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Assessment of aflatoxin M1 enrichment factor in cheese produced with naturally contaminated milk

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Abstract

Aflatoxin M1 (AFM1) is a well-known carcinogenic compound that may contaminate milk and dairy products. Thus, with the regulation 1881/2006, the European Union established a concentration limit for AFM1 in milk and insisted on the importance of defining enrichment factors (EFs) for cheese. In 2019, the Italian Ministry of Health proposed four different EFs based on cheese's moisture content on a fat-free basis (MMFB) for bovine dairy products. This study aimed to define the EFs of cheese with different MFFB. The milk used for cheesemaking was naturally contaminated with different AFM1 concentrations. Results showed that all the EF average values from this study were lower than those of the Italian Ministry of Health. Hence, the current EFs might need to be reconsidered for a better categorization of AFM1 risk in cheese.

Introduction

Aflatoxins are secondary metabolites produced by fungi of the genus *Aspergillus* and excreted during the logarithmic growth phase (Sarmast *et al.*, 2021). The best-known aflatoxins are B1, B2, G1, G2, and M1 (AFB1, AFB2, AFG1, AFG2, AFM1), and are all deemed as the most toxigenic mycotoxins (Ekwomadu *et al.*, 2022). The international agency for research on cancer includes all aflatoxins in class 1 carcinogenic compounds, with high hepatotoxicity and hepatic carcinogenicity (IARC, 2012). These mycotoxins occur as contaminants in a variety of foods and feed such as corn, cereals, peanuts, and pistachio nuts (Waśkiewicz and Goliński, 2015; Alshannaq and Yu, 2017). The intake of feed contaminated with AFB1 is a health hazard for cattle as it impairs reproduction, compromises the immune system functioning, reduces milk production and liver function, and increases susceptibility to diseases (Jiang *et al.*, 2021). Moreover, AFB1 undergoes hepatic biotransformation by hydroxylation mechanisms generating AFM1, which is excreted through milk (Khaneghah *et al.*, 2021). Therefore, it may become a hazard to human health as well, since AFM1 can be retrieved from raw milk and dairy products (Prandini *et al.*, 2009). The European Union (EU) established the Commission Regulation 1881/2006 (European Commission, 2006), setting maximum levels for certain contaminants in foodstuffs. The limit for AFM1 in milk is set at 0.050 μg/kg, while it is stated that the specific concentration factors for the processed foodstuffs shall be provided and justified by the food business operator when the competent authority carries out an official control. If the food business operator does not provide the necessary concentration factor or if the competent authority deems that factor inappropriate, the authority should itself define that factor, based on the available information and with the objective of maximum protection of human health (European Commission, 2006). Accordingly, in 2013, the Italian Ministry of Health proposed two different enrichment factors (EFs) to establish a limit for AFM1 in milk-derived food and feed: an EF of 3.3 for soft cheese and whey-derived products, and an EF of 5 for hard cheese (Italian Ministry of Health, 2013). This recommendation was updated in 2019 for bovine dairy products, with a suggestion of four different EFs based on cheese's moisture content on a fat-free basis (MMFB): an EF of 3 for soft (MFFB≥68%), 4 for semisoft (68%>MFFB≥62%), 5 for both semi-hard (62%>MFFB≥55%) and hard (55%>MFFB≥47%), and 6 for very hard (MFFB<47%) (Italian Ministry of Health, 2019).

Italy exhibits a vast assortment of cheeses with more than 400 recognized varieties (Vizzardi and Maffaeis, 1990), including 56 acknowledged by the European Union with recognition of protected designation of origin or protected geographical indication (MIPAAF, 2022). This wide variety of cheeses is linked to diversity in production technologies, ripening periods, MFFB, and several other characteristics performed throughout the whole Italian territory (Vizzardi and Maffaeis, 1990). Altogether, these many features may influence the carryover of aflatoxin M1 from milk to cheese, leading to the need of defining EFs in different cheese categories (Campagnollo *et al.*, 2016). Some surveys tried to assess the distribution of AFM1 in specific cheese production of different hardness, such as Parmigiano Reggiano (Brackett and Marth, 1982; Pietri *et al*., 2016) and Grana Padano cheese (Manetta *et al*., 2009) or other hard cheeses (Škrbic *et al*., 2015; Krstović *et al*., 2018), caciotta semi hard cheese (Pecorelli *et al.*, 2019) as well as fresh cheeses such as Primosale, Robiola, Maccagno, Fior di Latte (Cavallarin *et al*., 2014; Chavarría *et al*., 2017; Pecorelli *et al*., 2020), starting from either artificially contaminated milk or naturally contaminated milk at concentrations above law limits. The present study aimed to determine AFM1 EFs of cheeses with different MFFB produced with AFM1-naturally contaminated milk both above and below regulatory limits.

