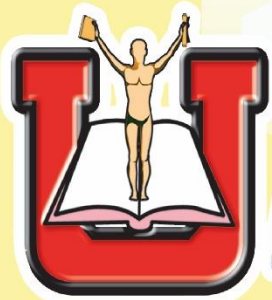




Aflatoxin M1 in Nicaraguan and locally made hard white cheeses marketed in San Salvador, El Salvador



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Abbreviations: AFM1, Aflatoxin M1; AFB1, Aflatoxin B1; AFs, Total Aflatoxins (B1+B2+G1+G2); ELISA, Enzyme-linked immunosorbent assay; %RSD, Coefficient of variation; HORRAT, Horwitz ratio.

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ABSTRACT

Aflatoxin M₁ is a carcinogenic and genotoxic metabolite of Aflatoxins present in food contaminated by fungi for lactating cattle, it is excreted through milk and when used to make cheese, the toxin will also be transferred to the dairy. The contamination of unripened hard white cheese with AFM₁ seems to vary according to the season of the year, possibly due to the change of foodstuff, from fresh pasture in the rainy season to dried foods in the dry season and vice versa. This research determined both the prevalence and contents of AFM₁ in cheeses of local and Nicaraguan origin marketed in El Salvador, as well as the changes occurred according to the season and the association between levels of AFM₁ with meteorological parameters. The significantly higher prevalence of AFM₁ contamination in both local cheeses and Nicaraguans, was found in the dry season and the lowest in the rainy season (41 % vs. 20 %; 31 % vs. 0%, respectively), the same trend was observed in AFM₁ contents (0.076 vs. 0.036 µg/kg; 0.050 vs. 0.021 µg/kg, respectively). A significant association was demonstrated between levels of AFM₁ with the averages of accumulated rainfall and relative humidity according to the sampled season. The prevalence of AFM₁ in cheeses indicate that El Salvador and Nicaragua are endemic to dairy contamination by that mycotoxin. Seasonal variation may be due to a lack of rainfall, that promotes the growth of aflatoxigenic fungi in the crops of raw materials, which will be used for feedstuff intended for dairy cattle, thus, the consumption of contaminated food will cause the temporary increase of AFM₁ in milk and their derivatives.

1. Introduction

Aflatoxin M₁ (AFM₁) is the main metabolite of Aflatoxin B₁ (AFB₁), which is one of the four most common variants of Aflatoxins (AFs) [1–6]. There is sufficient evidence for the carcinogenicity of AFM₁ alone or mixed with AFB₁ and Aflatoxin G₁ (AFG₁) [7], although of lower potency than AFB₁ [8,9]. When cattle ingest food or feed contaminated by AFs, it is estimated that up to 6.2 % of the content of AFB₁ is transformed into AFM₁ and excreted in milk [2–4,10,11]. If milk contaminated by AFM₁ is used to formulate cheese or other dairy products, the toxin is transferred from the raw material to the final product [12–14], a process enhanced by the affinity that AFM₁ has for casein [13,15–18]. This carry-over effect causes a higher concentration of that toxin content during the milk transformation process [14,15,19], reaching values up to 5.6 times the initial content of AFM₁ in soft cheeses [20] and up to 4.5 times in hard ones [15,21].

The prevalence of contamination and the contents of AFM₁ in milk and cheese vary seasonally, associated with changes in temperature, rainfall, relative humidity or events such as drought and floods [15,16, 22–24]. At the base of such seasonal fluctuation is the availability of green forage, as there is evidence that milk from animals that consume fresh pasture has lower prevalence values and AFM₁ contents [25–27]. The risk of milk contamination by AFM₁ is increased when cattle are fed mainly with feedstuffs, which are more susceptible to being colonized by aflatoxicogenic fungi due to inappropriate conditions during storage, and which occurs precisely in times with shortage of pasture [17,22,24, 25,27], thus, the use of contaminated food will cause the temporary increase in AFM₁ in milk and its derivatives [12–14,18].

