Pharmaceutica Analytica Acta

Rojas-Marín et al., Pharm Anal Acta 2018, 9:3
DOI: 10.4172/2153-2435.1000581

Commentary Open Access

Presence of Aflatoxin Carcinogens in Fresh and Mature Cheeses

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Abstract

Cheese has a high nutritional value so it is considered an essential food in the human diet. This review considered 27 countries with 59 different kinds of cheeses highly consumed per person, with France and the USA as the largest consumers with 26 kg and 15 kg per inhabitant per year, respectively. Mexico has a consumption of 2.1 kg per capita in a year. The presence of aflatoxins in cheeses represents a risk for human health because the International Agency for the Research of Cancer (IARC) classified them as Grade I, meaning that they are proven carcinogens to humans. Almost all of the countries reported aflatoxin M_1 in cheese, and only Mexico analyzed eight different aflatoxins and hydroxylates in this dairy product. It is also important to analyze the hydroxylate metabolites of aflatoxins because they are also carcinogenic and they increase the amount of ingested carcinogens in cheese samples, allowing us to obtain the real ingested dose.

Keywords: Aflatoxins; Cheeses; Food carcinogens; Human cancer

Introduction

Aflatoxins are undesirable mycotoxins that contaminate cheese directly through rennet or contaminate milk indirectly from oilseeds or cereals with the fungi *Aspergillus flavus* or *A. parasiticus* in animal feed. Cheese is produced by the addition of rennet or other enzymes to curdle the milk of certain mammals, such as cattle, sheep and goats, in the presence of lactic acid produced by spiked or natural microorganisms in milk; part of the humidity is eliminated when the curd is cut [1]. An accepted definition of cheese is "A fresh or refined product, solid or semisolid obtained by the coagulation of the entire milk, skimmed or partially skimmed due to the action of rennet…" [2]. Milk can be raw or pasteurized, and for the formation of cheese, two processes are required: curdling and acidification of the milk. Rennet is an aspartic protease enzyme extracted from the abomasum that is in the fourth stomach of bovines, and it acts by separating casein from its liquid phase, water, milk serum and carbohydrates. It is also produced in human babies to curdle the mother´s milk [3]. Acidification to pH 4.5 precipitates the insoluble protein via the action of bacteria or the addition of vinegar or lemon juice [4]. Bacteria convert milk carbohydrates into lactic acid, which plays an important role in the texture and organoleptic properties of cheeses [5]. The different flavors and textures of the cheeses depend on the kind of milk employed, the additives, the curdle time and the modification of proteins and fats by the different microorganisms employed, such as the lactic acid and propionic bacteria [6]. Additionally, some fungal strains are utilized, such as *Penicillium roqueforti* for making blue cheese or Roquefort and *Penicillium camemberti* for making Camembert and Brie cheeses [7]. Cheese is one of the most nutritive foods for humans as a source of calories; proteins; carbohydrates; saturated (AGS), unsaturated (AGM) and poly-unsaturated (AGP) fats; cholesterol, vitamin D; calcium; and sodium [3].

Production of Milk and Cheese in Mexico and Worldwide

The bovine milk production worldwide in 2016 was 488,812,000 tons, of which 189,080,000 tons (39%) was produced in the European Union and East Europe. The American continent was ranked in second place with 155,637,000 tons (32%). Asia produced 113,340,000 tons (23%), and Oceania registered a production of 30,755,000 tons (6%) [8]. In 2013, the European Union cheese production was 645,776,815 tons, Saudi Arabia produced 231,341,350 tons, New Zealand produced 269,306,317 tons, the United States of America produced 170,262,254 tons, Egypt produced 160,111,100 tons, and Australia produced 163,163,170 tons [8].

The USA was ranked first in bovine milk production in 2016, with 62%, followed by Brazil, which produced 17% and Mexico was in third place with 8%. Argentina and Canada produced 6% and 7% of bovine milk, respectively [8]. The total cheese production in the world is 18 million tons, with the USA generating 30% of the world´s production, followed by Germany with 13%, France with 12% and Italy with 7.5%. The remaining production is generated by The Netherlands, Poland, Brazil, Egypt and Argentina, among others. Mexico produced 282,000 tons of cheese in 2015, and it exported 4,112 tons and imported 116,117 tons [9].

Aflatoxins

Aflatoxins (AF) are a group of toxic secondary metabolites produced mainly by the fungi *Aspergillus flavus*, *A. parasiticus* and *A. nomius* [10]. These species are ubiquitous in nature, and they can contaminate dry fruits, oilseeds, cereals and spices. These contaminants can affect plants in the field, crops, transportation or warehouses [11].

