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Dietary Exposure and Health Risk Assessment of Aflatoxin M_1 in Dairy Products Consumed by Population of North Macedonia

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HIGHLIGHTS

- The highest Aflatoxin M_1 (AFM_1) incidence was detected for yogurt samples (93.8%) with an average of 35.1±40.4 ng/kg.
- Upper bound estimated daily intake of AFM_1 for high consumption population was 0.456 ng/kg body weight (bw)/day.
- This study revealed a potential risk for the population of North Macedonia exposed to AFM_1.

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Acronyms and abbreviations
AF=Aflatoxin
EDI=Estimated Daily Intake
ELISA=Enzyme-Linked Immunosorbent Assay
FLD=Fluorescence Detection
HI=Hazard Index
HPLC=High Performance Liquid Chromatography
LOD=Limit of Detection
LOQ=Limit of Quantification
MoE=Margins of Exposure
TDI=Tolerable Daily Intake
UHT=Ultra-High Temperature

ABSTRACT

Background: Aflatoxins (AFs), as secondary metabolites, are mainly produced by fungi of *Aspergillus* genus. The determination of contamination rate, dietary exposure, and health risk assessment for aflatoxin M_1 (AFM_1) was conducted aimed to estimate potential health risks for the population of North Macedonia.

Methods: A total of 974 dairy samples, including 404 Ultra-High Temperature (UHT) milk, 291 ice cream, 178 yogurt, and 101 cheese were collected from the markets in North Macedonia. Analysis of AFM_1 was done using Enzyme-Linked Immunosorbent Assay and High-Performance Liquid Chromatography with Fluorescence Detection.

Results: The AFM_1 incidence was highest in yogurt samples (93.8%) and lowest in UHT milk samples (67.8%). AFM_1 concentrations were 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. The Estimated Daily Intake (EDI) for the average population and high consumers (upper bound; samples with AFM_1<Limit of Detection (LOD) were 0.150 and 0.456 ng/kg body weight (bw)/day, respectively. The Hazard Index (HI), Margin of Exposure (MoE), and the fraction of Hepatocarcinoma (HCC) cases per 100,000 inhabitants for the average population reached values of 0.33, 8533, and 0.004, respectively.

Conclusion: To our best knowledge, this is the first report of dietary exposure and risk assessment of AFM_1 in dairy products of North Macedonia, revealing a potential risk of AFM_1 in population of this country.

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Introduction

Aflatoxins (AFs), as secondary metabolites, are mainly produced by three fungi species, *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* (Hassan et al., 2018). These fungi are frequent contaminants of

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several agricultural commodities, especially during adverse weather conditions, drought, and insect damage (Milićević et al., 2017). Among the AFs produced by the fungi, AFB_1 is the most abundant and most toxic one (IARC, 2002). They may exert immunosuppressive, teratogenic, mutagenic, and carcinogenic effects, especially on the liver. After ingesting contaminated feed by milk-producing animals, the AFB_1 is metabolized by microsomal cytochrome P450 to produce AFM_1 that can be excreted in the urine and milk (Montagna et al., 2008). Detectable concentrations of AFM_1 are measurable 12-24 h after ingestion, with excretion peak in 24-48 h after consuming highly contaminated feed (Serraino et al., 2019). Since 2002, due to its teratogenic, mutagenic, genotoxic, and carcinogenic properties, AFM_1 was classified as Group 2B human carcinogen by the International Agency for Research on Cancer (IARC, 2002). It is considered that the carcinogenic potency of AFM_1 is approximately 2-10% in relation to AFB_1 (Udovicki et al., 2019). However, since there is no recommended tolerable daily intake (TDI) for AFM_1, accounting for its carcinogenic potential, there is a possibility that daily exposure to even less than 1 ng/kg body weight (bw) per day might contribute to the risk of liver cancer (Duarte et al., 2013).

AFM_1 has a binding affinity for milk proteins, particularly with casein, thus an important portion of 40-100% could bind this protein. Therefore, the excreted AFM_1 is also present in dairy products due to its stability towards heat processing and storage at low temperature (Benkerroum, 2016). Additionally, the concentration of the toxin in cheese may also depend on the production technology, cheese type, and the dry matter content in the final product (Montagna et al., 2008).

