA Test Kit. Table 1 shows the absorbance values A450, B/B0 ratios, and CV% values for different concentrations of Aflatoxin M1 standards used in the validation.

Table 1. *Aflatoxin M1 ELISA test Kit validation results*

Aflatoxin M_1 (ng/kg)	A_{450}	B/B_0	CV%
Negative control			
0	1.836	100.0	2.1

Table 1. Continued

15	1.410	76.2	4.2
45	0.901	49.1	3.7
150	0.356	19.4	0.4
500	0.090	4.9	3.1

Notes: *A_{450} = Average absorbance using 450 nm primary filter and 630 nm differential filter*
Source: *developed by the authors*

CV% (Coefficient of Variation) is calculated as:

$$CV = (SD/\mu) \times 100\%, \quad (1)$$

where SD is the standard deviation of the replicates (two replicates for each concentration of the standard), and μ is the average of replicates.

Statistical analysis. Statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS). The data were categorized into fifteen groups based on the origin of the cheese. The AFM1 content was reported as mean values with standard deviation (SD), median, minimum, and maximum concentration. To determine if there were significant differences between groups, statistical tests were employed. To explore variations in AFM1 levels between different groups, the dataset was categorized based on the concentration of AFM1. These classifications were established according to their adherence to a designated threshold value falling within the range of >0.050 and <0.051 μg/kg. For comparisons between two groups, an Independent Sample t test was employed to assess differences in AFM1 levels within this defined range. One-way Analysis of Variance (ANOVA) was used to assess the influence of AFM1 levels on the consistency, starter cultures, and ageing time of different types of cheeses. The grouping of cheese samples into three or more categories was based on relevant factors such as cheese variety, production methods, and geographical origin. A significance level of $p<0.05$ was used for the test.

RESULTS AND DISCUSSION

Comparative analysis of cheese production among countries. In relation to the presence of Aflatoxin M1 in a total of 93 cheese samples, the outcomes (Table 2) revealed noteworthy findings. The mean concentration of Aflatoxin M1 detected in Italian cheese samples was recorded as 0.061±0.072 μg/kg, with the range spanning from 0.01 to 0.34 μg/kg. Out of these samples, 4 (14.81%) exceeded the LOD (Limit of Detection) and 12 (44.4%) exceeded the maximum level of 0.05 μg/kg.

Table 2. *Overview of cheese sample characteristics: a general analysis*

Origin of Cheese	N	% of total samples	x±σ	Median μg/kg	Min μg/kg	Max μg/kg	No. (%) of samples exceeding LOD**	No. (%) of samples exceeding the maximum level of 0.05 μg/kg*
Italy	27	29.0%	0.061±0.072	0.048	0.01	0.34	4(14.81%)	12(44.4%)
France	5	5.4%	0.191±0.334	0.334	0.02	0.79	2(40%)	2(40%)
Croatia	5	5.4%	0.036±0.016	0.016	0.02	0.06	0(0%)	1(20%)
Netherlands	3	3.2%	0.066±0.011	0.011	0.06	0.08	1(33.33%)	3(100%)
Germany	7	7.5%	0.063±0.022	0.022	0.03	0.09	4(57.14%)	4(57.14%)
Slovenia	3	3.2%	0.023±0.020	0.020	0.00	0.04	0(0%)	0(0%)
Turkey	3	3.2%	0.056±0.005	0.005	0.05	0.06	0(0%)	2(66.66%)
Greece	5	5.4%	0.152±0.195	0.195	0.04	0.50	2(40%)	3(60%)
Albania	3	3.2%	0.597±0.342	0.342	0.20	0.83	3(100%)	3(100%)
North Macedonia	6	6.5%	0.072±0.036	0.036	0.04	0.12	3(50%)	5(83.33%)
Hungary	5	5.4%	0.048±0.024	0.024	0.01	0.08	1(20%)	2(40%)
Lithuania	3	3.2%	0.046±0.005	0.005	0.04	0.05	0(0%)	0(0%)
Austria	9	9.7%	0.056±0.045	0.045	0.02	0.16	2(22.22%)	4(44.44%)
Denmark	3	3.2%	0.030±0.016	0.016	0.01	0.04	0(0%)	0(0%)
Kosovo	6	6.5%	0.062±0.007	0.007	0.06	0.07	2(33.33%)	6(100%)
Pulled	93	100%	0.086±0.145	0.145	0.00	0.83	24	47(50.53%)