Materials and Methods

Cheese production

Naturally contaminated milk was provided by 10 different farms from Lombardy region after specimens displayed valuable AFM1 concentrations. A quantity of 20 to 30 L of milk was taken directly from farms and immediately transported to the experimental dairy of the Experimental Zooprophylactic Institute of Lombardy and Emilia Romagna (Brescia, Italy). The cheesemaking procedure is described as follows: first of all, 20-30 L of milk was poured into the heating tank and slowly mixed until reaching a temperature of 35°C. Then, starter cultures (*i.e.*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*) were added at a concentration of 2%, followed by milk heating to 40°C. After 20 minutes of continuous stirring, rennet (Alce International S.R.L., Quistello, IT) was added at a ratio of 0.3 mL per L of milk, and mixing was stopped. Thirty minutes of coagulation was followed by 3 steps of curd cutting (*i.e.*, one cut every 10 minutes) for whey drainage. Then, curd pieces were collected in 1 to 4 plastic molds and settled at room temperature for almost 4 hours, turned over thrice. Curd was then salted by a direct method, sprinkling the surfaces with salt four times, once a day. Lastly, ripening was performed at 4-8°C, >85% of humidity for up to 56 days, turning up blocks every week.

Sampling

Milk was sampled for AFM1 concentration testing prior to cheesemaking. One more sample was taken for quality parameters tested with infrared spectrometry (data not shown) using MilkoScan 6000 (Foss-Electric, Hilleroed, DK) according to ISO (2013). Whey samples were taken immediately after cutting the curd and tested for AFM1 concentration. Lastly, cheese sampling was performed from the 1st week up to the 8th week, once every two weeks: each time, 10 g of cheese was sampled from each block for AFM1 aflatoxin examination and 100 g for quality parameters. The aflatoxin aliquot was grated and 2 g was weighted as a first step of the extraction procedure for cheese AFM1 ELISA test.

Milk and whey aflatoxin M1 concentration test

AFM1 quantification in milk and whey was determined with ELISA BIO SHIELDM1 ES kit (ProGnosis Biotech A.E., Larissa, GR). Milk samples were slowly stirred and tested without dilution. Analyses were performed according to the manufacturer's instructions, and results were evaluated photometrically by the means of BioTek EL×808 plate reader and Gen5 Data Analysis Software (Agilent Technologies, Inc., Santa Clara CA, US). AFM1 levels in samples were calculated with the use of a 6-level calibration curve, covering a range of quantification between 0.010 μg/kg and 0.10 μg/kg, with a limit of quantification of 0.010 μg/kg. Samples with percentage of absorbance below 20% were diluted 1:4 to be identified.

Cheese aflatoxin M1 concentration test

The AFM1 detection in cheese was performed by ELISA kit Immunoscreen AFM1 (Tecna, S.R.L., Trieste, IT), as reported by Biancardi and Moretti (2020). Briefly, 20 mL of dichloromethane was added to 2,00 g of milled sample. After shaking for 30 minutes, the raw extract was filtered through a folded standard filter paper. A 5 mL aliquot was then dried under a gentle nitrogen flow at 40°C and the residue was reconstituted in 750 μL of extraction buffer provided in the kit ELISA. Then, a defatting step was performed by a liquid-liquid partition with 2 mL of n-hexane. After centrifugation (10 minutes at 3000 rpm), the upper organic layer was eliminated by a vacuum pump, and 50 μL of aqueous defatted phase was diluted with 450 μL of milk diluent, provided in the kit as well. After mixing, the solution was centrifugated at 2000 rpm for 5 minutes. The dilution factor was 15. The sample was ready to undergo the ELISA protocol, as reported in the manufacturer's instructions (Tecna, S.R.L., Trieste, IT).