For obtaining reliable survey data, it is important to use suitable analytical methods in terms of their accuracy, precision, and sensitivity. Enzyme-Linked Immunosorbent Assay (ELISA) as a rapid and simple method was commonly used for the analysis of AFM_1 in milk and dairy products (Anfossi et al., 2012; Bahrami et al., 2016; Ertas et al., 2011; Hassan et al., 2018; Kazemi Darsanaki et al., 2013). Another commonly used method, especially for complex dairy products matrices, like cheese, is High-Performance Liquid Chromatography with Fluorescence Detector (HPLC-FLD) (Bahrami et al., 2016; Iha et al., 2011a; Iqbal et al., 2017; Santini et al., 2013; Škrbić et al., 2015).

In the European Union (EU), the established maximum permissible level for AFM_1 in raw, heat-treated milk, and milk for manufacture of milk-based products was 0.050 µg/kg (European Commission, 2006b). For infant formulae, the limits are stricter and the maximum acceptable limit is established at 0.025 µg/kg. Several European countries, including Switzerland, Austria, Turkey, and France have issued strict regulations concerning the maximum permissible AFM_1 levels in cheese at 0.250 µg/kg (Montagna et al., 2008; Škrbić et al., 2015). Currently, the regulatory limits for AFM_1 in raw, heat-treated, and infant formulae in North Macedonia are fully in line with EU legislation. However, regarding other dairy products, there are no regulated permissible AFM_1 levels, and this fact could imply the risk of exposure for the population. In general, the absence of established maximum level for AFM_1 in cheese samples is a serious difficulty for compliance decision, as well as the intake assessment. The lack of setting maximum limits for dairy products could be related to difficulties in determining a standardized milk-cheese conversion factor (Montagna et al., 2008).

The presence of AFM_1 in milk and dairy products, even at very low levels, represents a concern for public health, especially for infants who are more susceptible to the AF toxicity (Serraino et al., 2019). Risk characterization is the assessment of the occurrence of probable adverse effects on a consumer`s health. Considering the hepatotoxicity and likely carcinogenicity of AFM_1, the regulatory authorities established maximum levels following the concept of "as low as reasonably achievable" (ALARA), accounting for the unavoidable contamination of feed with AFB_1 (Serraino et al., 2019). The parameters that are recommended for risk characterization are Hazard Index (HI), Margins of Exposure (MoE), and the fraction of Hepatocarcinoma (HCC) cases (Cano-Sancho et al., 2010; EFSA, 2020; Serraino et al., 2019).

Accordingly, the present work was aimed to assess the contamination, intake, and the potential health risks from consumption of dairy products such as Ultra-High Temperature (UHT) milk, yogurt, ice cream, and cheese present on the market in North Macedonia.

Materials and methods

Study area

The study was performed in North Macedonia, accounting for the representativeness of the products supplied from big and small retailers across the country. Having in mind the area and the population number (around 2,000,000), we assumed that an approximate number of 1,000 samples would be sufficient for obtaining a realistic conclusion about the contamination of dairy products with AFM_1.

Sample collection

A total of 974 dairy products were collected in original packaging from sales markets in North Macedonia between February and December 2013. The tested samples were divided into 5 dairy products groups: ice cream

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(291), yogurt (178), UHT milk (404), brined white cheese (36), and hard yellow cheese (65). After collection, ice cream samples were stored at -20 °C, while the other samples were stored at +4 ºC, until analysis.

Analytical standards and reagents

Acetonitrile, water, and methanol used for the HPLC-FLD analysis and sample preparation were with HPLC grade, purchased from Carlo Erba (Milan, Italy). For calibration we used stock standard solution with concentration 0.5 µg/ml (R-Biopharm, Darmstadt, Germany). From this stock solution, eight calibration standards (0.075, 0.125, 0.25, 0.5, 1.25, 2.5, 5.0, and 10.0 µg/ml) prepared with suitable dilution in 10% acetonitrile in water were used for HPLC calibration curve.