Hard unripened cheeses in El Salvador, such as hard white, had an apparent consumption of 30821.8 metric tons in 2005, of all that imported volume, 71.8 % came from Nicaragua [28]. The import of hard white cheese continues to grow, from 11,240 to 13,266.7 metric tons, and from 29.7–35.2 million dollars between 2014 and 2017 [29,30]. The previous data provides relevance to three aspects, the consumption of hard white cheese is high and is growing among the Salvadoran population; due to this demand the dairy processing plants in Nicaragua (industrial, semi-industrial and artisanal) export their products either legally or illegally to El Salvador [31]. In addition, there are no local or Nicaraguan reports related to the monitoring of AFM₁ in that type of cheese. In view of the above, it is necessary to monitor the white hard cheese marketed in El Salvador, to determine the prevalence of contamination and the contents of AFM₁ in cheeses of local and Nicaraguan origin, the changes occurred according to the time of the year and the association between AFM₁ contamination levels with meteorological parameters.

2. Material and methods

2.1. Cheese specimen, manufacturing and storing

Unripened artisanal hard white cheese, also known as Morolique, is semi-dry and it has a firm soft texture due to its relative low moisture content and does not melt with heat, it has similarities to homemade Feta in appearance but not it is so salty and not crumbly.

Artisan cheese processing plants have up to 12 employees, they process from 150 to 1500 L per day with minimal equipment to do so; in addition, they are not legally obliged to pasteurize the milk for the amount they process daily [32].

Manufacturers do not have their own dairy herd but depend on intermediary traders with collection routes for the provision of milk that comes from various farms [31,32]. Ninety-five plants operate in El Salvador, according to 2014 data [32], and one hundred operate in Nicaragua, although the available data is from 1999 [31]. In both countries, there is an underestimation of artisanal plants since Salvadorans process up to 65 % of milk production [32] and Nicaraguans up to 55 % [31].

The white hard cheese manufacturers process the milk between 3 and 6 h after receiving it, they curdle, salt, mold and press it during the following 24 h in the form of 25 kg models, which is ready for distribution to the market, as an option, it can be left to dry for 3 or 5 days more depending on the demand [32]. In the plant, the cheese is stored only for a few hours before distributing it to municipal markets and the shelf life is 30–35 days in refrigeration (if any) or in display cases at room temperature, depending on the equipment of the retailer [32]. According to the previous data, it is estimated that 5–7 days' elapse between milking and the availability for sale of the cheese in the municipal market and it must be sold before it reaches its useful life (shelf life) because the product usually it is not pasteurized or vacuum packed.

2.2. Type of study and sampling

During a 13-month surveillance of white hard cheese from local and Nicaraguan manufacturing, 10 retail stores from municipal markets of San Salvador metropolitan area were sampled repeatedly. Samplings were spaced three months apart, so that the collection periods were July-August 2018 (rainy season), November-December 2018 (rainy to dry transitional season), February- March 2019 (dry season) and June-July 2019 (rainy season). The total samples of hard white cheese collected and analyzed were 130, 65 processed in El Salvador and 60 from Nicaragua, all marketed in El Salvador. Each sample collected weighed 1 kg, thus complying with the sampling specifications for the official control of mycotoxins in foods established by the Commission of European Communities [33] and were kept cold during transport to the laboratory. The samples were stored at 2– 4 °C in a horizontal freezer until processing and analysis.

2.3. Sample preparation, extraction, and analysis of AFM₁

Each 1 kg sample of hard white cheese was homogenized using a food processor. For AFM₁ extraction, a procedure according to NEOGEN® Corporation (Lansing, Michigan, USA) was used [34]. The concentration of AFM₁ was determined in the cheese samples using the VERATOX kit for AFM₁ with a quantification range between 0.005 and 0.100 µg/kg, agreeing

to NEOGEN® Corporation [34]. All reagents were acclimatized at room temperature (24 ± 2 °C) prior to use.

2.4. Validation of analytical method

The validation of the analytical method was done by applying two evaluation criteria, the average recovery and the intermediate precision [35]. The procedure to evaluate the average recovery, consisted of spiking hard cheese samples with AFM₁ standards at concentrations of 0.050, 0.100, 0.200, 0.300, 0.400, 0.500 and 0.600 µg/kg, prepared in HPLC grade acetonitrile (Avantor™, Ecatepec de Morelos, Mexico), prior to their analysis. Extraction of the toxin and its quantification was done in the same way described for the cheese samples [34]. The procedure is similar to other previous validations [15–17]. The spiking of AFM₁ was carried out in quadruplicate for each level and the analyzes were carried out with the same method, the same type of cheese and the same reactive kits, while the instruments and analysts were different during the five-day trial, as specified to evaluate the inter-run precision tests [35].