Notes: **Kosovo regulation; **LOD – Limit of detection (0.06 μg/kg)*
Source: *developed by the authors*

Conversely, in the case of French cheese samples, the mean Aflatoxin M1 concentration was determined to be 0.191±0.334 μg/kg, and the range of detected values varied from minimum 0.02 to 0.79 μg/kg. According to the study, 2 (40%) of the French cheese samples tested exceeded the LOD for AFM1 and 2 (4%) exceeded the maximum level of 0.05 μg/kg. Croatian cheese samples exhibited a mean Aflatoxin M1 concentration of 0.036±0.016 μg/kg, with the range observed between 0.02 and 0.06 for the entire sample set. Out of the samples tested, none of the samples exceeded the LOD while, 1 (20%) surpassed the maximum level of 0.05 μg/kg. In the case of Netherlands cheese samples, the mean concentration of AFM1 was found to be 0.066±0.011 μg/kg, with a range of 0.06 to 0.08 μg/kg for the entire sample set. Conversely, for Netherlands cheese samples, 1 (33.33%) exceeded the LOD and 3 (100%) exceeded the maximum level of 0.05 μg/kg. Additionally, Dutch cheese samples exhibited a mean Aflatoxin M1 concentration of 0.063±0.022 μg/kg, with the range falling between 0.03 and 0.09 μg/kg. Out of all the samples, 4 (57.14%) exceeded the LOD of AFM1 and 4 (57.14%) exceeded the maximum level of 0.05 μg/kg. In the context of additional countries, Slovenia exhibited Aflatoxin M1 concentrations with a mean value of 0.023±0.020 μg/kg. The range of observed values spanned from 0.00 to 0.04 μg/kg. Notably, none of the samples tested exceeded the LOD and the maximum level of 0.05 μg/kg. These findings suggest that the levels of AFM1 in Slovenian cheese samples are within acceptable limits.

Turkish cheese samples had a mean Aflatoxin M1 concentration of 0.056±0.005 μg/kg, with the lowest recorded content being 0.05 and the highest 0.06 μg/kg, based on the results (Table 2) none of the samples tested exceeded the LOD, while 2 (66.66%) surpassed the maximum level of 0.05 μg/kg. On the other hand, Greek cheese samples displayed a mean Aflatoxin M1 content of 0.152±0.195 μg/kg, with the range varying from 0.04 to 0.50 μg/kg. Out of all the samples, 2 (40%) exceeded the LOD and 3 (60%) exceeded the maximum level of 0.05 μg/kg. For Albanian cheese samples, the mean Aflatoxin M1 concentration was determined as 0.597±0.342 μg/kg, and the recorded minimum and maximum values spanned from 0.20 to 0.83 μg/kg. Notably, all the samples tested 3 (100%) exceeded the Limit of Detection and the maximum level of 0.05 μg/kg. These findings reveal that the levels of AFM1 in Albanian, Greece, and France cheese samples analysed notably exceed acceptable limits.

In relation to cheese samples from North Macedonia, the analysis revealed a mean Aflatoxin M1 content of 0.072±0.036 μg/kg, with a range spanning from 0.04 to 0.12 μg/kg, from the total sample tested 3 (50%) exceeded the LOD, whereas 5 (83.33%) exceeded the maximum level of 0.05 μg/kg. Hungarian cheese samples exhibited a mean Aflatoxin M1 content of 0.048±0.024 μg/kg, characterized by a minimum content of 0.01 and a maximum content of 0.08 μg/kg, while 1 (20%) of the sample tested exceeded the Limit of Detection, while 2 (40%) surpassed the maximum level of 0.05 μg/kg for AFM1. In contrast, Lithuanian cheese samples displayed a mean Aflatoxin M1 content of 0.046±0.005 μg/kg, with the detected values ranging from 0.04 to 0.05 μg/kg. The results of the study indicated that none of the samples tested exceeded the Limit of Detection (LOD) and exceeded the maximum level of 0.05 μg/kg. However, Austrian cheese samples indicated a mean Aflatoxin M1 content of 0.056±0.045 μg/kg, and the minimum and maximum values observed were 0.02 and 0.16 μg/kg, respectively. Results showed that 2 (22.22%) exceeded the LOD, whereas 4 (44.44%) exceeded the maximum level of 0.05 μg/kg. In terms of Danish cheese samples, the mean Aflatoxin M1 content was determined to be 0.030±0.016 μg/kg, while the range of detected values varied from 0.01 to 0.04 μg/kg. The results showed that none of the samples tested exceeded the Limit of Detection (LOD) and exceeded the maximum level of 0.05 μg/kg. Lastly, the analysis of Kosovo cheese samples demonstrated a mean Aflatoxin M1 content of 0.062±0.007 μg/kg, with the range of observed values falling between 0.06 and 0.07 μg/kg. Resulting that 2 (33.33%) of the Kosovar cheese samples tested exceeded the Limit of Detection (LOD) for AFM1.