ELISA method for AFM_1 analysis

Samples of UHT milk and yogurt were prepared for ELISA testing following the producers' manual for the AFM_1 kit (Tecna, s.r.l.,Trieste, Italy). Since there was no prescribed method in the producer's manual for ice cream samples, we used the same sample preparation method as for yogurt samples. All sample preparation procedures used for different matrices were internally validated for the Limit of Detection (LOD), the Limit of Quantification (LOQ), precision, and recovery. Optical Density (OD) of the standards and samples was measured at 450 nm using micro-plate reader (Bio-Rad Model 680, Philadelphia, USA). The constructed six-point calibration curve in the concentration range 0.005-0.250 µg/l was used for the calculation of AFM_1 concentrations in the samples.

HPLC method for AFM_1 analysis

The sample preparation of the cheese samples was performed according to the proposed method by Iha et al. (2011a). Briefly, homogenized samples were extracted with mixture of methanol and deionized water, and further purified with Aflaprep M_1 immunoaffinity columns (R-Biopharm, Darmstadt, Germany). The AFM_1 concentration was determined with Waters Alliance 2695 HPLC system (Waters, Milford, MA, USA) equipped with 2,475 multi-wavelength FLD (365 nm excitation and 435 nm emission). Chromatographic separation of AFM_1 was isocratic with acetonitrile : water mixture (25:75, v/v) at 1 ml/min flow rate through 150 mm C18 analytical column (MERCK, Darmstadt, Germany), at ambient temperature.

Exposure assessment and health risks

To estimate the AFM_1 dietary intake, we applied the deterministic approach, accounting for the normalized dairy product daily intake (per 60 kg bw) and the mean AFM_1 concentration. The individual AFM_1 exposure for each commodity was calculated by the previously proposed equation (Cano-Sancho et al., 2010): AFM_1 exposure (ng AFM_1/kg bw/day)=(daily food intake/bw)×(mean concentration of AFM_1 in food). The consumption data for UHT milk, yogurt, ice cream, and cheese published by the Statistical Office of North Macedonia (State statistical office of Republic of Macedonia, 2014) were used. Since the obtained data were left censored, we applied the European Food Safety Authority recommendation for left censored data management with more than 80% non-quantified, thus the data <LOD=LOD (upper bound).

In addition to exposure assessment, we estimated HI, MoE, and fraction of HCC, as indicators for risk characterization. The HI was calculated according to the proposed approach by Kuiper-Goodman (1990), who had estimated TDI at the value of 0.2 ng/kg bw/day, which was equivalent to a risk level of 1:100,000. The MoE was calculated from the ratio between the reference dose and the Estimated Daily Intake (EDI), and as a reference dose, the value of 0.00057 mg/kg bw/day was used (JECFA, 2001). The fraction of HCC was calculated according to Serraino et al. (2019), taking into account that North Macedonia previously was a country with low AFM_1 incidence, and assuming that 2% of the population was the potential carrier of Hepatitis B.

Statistical analysis

The obtained data for AFM_1 concentrations in the dairy products were processed for analysis of variance (ANOVA) using OriginPro 8 SR4 v8.0951 software package (OriginLab Corporation, Northampton, MA, USA). The results obtained for the AFM_1 occurrence were expressed as positive mean±standard deviation (SD), concentration range (min-max), median, and skewness. Differences in AFM_1 concentrations between sample groups were analyzed applying Kruskal-Wallis test and Mann-Whitney test, non-parametric methods for analysis of two or more non-equal by number data series with unknown distribution. The accepted confidence level required for significance was set at 95% (p<0.05). The calculations of percentiles for different risk-assessment scenarios were performed using MS Excel (MS Office 2007, Redmond, WA, USA).

Results

Validation data for ELISA and HPLC-FLD

The applied methods for AFM_1 determination were validated according to Commission Regulation No.