The recovery% was calculated by dividing the measured content of a sample by the spiking concentration and the resulting ratio is multiplied by 100; whereas, mean recovery% is the simple average of the set of recovery values obtained per day and per concentration of spiking [35]. The acceptable range for average% recovery values of an analyte in concentrations equal to or less than 1 µg/kg is 40–120%, as established by AOAC International [35]. To evaluate the inter-assay accuracy, the coefficient of variation of the average recovery (% RSD) and the Horwitz Ratio (HORRAT) of spiked samples were calculated [35,36]. To consider that an at concentrations equal to or less than 1 µg/kg, according to the threshold established by AOAC International [35], while HORRAT values must be between 0.3 and 1.3 [36].

2.5. Maximum aflatoxin M₁ level

The regulations of the quality standard of unripened hard cheeses, both from El Salvador and Nicaragua, does not have specified maximum permissible limits for AFM₁ [37,38] for that reason the maximum level of 0.050 µg/kg setting through Commission Regulation (EC) No 1881/2006 issued by the European Community was adopted in this work, applied it to both fluid milk and dairy products, considering the effect of drying and processing of milk on the concentration of that mycotoxin [8,9,27]. This limit is based on the ALARA principle "As low as reasonably achievable", because AFM₁ is a genotoxic carcinogen and that exposure to any level of that toxin will put consumers at risk [9]. As there is no legal limit established for AFM₁ in cheeses by the EU through any consensus, other maximum levels have been established on the initiative of five European countries [15,21,27], allowing fulfill requirements of AFM₁ maximum permissible content and preventing unwanted economic consequences by a very strict regulatory limit for dairy products to be marketed [13,23].

2.6. Meteorological parameters

To establish the association between the average contents of AFM₁ in cheeses of Salvadoran and Nicaraguan origin, with some meteorological parameters, the average monthly

accumulated rainfall (mm), temperature (°C) and relative air humidity (%), were obtained from the reports generated by the Environmental Observatory of the Ministry of Environment of El Salvador and by the General Directorate of Meteorology of the Nicaraguan Institute of Territorial Studies, available on the following websites <https://www.ineter.gob.ni/met.html> and <http://www.marn.gob.sv/informes-sequia-meteorologica/>. The data of aforementioned parameters for El Salvador were taken from 18 stations distributed throughout the territory, while the measures corresponding to Nicaragua were taken from the stations located in the Western Pacific Zone and the Northern Region, border territories with El Salvador and Honduras, where there is livestock commerce, from which milk is collected and processed to produce the cheeses that are exported to El Salvador [31].

2.7. Statistical analysis

Statistically significant differences among AFM₁ prevalence values or average contents, were determined by means of Chi Square test and Student t test, respectively. In all tests, a significance value of $p < 0.05$ was specified. The association between variables was determined by means of Pearson's r coefficient. The tests and figures were made with the IBM SPSS Statistics v.24 for Windows program.

2.8. Ethical considerations

In this study, only samples of cheese available for sale to the public were used, no data from vendors nor obtaining living animal tissue were needed, therefore, the consent of informants or the application of a guide for experimentation with animals were not required.

3. Results

Table 1. Method performance parameters for Aflatoxin M₁ (AFM₁) in spiked pooled samples of hard white cheese.

Spiked level µg/kg	Day 1 repeatability (n=4 per level)		Day 2 repeatability (n=4 per level)		Day 3 repeatability (n=4 per level)		Day 4 repeatability (n=4 per level)		Day 5 repeatability (n=4 per level)		Average of five day trial		Predicted coefficient of variation under intermediate precision conditions	Ratio of average trial RSD% to RSD predicted from Horwitz equation (n=20 per level) [33]	Accepted values
	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%) ¹			
0.050	147.15	23.71	215.10	22.38	191.75	22.78	191.00	22.79	147.70	23.69	178.54	23.07	25.12	0.92	0.3 to 1.3
0.100	112.73	22.23	129.95	21.76	140.75	21.49	140.73	21.49	95.30	22.79	123.89	21.95	22.63	0.97	
0.200	89.84	20.74	84.98	20.92	93.29	20.61	93.91	20.59	112.90	20.04	94.98	20.58	20.39	1.01	
0.300	75.45	20.01	113.53	18.82	80.28	19.82	80.27	19.82	101.21	19.14	90.15	19.52	19.18	1.02	
0.400	119.30	17.88	85.47	18.81	83.71	18.86	83.87	18.86	82.99	18.89	91.07	18.66	18.37	1.02	
0.500	119.33	17.30	112.04	17.46	84.93	18.20	84.01	18.23	90.87	18.02	98.23	17.84	17.76	1.00	
0.600	44.54	19.52	121.29	16.78	89.62	17.57	89.47	17.57	70.97	18.19	83.18	17.93	17.28	1.04	