The homogeneity of variances was tested using Levene's test, which revealed a statistically significant result (Levene Statistic=6.677, df1=14, df2=78, p=0.000). This indicates that the variances of the dependent variable differed significantly across the groups. The current study aimed to investigate the variations in average levels of Aflatoxin M1 (AFM1) in cheese across fifteen countries included in the study. There is a significant difference between groups as determined by one-way ANOVA (F (14.78)=5.26, p=0.000). A Tukey post hoc test, One Way Anova, revealed that the cheese with Albania origin content is statistically significantly higher level of AFM1 (0.59±0.34) compared to other countries as Italy (0.061±0.072), France (0.191±0.334), Croatia (0.036±0.016), Netherlands (0.066±0.011), Germany (0.063±0.022), Slovenia (0.023±0.020), Turkey (0.056±0.005), Greece (0.152±0.195), North Macedonia (0.072±0.036), Hungary (0.048±0.024), Lithuania (0.046±±0.005), Austria (0.056±0.045), Denmark (0.030±0.016), and Kosovo (0.062±0.007).

However, no significant differences (p>0.05) were observed among the remaining countries, indicating comparable levels of AFM1 in cheese samples from Italy, France, Croatia, Netherlands, Germany, Slovenia, Turkey, Greece, North Macedonia, Hungary, Lithuania, Austria, Denmark, and Kosovo. The mean levels of Aflatoxin M1 (AFM1) in cheese samples collected from countries compliant with the maximum allowable limit set by the European Commission (2006) (0.05) were as follows: Slovenia (0.023±0.020), Denmark (0.030±0.016), Croatia (0.036±0.016), Lithuania (0.046±0.005), and Hungary (0.048±0.024) as illustrated in Figure 1. The results of the research indicate that most of the countries have exceeded the permitted limit (0.05 μg/kg) of AFM1 in cheese products. These countries include Albania, France, Greece, North Macedonia, The Netherlands, Germany, Kosovo, Italy, Austria, and Turkey.