Cheese quality parameters, moisture content on a fat-free basis, and enrichment factors evaluations

The following cheese quality parameters were determined by near-infrared spectroscopy (buchi labortechnik, Flawil, CH): protein, fat, saturated fatty acid, carbohydrate, sodium chloride, ash, and moisture contents. Cheese's hardness was classified based on MFFB as reported by the Italian Ministry of Health and already described in the introduction (Italian Ministry of Health, 2013, 2019). The following formula was used for MFFB estimation:

$$\text{MFFB (\%)} = [\text{moisture content}/(\text{total weight - fat content})]\times 100.$$

Based on the low number of samples obtained from hard (55%>MFFB≥47%) and very hard (MFFB<47%) groups, the authors decided to merge the two categories into a single >55% MFFB hardness category. Thus, the following hardness categories were established: soft (MFFB≥68%), semisoft (68%>MFFB≥62%), semi-hard (62%>MFFB≥55%), and hard/extra hard (MFFB<55%).

Lastly, EF of AFM1 was determined using cheese AFM1 concentration and starting milk AFM1 concentration as follows:

EF = [aflatoxin M1 in cheese/aflatoxin M1 in milk].

Statistical analyses

The correlation between milk concentration and whey to milk ratio was calculated with Pearson correlation coefficient in R software with a confidence interval of 95%. Moreover, the standard deviation was determined for cheese samples along with the average EF for hardness categories. A student's t-test was performed to compare the proposed EF by the Italian Ministry of Health (2019) for different cheese hardness with the average EF obtained in the present study. Furthermore, cheese samples were divided into two categories, whether they originated from cheesemaking produced with milk above the regulatory limit (AFM1 in milk >0.050 µg/kg) or cheesemaking from milk below the regulatory limit (AFM1 in milk <0.050 µg/kg). A student's t-test was executed to identify statistical differences between these two categories.

Results

Milk and whey

Ten different batches of milk were analyzed in the present study. AFM1 concentration in milk ranged between 0.018 µg/kg and 0.384 µg/kg (Table 1). Milk samples used for cheesemaking number 1, 2, 6, 8 were below the 0.050 µg/kg regulatory limit, while those of cheesemaking 3, 4, 5, 7, 9, 10 were at least twice the abovementioned regulatory limit. Whey analyses showed an AFM1 concentration recurrently lower than starting milk, except for cheesemaking n. 1, where the AFM1 values were equivalent. A negative correlation was identified with the Pearson correlation coefficient between AFM1 concentration in milk and the whey to milk ratio (Figure 1).

Table 1. Aflatoxin M1 concentration in milk and whey samples, and whey to milk ratio.

N. cheesemaking	AFM1 concentration milk (µg/kg)	AFM1 concentration whey (µg/kg)	Whey/milk ratio
1	0.018	0.018	1
2	0.032	0.026	0.81
3	0.384	0.212	0.55
4	0.111	0.069	0.62
5	0.124	0.104	0.84
6	0.046	0.036	0.78
7	0.134	0.083	0.62
8	0.035	0.031	0.89
9	0.18	0.125	0.69
10	0.199	0.091	0.46

N, number; AFM1, aflatoxin M1.