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401/2006/EC (European Commission, 2006b). The LOD and LOQ values for ELISA were calculated from the obtained signals from 20 blank samples for each food commodity. Accordingly, determined LOD and LOQ were 9.7 ng/kg, and 32.0 ng/kg for UHT milk, 13.9 ng/kg and 45.9 ng/kg for ice cream, 25 ng/kg and 50 ng/kg for yogurt. The obtained validation data for the non-standardized sample preparation method for ice cream, at 50 ng/kg level, was 17.53% and 93.46%, for between-day precision and recovery, respectively. Regarding the cheese samples, the LOD and LOQ were calculated using the residual standard error of the calibration curve, determined within the calibration range. Thus, LOD and LOQ were calculated to be 0.05 ng/ml and 0.15 ng/ml, respectively, corresponding to 11 ng/kg and 33 ng/kg of AFM_1 in cheese samples. The validation data for accuracy and precision, both for the ELISA and HPLC-FLD method, were in accordance with the requirements arising from the Regulation (European Commission, 2006a), which, for recovery, should be in the range of 60-120% and ≤20% for between day precision.

AFM_1 in UHT milk, ice cream, and yogurt samples

For UHT milk, ice cream, and yogurt samples; ELISA was used due to shorter assay time, simplified sample preparation, and comparable detection limits with the HPLC-FLD method. In total, 873 samples were analyzed with the ELISA method for the presence of AFM_1, divided into three commodity groups: UHT milk (404), ice cream (291), and yogurt (178). The AFM_1 contamination levels ranged from LOD to 334.0 ng/kg, with an average of 37.5±49.4 ng/kg (Table 1). The contamination incidence was quite high since 78.7% of the samples contained detectable AFM_1 levels. Furthermore, in 19.0% of the contaminated samples, the revealed concentrations were above 50 ng/kg, with the highest maximum level exceeding observed for UHT milk (24.2%), followed by ice cream (16.2%), and yogurt (11.8%). Overall, in 60.9% of the tested samples, the AFM_1 level was between LOD and 50 ng/kg.

The variations in AFM_1 concentrations between the three sample groups were analyzed applying Kruskal-Wallis test and Mann-Whitney test. The tests revealed that at the probability level of 95%, there was no statistical difference in the mean value and variability between ice cream and yogurt data. However, compared to yogurt and ice cream samples, the AFM_1 data for UHT milk were significantly different in terms of mean and the variability (p<0.05). Positive skewness values indicate that the data were left-censored, this was important for data treatment during the intake assessment.

AFM_1 in cheese samples

The data from Table 2 showed that AFM_1 was detected in 86.1% of the brined white cheese samples, only one of which (2.8%) had exceeded the level of 100 ng/kg. The contamination incidence was similar for hard cheese samples, i.e., 89.2% of the samples were positive for AFM1 and five of them (7.7%) with AFM_1 concentration exceeding 100 ng/kg. Out of 101 analyzed samples of white and hard cheese, we detected 88.2% contaminated samples. The high standard deviation values reflected the high data variability within the populations (Table 2). Regarding AFM_1 levels, the Kruskal-Wallis test, and Mann-Whitney test confirmed that there is no significance of the mean values and variability between the two sample populations at 95% probability level (p>0.05).

Exposure assessment and risk characterization

EDI of AFM_1 from the most frequently consumed dairy products was determined at 0.150 ng/kg bw/day (Table 3). The EDI was calculated for an average Macedonian adult with 60 kg bw. As expected, the highest intake was arising from the consumption of UHT milk due to the highest AFM_1 contamination rate and highest daily consumed amount. Depending on the various risk scenarios, the EDI values varied from 0.050 to 0.456 ng/kg bw/day (Table 4). The calculated 95th percentiles for HI, MoE, and fraction of HCC, being 2.28, 1248.9 and 0.011, respectively, imposed that the consumed dairy products for high food consumers could pose a risk for the population.