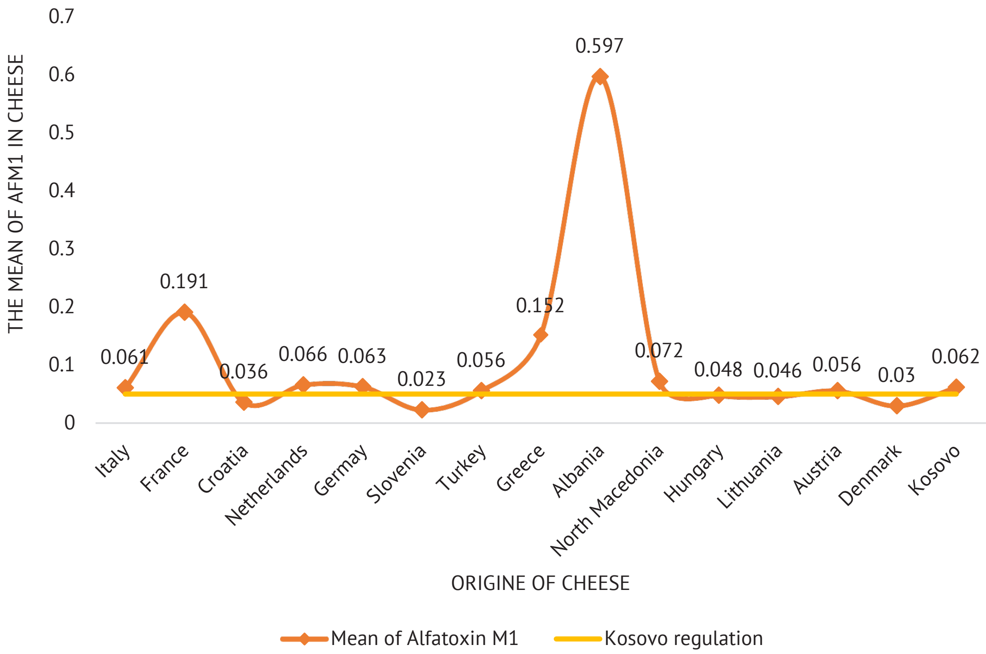

Figure 1. *Mean AFM1 concentration in cheese samples from different countries*
Source: *developed by the authors*

In relation to the Balkans, there are numerous studies that document the presence of AFM1 in dairy products. A research project, conducted by G. Ilievska *et al.* (2022), studied 974 dairy samples from North Macedonia's markets. The samples included 404 ultra-high temperature (UHT) milk, 291 ice cream, 178 yogurts, and 101 cheese, all of which were scrutinized for Aflatoxin M1 contamination. The study found that AFM1 concentrations averaged 49.1±68.4, 30.9±30.0, 35.1±40.4, and 40.1±90.1 ng/kg for UHT milk, ice cream, yogurt, and cheese samples, respectively. On a similar note, a separate study in Albania by D. Topi *et al.* (2022), examined 1,200 milk samples from various Albanian regions over a span of two years. Their findings indicated that 82.3% of the samples contained AFM1, with an average concentration of 0.051 μg/kg. Moreover, they found that 10.8% of the milk samples exceeded the European Union's maximum permissible limit of 0.05 μg/kg for AFM1. These findings underscore the widespread nature of AFM1 contamination in the region and the need for rigorous monitoring and control measures. A global systematic review and meta-analysis by T. Mahmudiono *et al.* (2023) which included thirty-one scientific papers with 34 data reports (Sample size=2,277) in their study, concluded that the lowest and highest prevalence of AFM1 in cheese was related to El Salvador (12.18%) and Serbia (100.00%). The pooled prevalence of AFM1 was 49.85%, 95% CI (37.93-61.78%). In our study, there were not analysed the cheese samples originated from Serbia. Cheese produced in Serbia, because of the political issues, it is not common in Kosovo's markets even though it is a neighbouring country.

Comparative Analysis: Examining AFM1 Levels in Cheese Alongside Nutritional Parameter. The following Table 3 presents a comprehensive overview of various nutritional parameters intrinsic to cheese. These parameters include total fat content, complete carbohydrate composition, protein constitution, and sodium chloride content. The empirical findings evince that, with regard to the aggregate fat content, the mean attains a value of 24.10 g per 100 g. Notably, the lower and upper bounds of this continuum manifest at 1.50 g per 100 g and 35 g per 100 g, respectively. Conversely, in relation to the composite carbohydrate content encompassing the entire cheese sample set, the mean is ascertained at 1.89 g per 100 g. The minimum and maximum values are reported 1.00 g per 100 g and 12.50 g per 100 g. Pertaining to the protein profile of cheese, the total mean registers at 19.87 g per 100 g, accompanied by an amplitude stretching from 1.50 g per 100 g to 37 g per 100 g. The ultimate nutritional parameter, namely sodium chloride content, is reported at 2.11 g per 100 g, characterized by a range from 0.50 g per 100 g to 5.60 g per 100 g in terms of minimum and maximum values, respectively.

***Table 3.** The characteristics of various nutritional parameters of cheese*

Nutritional parameters	Mean±SD	Min	Max
Fat-total (g/100g)	24.10±6.17	1.50	35
Carbohydrates-total(g/100g)	1.89±2.45	1.00	12.50
Protein (g/100g)	19.87±7.46	1.50	37
Salt(g/100g)	2.11±.93	0.50	5.60
Other parameters	Subcategory	N (Percentage)	
Consistency	Hard Semi hard Semi soft; semi soft creamy soft; soft-creamy	9(9.9%) 24(26.4%) 4(4.4%) 54(59.3%)	
Starter cultures	Bacterial cultures Bacterial-rennet Bacterial-rennet-fungal Rennet Bacterial-rennet-processed Rennet-processed	11(12.5%) 38(43.2%) 6(6.8%) 17(19.3%) 4(4.5%) 12(13.6%)	
Ageing time	None 1-3 months 4-6 months 6-12 months >13 months	35(40.2%) 34(39.1%) 4(4.6%) 11(12.6%) 3(3.4%)	