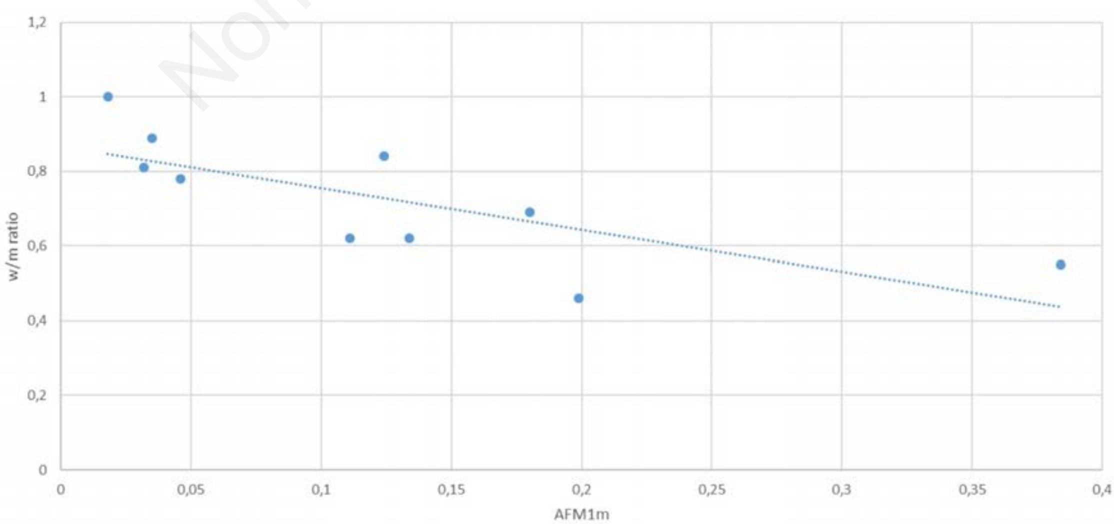

Figure 1. Negative correlation between whey to milk ratio (w/m ratio) and aflatoxin M1 concentration in milk. r=- 0.74, P=0.014 (confidence interval 95%; -0.93, -0.21). AFM1m, aflatoxin M1 concentration in milk.

Cheese

A total of 27 blocks were prepared and ripened for different periods, for 74 specimens overall. In detail, 16 were deemed as soft, 37 as semi-soft, 15 as semi-hard, and 6 as hard/extra-hard (Table 2). EF average values were, from soft to hard/extra-hard: 1.71, 1.95, 3.22, and 4.26. AFM1 concentration was always higher than the corresponding starting milk, except for a sample of a cheese block from cheesemaking n. 6, where an EF of 0.77 was obtained. Moreover, the EFs proposed by the Italian Ministry of Health in 2019 are reported in Table 2 as a yardstick, along with the student's t-test results of the correlation between the EFs from the present study and the EFs by the Italian Ministry of Health.

In addition, a comparison between samples originating from cheesemaking n. 1, 2, 6, 8 (AFM1 in milk <0.050 µg/kg, group 1) and cheesemaking n. 3, 4, 5, 7, 9, 10 (AFM1 in milk >0.050 µg/kg, group 2) was also assessed. In each hardness category, the average EF from group 1 samples was lower than EF from group samples (Table 3). The student's t-test did not highlight statistically significant differences between EF categories.

Discussion

Aflatoxins are well-known cancerogenic mycotoxins that may contaminate many different foods and feed, such as nuts (AFB1), and milk or dairy products (AFM1) (Campagnollo *et al*., 2016). Countries around the world established regulatory limits for AFM1 concentration in milk, and the EU provided a limit of 0.050 µg/kg (European Commission, 2006). Despite that, no limits are determined for cheese, and EFs are proposed by the food business operator or competent authority. Some European countries such as Denmark or the Netherlands have a narrow diversity of cheese varieties, and might easily select EFs that cover the whole national market. On the other hand, countries like France and Italy have a much wider cheese variety, which brings along higher difficulty in establishing proper EFs. Thus, the present study aimed to assess the AFM1 EFs of cheeses produced with AFM1-naturally contaminated milk both below and above regulatory limits, based on different MFFB. To begin with, a comparison between AFM1 concentration in whey and milk was determined. The concentration in whey was recurrently lower than the concentration in the corresponding starting milk, and only one batch of milk (n. 1) had the same concentration for both. This result is in line with many studies from the literature, where AFM1 concentration in whey is commonly lower than AFM1 in milk (Krstović *et al*., 2018; Chavarría *et al*., 2017). As a matter of fact, part of AFM1 is retained by curd and cheese and the difference might be related to the kind of cheesemaking and cutting of the curd (Campagnollo *et al*., 2016). Moreover, a negative correlation was assessed between whey to milk ratio and AFM1 concentration in milk. This kind of association could be determined because only one kind of cheese was produced in the present study, thus excluding any manufacturing procedures-related variable (Pietri *et al*., 2016). The trend displayed in Figure 1 could be explained by the fact that a higher concentration of AFM1 in milk might be retained in cheese, as AFM1 is mostly linked to casein, lowering the AFM1 whey to milk ratio (Prandini *et al*., 2009).