Table 1: Occurrence of Aflatoxin M_1 (AFM_1) in Ultra-High Temperature (UHT) milk, ice cream, and yogurt samples in North Macedonia

Sample	Size	Range (ng/kg)	Mean±SD*** (ng/kg)	Median (ng/kg)	Skewness	Distribution (%)		
						<LOD	LOD-50 ng/kg	>50 ng/kg
UHT milk	404	<9.7-319.7	49.1±68.4**	16.8	+1.9	33.2	42.6	24.2
Ice cream	291	<13.9-334.0	30.9±30.0*	23.6	+6.1	12.4	71.4	16.2
Yogurt	178	<25.0-285.8	35.1±40.4*	24.2	+4.2	6.2	82.0	11.8
Total	873	<9.7-334.0	37.5±49.4	22.1	+2.9	20.1	60.9	19.0

*No significant difference between the positive mean and the variability between ice cream and yogurt (p>0.05)
**Significant difference between the positive mean and the variability in comparison to ice cream and yogurt (p<0.05)
***SD – standard deviation
LOD=Limit of Detection

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Table 2: Aflatoxin M_1 (AFM_1) incidence in white and hard cheese in North Macedonia

Sample	Size	Range (ng/kg)	Mean±SD (ng/kg)	Median (ng/kg)	Skewness	Distribution (%)		
						<LOD	1-100 ng/kg	>100 ng/kg
White cheese	36	<11.0-795.0	42.7±129.9	15.1	+5.6	13.9	83.3	2.8
Hard cheese	65	<11.0-366.0	44.8±61.4	25.3	+3.9	10.8	81.5	7.7
Total	101	<11.0-795.0	43.6±51.5	19.1	+4.8	11.8	82.2	6.0

LOD=Limit of Detection

Table 3: Estimated Daily Intake (EDI) from aflatoxin M_1 (AFM_1) from consumption of Ultra-High Temperature (UHT) milk, yogurt, ice cream, and cheese in North Macedonia

Dairy product	UHT milk	Yogurt	Ice cream	Cheese**
Mean concentration (ng/kg)*±SD***	50.6±67.5	39.5±38.4	31.2±29.8	40.1±90.1
Mean daily consumption (kg)	0.101	0.050	0.023	0.030
EDI mean (ng AFM_1/kg bw/day)	0.085	0.033	0.012	0.020
Total EDI (ng AFM_1/kg bw/day)	0.150			

*Calculated by substitution method (values <LOD are set at LOD value)
**Combined data for white and yellow cheese
***SD–standard deviation
EDI=Estimated Daily Intake

Table 4: Estimated total daily intake from aflatoxin M_1 (AFM_1) and risk characterization for different risk scenarios in North Macedonia

	EDI* (AFM_1 ng/kg bw/day)	HI** values	MoE*** values	Fraction of HCC****
Mean	0.150	0.75	3800.0	0.004
25^{th} percentile	0.050	0.25	11348.2	0.001
50^{th} percentile	0.067	0.33	8533.2	0.002
75^{th} percentile	0.144	0.72	3955.3	0.004
95^{th} percentile	0.456	2.28	1248.9	0.011

*EDI – estimated daily intake
**HI–hazard index
***MoE–Margins of Exposure
****HCC–hepatocarcinoma cases per 100,000 habitants

Discussion

The presence of AFM_1 in dairy products is a public hazard due to the many proven adverse effects on human health (IARC, 2002). The mycotoxin regulation (European Commission, 2006b) has set maximum level value only for raw and processed milk at 50 ng/kg. However, no official maximum level values for ice cream, yogurt, and cheese were established. Due to direct raw milk treatment for UHT milk and yogurt production, practically there is no concentration of the toxin in the final products. During the ice cream production, the AFM_1 concentration could be partially diluted by the addition of some flavorings. Therefore, for compliance assessment of these dairy products, the approximation that there is no technological concentration or dilution of AFM_1 could be accounted. Accordingly, from the theoretical point of view, the maximum level value for raw milk might also apply to all these food commodities.