***Source:** developed by the authors*

Table 3 presents a range of descriptive data regarding the complete collection of cheese samples, concerning diverse factors like texture, starter cultures, and cheese maturation duration. Drawing from the outcomes of the entire cheese sample pool, if was identified 9 samples as hard (constituting 9.9%), 24 as semi-hard (accounting for 26.4%), 4 as semi-soft/semi-soft creamy (equivalent to 4.4%), and 54 as soft/soft-creamy (comprising 59.3%) cheeses. Among the starter cultures analysed in the total sample size, the distribution was as follows: 11 samples (12.5%) were identified as bacterial cultures, 38 samples (43.2%) were categorized as bacterial-rennet cultures, 6 samples (6.8%) were classified as bacterial-rennet-fungal cultures, 17 samples (19.3%) were attributed to rennet cultures, 4 samples (4.5%) were characterized as bacterial-rennet-processed cultures, and 12 samples (13.6%) were associated with rennet-processed cultures. Regarding the duration of ageing, out of the total sample of 93, 40.2% (n=35) were categorized as "none." In the 1-3 months range, there were 34 samples, accounting for 39.1% of the total. The 4-6 months bracket had 4 samples, representing 4.6% of the group. For the 6-12 months category, there were 11 samples, making up 12.6%. Additionally, 3 samples (3.4%) were recorded as having an ageing duration of more than 13 months. To assess the potential differences of the examined nutritional parameters on the levels of Aflatoxin M1 (AFM1) in cheese, an Independent Sample T-test was employed (Table 4).

***Table 4**. Comparison of nutritional parameters in different concentrations of Aflatoxin M1 in cheese samples*

	AFM1 concentration >0.050 µg/kg	AFM1 concentration <0.051 µg/kg	Mean Difference	t-value	Sig.
Nutritional parameters	Mean	Mean			
Fat total	24.90	24.75	0.151	0.124	0.902
Carbohydrates	2.14	1.95	0.19	0.331	0.741
Protein	21.65	19.92	1.72	1.04	0.298
Salt	1.90	2.26	-0.35	-1.56	0.122

***Source:** developed by the authors*

The ensuing Table 3 presents the outcomes of a thorough comparative analysis pertaining to the concentrations of AFM1 in discrete classifications of cheese samples. These classifications are established according to their adherence to a designated threshold value falling within the range of >0.050 and <0.051 µg/kg. In relation to the concentration of AFM1 concerning the total fat content, the average concentration observed in cheese samples surpassing the specified threshold of 0.050 µg/kg is measured at 24.90 µg/kg. In contrast, samples that fall below the threshold exhibit a slightly lower average concentration of 24.75 µg/kg. Consequently, the results collectively suggest an absence of statistically significant (p=0.902) differentiation in

AFM1 concentration based on the regulated total fat content within the cheese. In the case of AFM1 concentration of carbohydrate content, the mean concentration variated from 2.14 µg/kg in cheese samples to the predefined threshold, while a slightly diminished mean of 1.95 µg/kg characterizes samples situated below the threshold. The results show no significant differences (p=0.175) between the two groups in the concentration of carbohydrate content. In terms of protein, the mean concentration is 21.65 µg/kg in samples exceeding the predefined threshold, while a slightly diminished mean of 19.92 µg/kg characterizes samples situated below the threshold. The findings revealed no statistically significant (p=0.298) differences across AFM1 level for protein concentration. Turning attention to AFM1 concentration with respect to salt content, samples surpassing the established threshold exhibit a mean concentration of 1.90 µg/kg, whereas their sub-threshold counterparts display a marginally higher mean concentration of 2.26 µg/kg. The results indicate an absence of statistically significant (p=0.122) divergence in AFM1 concentration, predicated upon the regulated salt content within the cheese.