¹ Acceptable recovery percentages from 40% to 120%, and acceptable values of RSD% are ≤45.3 after Horwitz, and ≤30.0 after AOAC International [32].

3.1. Method validation parameters to determinate AFM₁ in pooled samples of hard white cheese

The values of the mean recovery and the inter-test precision as validation parameters of the method to analyze AFM₁, are presented in Table 1. The average recovery for spiking greater than 0.050 µg/kg, obtained during the five-day test, were better adjusted to the established

range. The mean recovery values did not vary significantly between the five days of the validation test or between the spiking concentrations ($F = 1,804$, 139 df, $p = 0.132$). Regarding the coefficient of variation under reproducibility conditions (% RSD), it presented an average range of values from 17.84 to 23.07, regardless of the six spiking concentrations used, therefore it did not exceed the limit value threshold of 30 % for contents equal to or less than 1.0 $\mu\text{g}/\text{kg}$. The HORRAT presented a range of values from 0.92 to 1.04 (Table 1), adjusting to the limits established between 0.3 and 1.3. The HORRAT values also did not vary significantly between the five days of the validation test or between the spiking concentrations ($F = 1,952$, 139 df, $p = 0.105$).

3.2. Sample locations, and results by location

Sampled sales location and origin of cheese.

SAN SALVADOR- EL SALVADOR

Sampled dates

	Feb-Mar-2019 Mean	Jul-Aug-2018 Mean	Jun-Jul-2019 Mean	Nov-Dec-2018 Mean
Mean content μg per kg	,040	,036	,054	,082
Average temperature in $^{\circ}\text{C}$	26,6	25,7	27,5	25,0
Average relative humidity	63,9	77,8	76,5	80,4

The prevalence of AFM1 positive samples ($>0.005 \mu\text{g}/\text{kg}$) was slightly higher in the November-December sampling period, compared to July-August, with a total sum difference of 0.031 $\mu\text{g}/\text{kg}$ between both sampling periods. On average, the samples of national origin had a higher percentage of positive values for AFM₁, compared to the samples of Nicaraguan origin, with a difference of 0.063 $\mu\text{g}/\text{kg}$ in the total results, between both categories. The average values between the sampling period and the national and Nicaraguan categories varied significantly, with the exception of the town of San Miguel, in which case the variation is less defined.



Figure 1. AFM1 mean values of Salvadoran cheeses regarding four periods.

SAN SALVADOR-NICARAGUA				
Sampled dates				
	Feb-Mar-2019	Jul-Aug-2018	Jun-Jul-2019	Nov-Dec-2018
	Mean	Mean	Mean	Mean
Mean content µg per kg	,042	,030	,006	,015
Average temperature in °C	25,9	25,8	27,1	25,4
Average relative humidity	64,8	72,5	75,7	82,3

The average levels for San Salvador were constant in the first sampling period, with a slight difference of 0.004 µg/kg, for both categories (National-Nicaraguan). When analyzing the second sampling period, we can see a significant difference in the average values, with an increase in the case of the National category and with data that exceeded the reference values. Conversely, for the Nicaraguan category, where the values remained low, the difference between both categories in the same sampling period was 0.056 µg/kg.