In this study, the reported AFM_1 contamination of UHT milk and dairy products was similar to the reported findings for worldwide regions with suitable climate conditions for AFs production (Bahrami et al., 2016; Cano-Sancho et al., 2010; Ertas et al., 2011; Iha et al., 2011b; Iqbal et al., 2017). The revealed AFM_1 incidence and concentration range in UHT milk (66.8% positivity, range <LOD-319.7 ng/kg) (Table 1) corresponds with the data reporting high occurrence of this mycotoxin for the same dairy product (Cano-Sancho et al., 2010; Santini et al., 2013; Škrbić et al., 2014). Thus, the research

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conducted in Serbia during the high AFM_1 incidence revealed similar positivity for UHT milk (76%) as our study, but, with significantly higher concentration levels (up to 1,440 ng/kg) (Škrbić et al., 2014). Hence, similar contamination (94.4%) was reported by Cano-Sancho et al. (2010) in a research conducted for Catalonia province (Spain) in UHT milk samples. In addition, Santini et al. (2013) reported 48% incidence for UHT milk samples from Sicily (Italy). However, the reported findings were with AFM_1 average values bellow the maximum level. This was probably due to the already established continuous national monitoring programs for AFM_1 in raw milk in these two countries. Regarding North Macedonia and Serbia, the AFM_1 was not a food safety issue until 2013, when an incidence occurred as a result of high contamination of corn with AFB_1 (Dimitrieska-Stojković et al., 2016; Kos et al., 2014).

Ice cream contamination with AFM_1 is rarely investigated, so there are few publications reporting the contaminant occurrence in this dairy product. The detected AFM_1 incidence (87.6%) and average value (30.9±30.0 ng/kg) found for 291 ice cream samples are comparable to the results presented by Kazemi Darsanaki et al. (2013) and Lee and Lee (2015). The research conducted by Kazemi Darsanaki et al. (2013) for the Guilan Province (Northern Iran) on 90 ice cream samples revealed 68.88% incidence, with an average positive mean of 40.36 ng/kg (range 8.4-147.7 ng/kg). Lee and Lee (2015) have reported 36% positivity, with mean value 33.16 ng/kg, from a study conducted for Seoul (Korea) on 50 ice cream samples. However, our study revealed significantly higher AFM_1 values (up to 334 ng/kg), which correspond to the level detected in UHT milk (Table 1). The higher AFM_1 values reported are probably as a result of the sampling period during the AF incidence in the Balkans. In addition, the number of tested samples significantly differs and the data series may not be fully comparable. However, the published studies of Cadirci et al (2011) and Nilchian and Rahimi (2012) reported low AFM_1 contamination rates. In the report from Cadirci et al. (2011) accounted for 120 samples from Samsun, Turkey, the revealed positive samples were 26.08% with detected range of 6.12-32.15 ng/kg and average of 14.67 ng/kg. In the study published by Nilchian and Rahimi (2012), for 40 tested ice cream samples with origin from Shahrekord in Iran, 29% positive samples were detected with a mean 65.1 ng/kg and detected maximum of 197.4 ng/kg. When compared to our findings, the lower contamination rate revealed for these two studies can be partially explained due to different contamination levels in milk samples, different number of tested samples, and pH value of the raw milk (Nilchian and Rahimi, 2012).

Similarly, for ice cream samples, we also found high contamination rate of AFM_1 for the yogurt samples (93.8%), although only 11.8% of the samples exceeded 50 ng/kg (Table 1). In addition, we determined and average of 35.1 ng/kg with maximum determined concentration of 285.8 ng/kg. Among the studies, a significantly higher AFM_1 incidence of 76.1% on 21 yogurt samples was reported for Qatar consumers, with the mean value of 31.32 ng/kg (Hassan et al., 2018). This result corresponds to the mean value detected within this study, although the maximum detected value was 31.32 ng/kg. In a report from Nilchian and Rahimi (2012) for 40 yogurt samples, 35% incidence was detected with average of 130.5 ng/kg and maximum detected concentration 115.8 ng/kg. Iha et al. (2011b) reported very low AFM_1 levels in a study conducted for Ribeirão Preto-SP, Brazil (all below 50 ng/kg). However, the findings accounted only 6 tested samples, which could be the main reason for such difference in the contamination level. A decade ago, there were some opinions that the AFM_1 content in yogurt was affected not only by the contamination of milk used for the process (Iha et al., 2011b), but also by the pH value, the formation of organic acids, or the presence of lactic acid bacteria (Nilchian and Rahimi, 2012). Despite the assumptions that some strains of lactic acid bacteria for yogurt production might influence AFM_1 decontamination in milk, later data showed that the fermentation process for yogurt had a negligible impact on AFM_1 levels (Udovicki et al., 2019).