The current investigation utilized a One-way ANOVA to aspects compare the consistency, starter cultures and ageing time of cheese in the levels of AFM1 in different types of cheeses. The results represented in Table 5 indicate that there was no statistically significant (p=0.926) difference in the average levels of AFM1 across consistency for hard (0.077±0.10 µg/kg), semi-hard (0.078±0.14 µg/kg), semi-soft, semi-soft cream (0.061±0.019 µg/kg), soft, and soft-creamy (0.095±0.159 µg/kg) cheese.

Table 5. *Comparison of consistency, starter culture and ageing time parameters in Aflatoxin M1 concentration in Cheese Samples*

Variables	**Level of AFM1**	
Consistency	Mean±SD	
Hard	0.077±0.11[a]	0.02-0.34
Semi hard	0.078±0.15[b]	0.01-0.76
Semi soft; semi soft creamy	0.061±0.019[c]	0.04-0.08
Soft; soft-creamy	0.095±0.163[d]	0.00-0.83
Starter cultures		
Bacterial cultures	0.15±0.24[a]	0.04-0.83
Bacterial-rennet	0.10±0.16[b]	0.01-0.79
Bacterial-rennet-fungal	0.16±0.17[c]	0.06-0.50
Rennet	0.04±0.02[d]	0.01-0.08
Bacterial-rennet-processed	0.06±0.01[e]	0.06-0.08
Rennet-processed	0.05±0.03[f]	0.02-0.16
Ageing time		
None	0.09±0.15[a]	0.00-0.83
1-3 months	0.10±0.17[b]	0.01-0.79
4-6 months	0.01±0.00[c]	0.01-0.02
7-12 months	0.04±0.01[d]	0.02-0.08
<13 months	0.06±0.00[e]	0.06-0.07

Notes: *means with same superscript letters differ significantly at p<0.05. *Minimum and maximum levels of AFM1 in cheeses.SD, standard deviation*
Source: *developed by the authors*

According to the results represented in Table 5, the mean AFM1 concentration was highest in cheeses made with bacterial-rennet-fungal cultures (0.16 µg/kg), bacterial cultures (0.15 µg/kg), followed by those made with bacterial-rennet cultures (0.10 µg/kg), bacterial-rennet-processed cultures (0.06 µg/kg), rennet cultures (0.05 µg/kg), and rennet (0.04 µg/kg). The results also indicate that there were no statistically significant (p=0.472) differences in mean AFM1 concentration between the starter culture types. According to the results, the mean AFM1 concentration was highest in cheeses aged for 1-3 months (0.10 µg/kg), followed by those aged for less than 1 month (0.09 µg/kg), those aged for more than 13 months (0.06 µg/kg), those aged for 7-12 months (0.04 µg/kg), and those aged for 4-6 months (0.01 µg/kg). The results also indicate that there were no statistically significant (p=0.691) differences in mean AFM1 concentration between in the ageing time categories of cheese. These findings suggest the consistency, starter cultures, and ageing time of cheese do not have a significant impact on the levels of AFM1 present within it. These parameters are all important in determining the final characteristics of cheese and can be carefully controlled to produce cheeses with specific qualities.

Compared to other articles published in the last five years, some studies have identified a relationship between aflatoxin M1, nutritional parameters, and the manufacturing process of cheese. A study by D. Costamagna *et al.* (2019), concluded that the concentration levels of AFM1 increased both in curd and in long maturing cheese, suggesting that AFM1 concentration in milk could be a good predictor of its fate in milk products. A. Zinedine *et al.* (2021) and A. Vaz *et al.* (2022) studied the distribution of AFM1 during the production of two different cheeses, where the amount of the toxin was monitored on a wet and a dry basis. On the wet basis, there was a continuous increase in concentration. The same study on a dry basis concluded that under the tested conditions, there is no degradation of the toxin, and the concentration increase observed previously was in fact due to the loss of humidity. Current study results imply that the levels of AFM1 in cheese are not notably affected by factors such as consistency, starter cultures, and ageing time.