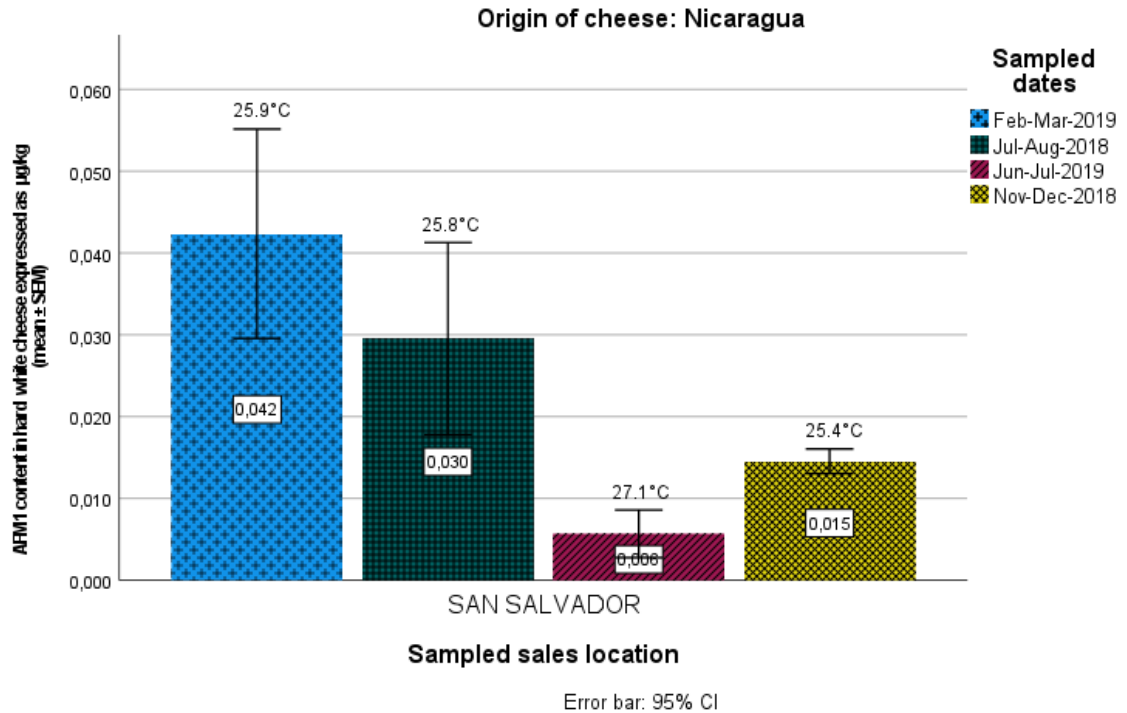


Figure 2. AFM₁ mean values of Nicaraguan cheeses collected at San Salvador, regarding four periods.

3.3. Seasonal occurrence of AFM₁ in Salvadoran and Nicaraguan hard white cheeses

The prevalence values of contamination by AFM₁ in cheeses of local origin and the predominant meteorological parameters are presented in Table 2. During February and March 2019, the highest prevalence of samples took place, which exceeded 0.050 µg/kg during the year sampled (41.0 %); this interval had the lowest average accumulated rainfall of the four quarters sampled (3.6 mm), a temperature of 26.6 °C and the lowest relative humidity overall (63.9 %).

The period between June and July 2019 had the significantly higher prevalence of AFM₁ negative samples (20.0 %, $\chi^2 = 16.71$, 6 df, $p < 0.05$), it had also an accumulated rainfall of 231.8 mm, average temperature of 27.5 °C and relative humidity of 76.5 %. The seasonal variation of the prevalence of contamination by AFM₁ in Nicaraguan cheeses, as well as the meteorological parameters, are shown in Table 3. The period from November to December 2018 presented the significantly higher prevalence of cases with levels between 0.005 and 0.050 µg/kg throughout the monitored year (94.9 %, $\chi^2 = 63.66$, 6 df, $p < 0.001$) although no sample exceeded the limit of 0.050 µg/kg; This period experienced the highest accumulated rainfall of the year (239.7 mm), average temperature of 25.4 and relative humidity of 82.3 %.

Table 2. Occurrences of AFM₁ in hard white cheese from El Salvador and meteorological parameters in this country.