With regard to cheese samples, the detected AFM_1 average concentration from combined data for both cheese types were 40.1±90.1 ng/kg with 88.1% of the values over LOD (Table 2). If we compare the detected AFM_1 levels in cheese samples to the ones found for UHT milk (Table 1), we would expect higher AFM_1 levels in cheese samples. AFM_1 can bind to the protein fractions of the milk, particularly with casein, so its concentration in cheese is expected to be 2-5 times higher than in raw milk samples (Škrbić et al., 2015). A study regarding the distribution of AFM_1 in cheese obtained from contaminated milk revealed that 60% of the toxin is portioned into the whey fraction and the remaining 40% in cheese (Santini et al., 2013). However, the variations in AFM_1 concentration are dependable on the cheese type, moisture content, technologies for the production, and testing method (Iha et al., 2011b).

There are few possible explanations for the inconsistency between AFM_1 levels found in UHT milk and cheese, which were, however, not confirmed in this study. It is possible that the tested mature cheeses were produced before the incidence period or after the implementation of strict raw milk controls (Dimitrieska-Stojković et al., 2016). The other explanation could be due to the possible presence of certain lactobacilli strains with the ability to bind AFM_1, thus decreasing their levels in secreted milk (Montagna et al., 2008). However,

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in order to reach more plausible conclusions, such assumptions should be further studied.

Our findings for cheese samples differed from the study reported for Serbia in 2013, whereas the reported levels for 33 tested samples (23 soft and 10 hard cheese), ranged from 13 up to 2230 ng/kg (Škrbić et al., 2015). Among tested samples, the authors found AFM_1 in 54% of them, with 13% with levels above 250 ng/kg. Also, another research study carried out in Italy (Apulia region) on 265 samples revealed AFM_1 concentrations in cheese in the range of 50-250 ng/kg (Montagna et al., 2008). AFM_1 was detected on 16.6% of the tested samples among which the highest concentration was measured in long-term ripened cheeses from sheep and goat milk. Anfossi et al. (2012) reported the occurrence of AFM_1 in Italian cheese with reference to manufacturing practices, production season, type of milk production animals, and cheese maturity. Applying the ELISA testing method, detected concentrations were in the range 25–257.8 ng/kg and the revealed contamination was 83%. In a study for Grana Padano cheese collected from the province of Po valley (Italy) (Manetta et al., 2009), carried out on 25 cheese samples, it was determined that AFM_1 values were in the range 111-413 ng/kg. If we compare these findings with the data from our study (88.2% positivity; highest detected amount of 795 ng/kg), it could be concluded that the positivity rate was similar to that reported by Anfossi el al. (2012), but different from the one reported by Škrbić et al. (2015). The highest found concentration, we found, was higher than that reported for Italian cheeses (Anfossi et al., 2012; Manetta et al., 2009). This variability in the results for AFM_1 in cheese samples could be attributed to the differences in milk contamination with AFM_1, number of tested samples, sampling season, milk type, cheese maturity, and testing method.

As usual, the published reports on AFM_1 high content findings commonly referred to studies conducted in southern Europe (Anfossi et al., 2012; Cano-Sancho et al., 2010; Duarte et al., 2013), Turkey, middle East countries (Bahrami et al., 2016; Ertas et al., 2011; Hassan et al., 2018), and Brazil (Iha et al., 2011b; Prado et al., 2008). However, weather changes in the past decade could pose a reason for AF-related issues in the world regions with temperate climates (Dimitrieska-Stojković et al., 2016).