A study by G. Chavarría *et al.* (2017), aimed to investigate the distribution and fate of AFM1 in fresh cheese and whey throughout cheese manufacturing and storage. The research aimed to understand the interaction of this toxin with various proteins in milk, cheese, and whey. The findings revealed that during the production of fresh cheese, up to 70% of AFM1 levels present in milk spiked with 0.5 and 1.5 μg/L were released into whey. Additionally, a significant reduction in AFM1 concentration was observed during ripening, showing an inverse correlation with lactic acid bacteria plate counts and a direct correlation with the whey-draining process that occurs during the maturation or storage of fresh cheese. A study done by E. Sarmast *et al.* (2021), found that cheese production increased aflatoxin M1 (AFM1) levels by 77.2% ($P<0.001$) compared to milk. The order of increased AFM1 levels in cheese was hard (168%) > semi-hard (68.7%) >soft (60.6%). Cheese from milk without a starter culture had a higher increase than that with a starter culture. Overalls, there was a significant reduction in AFM1 levels after ripening/storage (-11%, $P=0.007$), particularly in cheese from milk with a starter culture (-21.1%, $P=0.009$). In our study, in contrast, the findings suggest that the ageing time of cheese does not have a significant impact on the levels of AFM1 present within it. Also, the findings of this study revealed no statistically significant ($p=0.298$) differences across AFM1 level for protein concentration. The results of this study also indicate that there were no statistically significant ($p=0.472$) differences in mean AFM1 concentration between the starter culture types.

In a recent study done by S. Sabatelli *et al.* (2023) in Italy, which aimed to define the enrichment factor (EFs) of cheese with different moisture content on a fat-free basis (MFFB). The milk used for cheesemaking was naturally contaminated with different AFM1 concentrations. This research found that the Enrichment Factor (EF) values for soft and semisoft cheeses were half of the suggested EFs established by the Italian Ministry of Health in 2019. They propose that a comprehensive examination of EF should also consider technological aspects and quality parameters that may influence EF variability. This approach could assist regulatory bodies in establishing an appropriate legal limit, whether based on EFs or concentration values, to protect public health and the diverse range of Italian cheese productions. European Commission (2006) emphasizes the significance of determining the enrichment factor (EF), a crucial parameter that needs to be specified for assessing the permissible level of the toxin in cheese. This is essential to guarantee that cheese is derived from milk that adheres to the regulatory standards. Similarly, I. Pecorelli *et al.* (2020) suggest that the grouping of cheeses into two wide categories (hard and soft cheeses), based on MFFB, and the consequent attribution of the suggested EF, may not be accurate because the experimental evidence shows that soft and semi-soft, as well as semi-hard and very hard cheese categories, may present different results addressing for different categorization. In Kosovo, there is a lack of institutional similar data. The data found in this study could be a significant aid in determining the Enrichment Factors (EF) for cheese, taking into consideration the factors suggested by the above-cited work.

CONCLUSIONS

The findings revealed considerable variations in AFM1 contamination levels among different countries. Notably, current research found that there were cheese samples from some countries that exceeded the concentration of 0.05 ug/kg for AFM1, like, Albania, France, Greece, North Macedonia, The Netherlands, Germany, Kosovo, Italy, Austria, and Turkey. This highlights the importance of continuous monitoring and stringent quality control measures to ensure the safety of cheese products for consumers. The countries with the mean levels of Aflatoxin M1 (AFM1) in cheese samples compliant with the level of 0.05 ug/kg were as follows: Slovenia, Denmark, Croatia, Lithuania, and Hungary. The occurrence of AFM1 in cheese samples was notable, with 47 out of the total 93 different cheese samples (50.53%) exhibiting concentrations higher than 0.05 μg/kg for AFM1.

To evaluate potential variations in Aflatoxin M1 (AFM1) levels in cheese associated with examined nutritional parameters, an Independent Sample T-test was applied. The collective results indicate the absence of statistically significant differentiation ($p=0.902$) in AFM1 concentration based on the regulated total fat content in the cheese. Similarly, no significant differences (p=0.175) were observed in AFM1 concentration between two groups regarding carbohydrate content. The findings further reveal no statistically significant differences (p=0.298) in AFM1 levels concerning protein concentration. Moreover, there is no significant divergence (p=0.122) in AFM1 concentration based on the regulated salt content in the cheese. In assessing the consistency, starter cultures, and ageing time of various cheese types in relation to AFM1 levels, a One-way ANOVA was employed. The results, indicate no statistically significant difference (p=0.926) in average AFM1 levels across different consistencies, including hard (0.077±0.10 μg/kg), semi-hard (0.078±0.14 μg/kg), semi-soft, semi-soft cream (0.061±0.019 μg/kg), soft, and soft-creamy (0.095±0.159 μg/kg) cheese. Examining the mean AFM1 concentration in cheeses produced with bacterial-rennet-fungal cultures, the results show the highest level at 0.16 μg/kg. However, no statistically significant differences (p=0.472) were observed between different starter culture types. Similarly, no significant differences (p=0.691) were found in mean AFM1 concentration across various ageing time categories of cheese, with the highest mean concentration observed in cheeses aged for 1-3 months at 0.10 μg/kg. Overall, these findings collectively suggest that the consistency, starter cultures, and ageing time of cheese do not exert a significant impact on the levels of AFM1 present within it.