Concerning AFM1 concentration in cheese, samples' results were distributed in categories based on their MFFB (Table 1). The merging of hard and extra hard categories in the present study was attributed to the low number of samples for both categories, generating a single, <55% MFFB category. The low number of samples for hard and extra hard categories is related to the cheesemaking technology, which doesn't frequently allow to reach such hardness in 8 weeks. Moreover, only one cheese sample had a lower AFM1 concentration in cheese than in starting milk. Hence, EFs were recurrently above 1 for all cheese categories, as described in Table 1. Many reports in the literature have described a higher concentration of AFM1 in cheese than in milk (Pecorelli *et al*., 2019; Krstović *et al*., 2018). It might be due to the water-soluble nature of AFM1 and its affinity to form a hydrophobic bond with the

Table 2. Enrichment factors of samples from the present study based on moisture content on a fat-free basis values (n. of samples, enrichment factors range, enrichment factors average and standard deviation), enrichment factors proposed by the Italian Ministry of Health in 2019 and the student's T-test results (P value).

Hardness categories	N. samples	EF range	EF average (SD)	EF (Italian Ministry of Health, 2019)	Student's t-test (P value)
Soft (MFFB≥68%)	16	1.01-2.45	1.71 (0.444)	3	6.95874E-09
Semisoft (68%>MFFB≥62%)	37	0.77-3.60	1.95 (0.706)	4	2.63935E-19
Semi-hard (62%>MFFB≥55%)	15	1.54-4.97	3.22 (0.875)	5	1.68517E-06
Hard (55%>MFFB≥47%) and extra-hard (MFFB<47%)	6	3.72-4.95	4.26 (0.485)	5 (hard) 6 (extra-hard)	0.01371743

N, number; EF, enrichment factors; SD, standard deviation; MFFB, moisture content on a fat-free basis.

Table 3. Comparison of enrichment factor averages of samples below and above regulatory limit, for each hardness category.

	<0.050 µg/kg samples		>0.050 µg/kg samples	
Hardness categories	N. samples	EF (average)	N. samples	EF (average)
Soft	8	1.60	8	1.82
Semisoft	9	1.68	28	2.04
Semi-hard	5	2.96	10	3.35
Hard and extra Hard	3	4.05	3	4.47

N, number; EF, enrichment factors.

hydrophobic part of casein that is subsequently concentrated in cheese (Sarmast *et al*., 2021). Moreover, the data in the literature concerning EFs range from similar to our results (Pecorelli *et al.*, 2019) to higher (Pecorelli *et al*., 2020; Krsotvić et al., 2018; Brackett and Marth, 1982). This diversity might be related to the manufacturing techniques used in different studies, along with differences in ripening periods. Results from the present research were compared only with investigations from the literature that implied the use of naturally contaminated milk. As a matter of fact, AFM1-spiked milk and spiked cheese may be different from those naturally contaminated, because AFM1 could be partially unbound, leading to a different chemical behavior (Pecorelli *et al.*, 2019). Thus, the research design for the present study was conceived only with the use of naturally contaminated milk. Furthermore, conversely to the 2019 recommendations of the Italian Ministry of Health, results from the present study highlighted how EF from soft and semi soft categories are similar to each other and therefore can be merged in a single value, while semi hard and hard have different values and can be therefore kept separated. The similarities of EFs from soft and semi soft categories might be related to the fact that MFFBs of soft cheese from the present study were close to the 68% limit, thus being close to an MFFB of a semi soft cheese. Moreover, all the EF average values were lower than those of the Italian Ministry of Health, with a relevant P value, especially for soft and semi-soft categories. At last, the authors tested if there was any difference in EFs between cheese produced with milk below AFM1 concentration regulatory limits (group 1) and cheese from milk above AFM1 concentration regulatory limits (group 2) (Table 3). Despite the absence of statistical significance, it can be seen that group 1 average EFs are recurrently lower than the average EFs of group 2. This feature could be related to the behavior of AFM1 in milk and its link to casein, and the loss of total AFM1 in whey during the cutting of the curd (Campagnollo *et al*., 2016; Sarmast *et al.*, 2021).

Conclusions

The present survey identified EF values for soft and semisoft cheeses of 1.71 and 1.95 respectively, which are half the proposed EFs from the Italian Ministry of Health in 2019. Thus, further studies on these two hardness categories should be carried on, to improve the knowledge about AFM1 concentration in soft and semisoft categories. Furthermore, it is essential an in-depth examination of EF not only based on MFFB but also related to technological features or quality parameters that may have any association with EF variability. This way, the competent authority might be able to identify a law limit, either with EFs or with concentration values, that can safeguard both public health and the vast number of different Italian cheese productions.

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