Country of origin of samples	Classification based on AFM ₁ level	Sampled months by year			
		July to August, 2018 (Rainy season)	November to December, 2018 (Rainy to dry transitional season)	February to March, 2019 (dry season)	June to July, 2019 (Rainy season)
El Salvador	< 0.005 µg/kg (< LOQ)	3 (7.9%) ^{a,b}	0 (0%) ^a	2 (5.1%) ^{a,b}	8 (20.0%) ^b
	0.005 – 0.050 µg/kg	24 (63.2%) ^a	28 (80.0%) ^a	21 (53.8%) ^a	23 (57.5%) ^a
	> 0.050 µg/kg	11 (28.9%) ^a	7 (20.0%) ^a	16 (41.0%) ^a	9 (22.5%) ^a
	Range of contents (µg/kg)	0 to 0.092	0.005 to 0.350	0 to 0.485	0 to 0.308
	Sample size	38	35	39	40
	Meteorological parameters				
	Average cumulative rainfall (mm)	205.8	234.3	3.6	231.8
	Average temperature °C	25.7	25.0	26.6	27.5
	Average relative humidity %	77.8	80.4	63.9	76.5

< LOQ: Under limit of quantitation of test.

^{a,b} Counts and percentages with distinct letter differ significantly between same AFM₁ level group per season of a year ($p < 0.05$, Chi Square test). Salvadoran cheese samples, $n = 152$.

Cumulative rainfall, temperature, and relative humidity data are averages of the sampled months, including the prior one to each collection period, if they are available. N.D. Not determined

February and March 2019 interval showed the significantly higher prevalence recorded in the four sampled quarters of specimen that exceeded the 0.050 µg/kg limit (31.3 %, $\chi^2 = 63.66$, 6 df, $p < 0.001$); the season in question had the lowest accumulated rainfall of the year (4.5 mm), temperature of 25.9 °C and average relative humidity of 64.8 %. The period between June and July 2019 had the significantly higher prevalence of negative samples (52.3 %, $\chi^2 = 63.66$, 6 df, $p < 0.001$) This sampled period had an accumulated rainfall of 153.3 mm, an average temperature of 27.1 °C and a relative humidity of 75.7 %.

3.4. Seasonal variation on AFM₁ contents in Salvadoran and Nicaraguan hard white cheeses

The levels of AFM₁ in samples of Salvadoran hard white cheese, obtained during the four monitored quarters are presented in Fig. 1. A significant difference was found between average contents of 2018's rainy and 2019' dry seasons ($t = -2.545$, 75 df, $p = 0.014$), partially coinciding with the trend shown by the prevalence of AFM₁ contamination cases in samples collected in the same periods, especially those that exceed 0.050 µg kg (Table 2). Additionally, the average contents of AFM₁ in hard white cheese samples have a low but significant association with the average cumulative precipitation values (Pearson $r = -0.226$, $p < 0.01$, $n = 152$) and relative humidity (Pearson $r = -0.225$, $p < 0.01$, $n = 152$), recorded during the four monitored periods.

The levels of AFM₁ in samples of hard white cheese from Nicaragua, obtained during the four monitored quarters are presented in Fig. 2. Significant differences were found among average contents of 2019's dry and 2018's rainy seasons ($t = -2.023$, 75 df, $p = 0.047$), also with the 2018's rainy to dry transitional season ($t = -3.975$, 85 df, $p < 0.001$), and 2019's rainy season as well is ($t = 2.550$, 90 df, $p = 0.012$). The significant differences found in the contents of AFM₁ coincide with the trend shown by the prevalence of samples contaminated by that mycotoxin, which exceed the limit of 0.050 µg/kg and were collected in the same periods (Table 3).

Table 3. Occurrences of AFM₁ in hard white cheese from Nicaragua and meteorological parameters in this country.

Country of origin of samples	Classification based on AFM ₁ level	Sampled months by year			
		July to August, 2018 (Rainy season)	November to December, 2018 (Rainy to dry transitional season)	February to March, 2019 (dry season)	June to July, 2019 (Rainy season)
Nicaragua, Western Pacific Zone and Northern Region	< 0.005 µg/kg (< LOQ)	3 (10.3%) ^a	2 (5.1%) ^a	2 (4.2%) ^a	23 (52.3%) ^b
	0.005 – 0.050 µg/kg	22 (75.9%) ^{a,b}	37 (94.9%) ^b	31 (64.6%) ^{a,c}	18 (40.9%) ^c
	> 0.050 µg/kg	4 (13.8%) ^{a,b}	0 (0.0%) ^b	15 (31.3%) ^a	3 (6.8%) ^b
	Range of contents (µg/kg)	0 to 0.162	0 to 0.044	0 to 0.241	0 to 0.415
	Sample size	29	39	48	44
Meteorological parameters					
	Average cumulative rainfall (mm)	118.6	239.7	4.5	153.3
	Average temperature °C	25.8	25.4	25.9	27.1
	Average relative humidity %	72.5	82.3	64.8	75.7

< LOQ: Under limit of quantitation of test.