Overall, this study underscores the need for ongoing efforts to address and mitigate AFM1 contamination in cheese production. Stringent monitoring, adherence to quality standards, and continuous research into effective control measures are crucial to ensure the safety and quality of cheese products consumed worldwide. As the findings have shown, a collaborative approach between regulatory bodies, producers, and researchers is essential to safeguarding public health and maintaining consumer confidence in cheese products.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Оцінка вмісту альфатоксину М1 та поживних речовин у зразках ринкового сиру в Косові

Арієта Камай Ібрагімі
Кандидат з харчових наук та технологій, доцент
Університет Хакші Зека
30000, вул. «UÇK», м. Печ, Республіка Косово
https://orcid.org/0000-0002-3560-3704
Байрам Беріша
Кандидат сільськогосподарських наук, професор, доктор габілітований
Приштинський університет
10000, вул. Джорджа Буша, 31, м. Приштина, Республіка Косово
https://orcid.org/0000-0001-9112-054X
Хата Дібрані Сопджані
Магістр наук, хімік
Агентство з питань продовольства та ветеринарії
10000, площа «Нана Тереза», м. Приштина, Республіка Косово
https://orcid.org/0009-0008-6245-9024
Афердіта Камай Іса
Кандидат хімічних наук, менеджер з контролю якості
Косовське агентство з лікарських засобів
10000, Ладжія та Спіталіт, м. Приштина, Республіка Косово
https://orcid.org/0000-0001-7044-8810
Шихрет Мурікі
Кандидат наук з агроекономіки, доцент
Університет Хаджі Зека
30000, вул. «UÇK», м. Печ, Республіка Косово
https://orcid.org/0000-0002-3389-0594

Анотація. Присутність та потужна канцерогенна дія афлатоксинів, групи мікотоксинів, становить значну загрозу глобальній безпеці харчових продуктів, особливо в молочному секторі. Це дослідження мало на меті оцінити поширеність і розподіл афлатоксину М1 у різних сортах сиру на ринках Косова у 2022 році та виявити потенційні кореляції з типами сиру, умовами переробки та поживними параметрами, щоб покращити розуміння та забезпечити безпеку молочних продуктів. У другій половині 2022 року загалом 93 зразки різних видів сирів з 15 різних країн були проаналізовані на вміст токсину афлатоксину М1 за допомогою методу імуноферментного аналізу. Результати дослідження вказують на те, що в кількох країнах було виявлено перевищення концентрації афлатоксину М1 у 0,05 мкг/кг, що є максимально допустимою межею для молока та молочних продуктів у багатьох країнах світу. Примітно, що зразки сиру з Албанії показали значно (p=0,000) вищі рівні афлатоксину М1 порівняно з іншими країнами. Результати показали, що концентрація афлатоксину М1 не мала статистично значущих відмінностей залежно від загального вмісту жиру (p=0,902), вуглеводів (p=0,741), білків (p=0,298) або солі (p=0,122) в сирі. Результати продемонстрували, що рівень афлатоксину М1 суттєво (p=0,926) не залежав від консистенції сиру. Хоча різні закваски були пов'язані з різними концентраціями афлатоксину М1, ці відмінності не були статистично значущими (p=0,472). Аналогічно, час визрівання не мав значного (p=0,691) впливу на рівні AFM1, оскільки концентрації залишалися відносно постійними протягом різних періодів визрівання. Ці результати дають цінне уявлення про варіації рівнів афлатоксину М1 у зразках сиру в різних країнах. Вони підкреслюють важливість регуляторних заходів і постійного моніторингу для забезпечення безпеки харчових продуктів і дотримання максимально допустимих меж

Ключові слова: типи сирів; ІФА; забруднення афлатоксином М1; безпечність молочних продуктів