Besides the assessment of AFM_1 contamination, this study was aimed at estimating the intake and the possible health risks for the consumers from highly contaminated dairy products. The found AFM_1 incidence in dairy products (>60%) is considered as highly critical for risk assessment. During the exposure assessment, the difficult step was handling of data reported below the LOD or LOQ. The amount of non-quantified data resulted in a left-censored distribution (Tables 1 and 2). Therefore, the substitution method was applied; hence, for the results below the LOD value, LOD was imputed (upper bound). This was the recommended approach by European Food Safety Authority for managing left-censored data, with more than 80% non-quantified (EFSA, 2010). Accordingly, the EDI was 0.150 ng/kg bw/day mainly originating from UHT milk consumption (Table 3).

As there was no recommended TDI value for AFM_1, we compared the data from this study with reports by other authors. Thus, the EDI was significantly higher than those published for Portugal (0.08 ng/kg bw/day; Duarte et al., 2013), Catalonia, Spain (~0.04 ng/kg bw/day; Cano-Sancho et al., 2010), and France (0.01 ng/kg bw/day; Leblanc et al., 2005). However, the published study for Serbia (Kos et al., 2014) reported higher EDI value, whereas the estimate was 0.21 ng/kg bw/day, accounted only for raw milk. This is to some extent comparable to our finding, which was expected due to the same sampling period and the same source of AF contamination in feed and milk (Dimitrieska-Stojković et al., 2016).

In this study, the risk characterization from exposure to AFM_1 was performed by calculating the HI, MoE, and the fraction of incidence of HCC per 100,000 of population (Table 4). For carcinogens such as AFM_1, TDI is generally not determined; therefore, as recommended, its level in food should be kept as low as reasonably achievable. The HI was used to facilitate the interpretation of the estimated EDI values (Serraino et al., 2019). Even though, the HI value below 1 is not considered as a cause of concern (mean HI=0.75), the estimated 95^{th} percentile (HI=2.28) in this study (Table 4), indicates the possibility of serious health consequences. The MoE as a risk characterization parameter is commonly used for hazard estimation from exposure to genotoxic and carcinogenic contaminants in food (Serraino et al., 2019). Our study, as well as the latest European Food Safety Authority data for MoE calculation for AFM_1 (EFSA, 2020), showed that the exposure estimates for the 95^{th} percentile are far below 10,000 which increases the health concern, in particular for younger age groups and high consumers. Other publications reporting HI and MoE revealed values comparable to those presented in this work. Hence, the reported HI and MoE from yogurt consumption in Greece were 1.0 and 2,808 (Udovicki et al., 2019), respectively. In addition, the HI from UHT milk consumption in Serbia was 0.75 (Miličević et al., 2017). When compared to European Food Safety Authority data (0.004-0.007), the calculated HCC value depending on the risk scenario, for the average adult population, was in the range 0.001-0.011, with a higher 95^{th} percentile value for high consumers (Serraino et al., 2019). For obtaining more reliable conclusions regarding the long-term intake and risk characterization, a multi-annual survey of the

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presence of AFs in feed, raw milk, and dairy products should be implemented by competent food authorities.

Conclusion

High AFM_1 incidence in UHT milk and dairy products marketed in North Macedonia was revealed in this study. The data from the exposure assessment and risk characterization showed an increased health risk from exposure to AFM_1, particularly from consumption of highly contaminated dairy products, for the younger population and high food consumers. Having in mind the fact that dairy products are consumed on daily basis and additionally considering the AFM_1 stability during the processing and storage, it is necessary to perform multi-annual monitoring of this mycotoxin in the overall food chain. The data shown emphasize the necessity to establish maximum levels for AFM_1 in non-regulated dairy products.

Author contributions

G.I. did the conceptualization, methodology, investigation, validation, resources, writing and editing; B.S.-D. did the conceptualization, investigation, critical reviewing, editing; D.K. carried out investigation, review and editing; G.S. did the formal analysis and data curation; A.A. carried out data analysis, writing, editing; E.D.-S. did the conceptualization, methodology, investigation, validation, resources, data curation, writing, editing and supervision. All authors read and approved the final manuscript.

Conflicts of interest

All reported work in this manuscript is original, and all authors have read and approved this version of manuscript. Neither the entire paper nor any part of its content has been published or accepted elsewhere. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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