^{a,b} Counts and percentages with distinct letter differ significantly between same AFM₁ level group per season of a year (p<0.05, Chi Square test). Nicaraguan cheese samples, n=160.

Cumulative rainfall, temperature, and relative humidity data are averages of the sampled months, including the prior one to each collection period, if them are available. N.D. Not determined

Additionally, the variation in the average contents of AFM₁ in hard white cheese samples, has a low but significant association with the average cumulative precipitation values (Pearson r =-0.266, p <0.01, n =160) and with the percentage relative humidity (Pearson r =-0.264, p < 0.01, n = 160), recorded during the monitoring period. Most of the datasets generated and analyzed during the current study are available at the Mendeley Data site: <https://data.mendeley.com/datasets/5hm687y7td/3> [39].

4. Discussion

According to the results obtained in the validation of the analytical method, the recovery averages for spiking equal to and greater than 0.100 µg/kg, are coincident with the acceptable values of mean recuperation for concentrations equal to or less than 1 µg/kg [35] and that are similar to the results obtained in other recovery trials of AFM₁ in cheeses [16,19,26].

Regarding the inter-run precision, none of the measurements of both average recovery and % RSD varied significantly between the five days of the trial; in addition, the %RSD values obtained in this work do not exceed the thresholds established by the Codex Alimentarius Commission (45.3 %) or by the AOAC International (30 %) [35]. On the other hand, the HORRAT averages calculated in the five-day trial meet the accepted inter-run precision values [36].

The average prevalence values of AFM₁ are high in both Salvadoran (91.7 %) and Nicaraguan (82 %) cheeses, indicating that both countries are endemic to dairy contamination by the aforesaid mycotoxin. Nevertheless, the highest prevalence of cases that exceeded the 0.050 µg/kg limit were detected in the dry season, while the lower prevalence values occurring in the rainy to dry transitional season.

Concerning the fluctuations described in the prevalence of hard white cheese contamination of both origins, several studies provide evidence about the seasonal variation of AFM₁ in milk and its derivatives [17,24,25,27,40]. It has been described that prolonged events or conditions of shortage of rain or drought and high temperatures are associated with increases in AFM₁ contamination in dairy products or in their raw material [15,22–24,41]. The increases in temperature and the decrease in rainfall upsurge the conditions for the growth of aflatoxicogenic fungi in corn [3,4,42,43], the main raw ingredient of feedstuff for cattle [22,42,44,45], therefore, the consumption of contaminated feed will cause the temporary increase of AFM₁ in milk and its derivatives [13,15,23]. The previous framework would explain the increase in the prevalence of samples of Salvadoran and Nicaraguan cheeses, with levels of AFM₁ that exceed the limit of 0.050 µg/kg during the dry season months, precisely when the use of supplementary food is used more before the shortage of grass for all the cattle, especially the lactating ones.

The proportion of AFM₁ positive cheeses of both origins, reported in this work, is similar or higher than the values reported in most other studies presented in Table 4. Differences on AFM₁ prevalence among collected values and those from Italy and Argentina are due to, such studies being conducted to estimate the incremental effect of the toxin concentration in cheese making [15] or the process of carryover of Aflatoxins in livestock feed to cheese [20].

In general terms, during the months of February and March 2019, the average levels of AFM₁ were significantly higher, both in Salvadoran and Nicaraguan cheeses, coinciding with the lowest averages of both accumulated precipitation and relative humidity for those zones of the Central American Isthmus. Significantly lower average AFM₁ contents were detected between July and August 2018 for Salvadoran cheeses, and between June and July 2019 for Nicaraguan dairy products, in both cases coinciding with relatively high averages of accumulated precipitation and relative humidity.

The increase in the mean contents of AFM₁ in Salvadoran and Nicaraguan cheeses during the time of the year with less accumulated rainfall, coincides not only with the higher prevalence of contamination by that mycotoxin, but with the evidence indicating that the scarcity or absence of rain is associated with the increase of AFM₁ contamination in milk and its derivatives [23,24,41].

Regardless of the time of year sampled, the ranges and averages of the levels of AFM₁ in both Salvadoran and Nicaraguan cheeses, presented in this work, are lower than the values obtained in most of the studies shown in Table 4, but similar to those reported in Spain, Iran, Italy and Argentina [12,18,20,26,46]. In any case, substantially higher levels of AFM₁ are reported in other geographical regions, for example, some countries in Northern Africa and the Middle East [49–53].

Understanding the seasonal fluctuation and the high prevalence of AFM₁ in cheeses, can provide the basis for selecting the best season and crop practices that are used for the

preparation of feed for livestock, thus preventing the contamination by toxicogenic fungi and, consequently, the transfer of AFM₁ to dairy products.

The risk estimate for Salvadorans of all ages within the frequent consumption of dairy products contaminated by AFM₁ should be addressed in future studies, as has been done in milk [47,48] and cheese [16].

Table 4. AFM₁ occurrences and levels in cheeses, after report's year, country and season.

Year and location	No. samples	Occurrence and season	Contents (range and/or average $\mu\text{g}/\text{kg}$)	Reference
1996, Spain	9	55.5%, Not determined	0.020 to 0.130, 0.074	10
2004, Libya	20	75%, Summer	0.110 to 0.520, 0.290 ± 0.160	46
2009, Italy	25	100%, Late autumn to early winter	0.111 to 0.413, 0.246 ± 0.095	13
2010, Turkey	304	71.1%, from autumn 2006 to late summer 2007	0.051 to 0.860, 0.263 ± 0.198 Spring 0.162 ± 0.024 Summer 0.051 ± 0.015 Autumn 0.053 ± 0.018 Winter 0.083 ± 0.020	47
2011, Iran	75	Total 65.3%	0.055 to 0.374, 0.083 ± 0.079 0.041 to 0.215, 0.024 ± 0.025	15
2012, Iran	50	80% in Winter 2008 40% in Summer 2009	Not available	16
2013, Iran	823	40 to 86.6%, Not determined	Spring: Not detected Summer 0.031 ± 0.005 Autumn 0.047 ± 0.006 Winter 0.119 ± 0.070	48
2014, Iran	20	Not determined	0.050 to 0.700, 0.130	24
2014, Turkey	40	50%, Not determined	0.13 to 0.22, 0.64	49
2015, Serbia	10	100%, Late spring to early summer	0.106 to 0.702, 0.211	11
2016, Turkey	100	52%, Autumn	0.153 ± 0.0003	12
2017, Iran	180	57.8%, Winter 50.0, Summer	0.121 ± 0.0023	14
2017, Iran	100	52%, Winter	0.052 to 0.424, 0.169 ± 0.032	50
2018, Italy	40	27.5%, Winter, spring and early summer	0.039 to 0.096	43
2019, Argentina	36	100%, Winter	0.084	18

5. Conclusions

The method to quantify AFM₁ used in this work proved to have adequate precision, because it has reached acceptable values of average recovery and intermediate reproducibility or inter-assay accuracy.

The high prevalence values of AFM₁ in cheeses of both origins, indicate that both El Salvador and Nicaragua are endemic to the contamination of dairy products by that mycotoxin; however, the average contents found in this work do not exceed those reported in other geographical areas such as the Middle East.

The prevalence of cases that exceed the limit of 0.050 $\mu\text{g}/\text{kg}$ and the average AFM₁ contents in cheeses vary seasonally, reaching higher measurements during the season with lower values of rainfall and relative humidity, while the lowest prevalence was recorded during the season with more accumulated rainfall and greater relative humidity, regardless of the country of origin of the dairy. This seasonal variation may be due to rain shortage conditions that are associated with the increase in dairy contamination by AFM₁, precisely because they promote the growth of aflatoxicogenic fungi in crops that serve as raw materials to produce feedstuffs for cattle. In this way, the consumption of contaminated food will cause the temporary increase in AFM₁ in milk and its derivatives.

Knowing the seasonal variation of AFM₁ in dairy products will allow informed decisions regarding the season and practices that represent less risk of contamination by toxicogenic fungi in crops used to feed livestock, thus preventing contamination by AFM₁ and its transfer to dairy.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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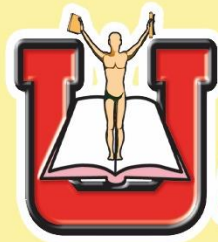
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