The main entrance of AFs in mammals is through the ingestion of contaminated foods and feeds, with the liver as the main affected target [12]. AFs damage animals and humans and are considered the most potent natural mutagens and carcinogens that can link to DNA and be stored for years [13]. Aflatoxin B_1 (AFB_1) is classified as a proven Group I carcinogen for humans [14]. Aflatoxin M_1 is possibly carcinogenic to humans and was classified as a Group 2B carcinogen by the IARC (1997) [15]. The acute symptoms related to AF consumption are vomiting, hemorrhaging, diarrhea and death. The chronic symptoms

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Received March 13, 2018; **Accepted** March 23, 2018; **Published** March 30, 2018

Citation: Rojas-Marín V, Carvajal-Moreno M, González-Villaseñor MC, García-Hernández EA, González–Mendoza A (2018) Presence of Aflatoxin Carcinogens in Fresh and Mature Cheeses. Pharm Anal Acta 9: 581. doi: 10.4172/2153-2435.1000581

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include immunosuppression, fetal malformations, cirrhosis, hepatitis, and several cancers such as hepatocellular carcinoma and colorectal, cervical, breast, pancreatic and lung cancers [16,17].

Consumption and Excretion of AFs in Cattle

In the case of domestic animals, such as bovines, goats and sheep, the feed ingestion of AFB_1 and aflatoxin B_2 (AFB_2) AFs is due to the ingestion of balanced feeds produced with contaminated cereals and poultry litter [18]. AFB_1 and AFB_2 are not very soluble in water, and thus, in order to eliminate them from the animal, the liver adds a OH-molecule to transforms these species into the hydroxylates aflatoxin M_1 (AFM_1), aflatoxin M_2 (AFM_2), aflatoxin P_1 (AFP_1) and aflatoxicol (AFL), which are very soluble in water and can be eliminated from the body in urine, maternal milk and litter. AFL is interconvertible with AFB_1 and remains stored, and although it is less carcinogenic than AFB_1, it is still very carcinogenic and cannot be considered a decontamination product [19]. AFM_1, AFM_2, AFP_1 and AFL are excreted into the milk of lactating cows in 1 to 3% of the original AFB_1 concentration consumed in the diet. Therefore, all of these AFs and their hydroxylated metabolites s with AFL are detected in the milk of cattle, goats and sheep, and from there, they are incorporated into cheeses highly consumed by humans [20].

Aflatoxins in Milk and Cheeses in Mexico and Worldwide

AFs have been identified in raw and pasteurized milk in Brazil, Turkey, Kuwait, Italy, Serbia [21-23] and Australia [24]. There are reports of AFs (AFB_1, AFM_1 and AFL) in 580 L of Mexican milk, where 13% of pasteurized and 8% of ultrapasteurized milk with different amounts of fat contained AFL (>0.05 μg L^{-1}) [25,26] and AFL was also found in artisanal Oaxaca cheese in Veracruz, Mexico [27,28]. There were only traces of AFB_1 (0 a 0.4 μg/L), and the amount of fat was not significant.

The detection of AFM_1 in the State of Hidalgo, Mexico, was reported in 2007 with a concentration from 167 to 332 μg L^{-1} [29]. Other reports from the central high plateau of Mexico in 2008 found 59% (26/44) of AFM_1 contamination (>0.05 μg kg^{-1}), which is the highest tolerable level in the European Community [30]. The detection of AFM_1 in 100% (50/50) of the milk samples was obtained from Guadalajara, Jalisco,

N°	Country	Cheese type	+/total samples or sample %	Aflatoxin	Concentration (μg kg^{-1})	Source
1	Australia	-	-	M1	-	[24]
2	Brazil	Prato, Parmesan	0/36		ND	[33]
3		Minas cheese	48/58		10 to 304	[34]
4			13/48		0.04 A 0.31	[35]
5		Artisanal	3.2%			[36]
6		Parmesan	18/30		0.16	[37]
7	Czechoslovakia	Edam-type	29, 20%		5-66	[38]
8	China	Artisanal white fresh cheese	4/4			[39]
9	Colombia		37/37		5 to 351	[40]
10	Costa Rica		10/70		31 to 276	[8,41]
11	Denmark,	Blue and white mould	0/10		ND	[42]
12	Ecuador	Artisanal white fresh cheese	7/33		0.04 to 0.83	[43]
13	Egypt	Kareish & Damietta	20/50		1.95 to 14.75	[44]
14	Finland	Blue and white mould	0/10		ND	[42]
15	France		0/10		ND	
16	Germany,		0/10		ND	
17	Greece	Feta & Teleme	0/127		ND	[45]
18	Iran	White cheese	8, 87%		NRb	[46]
19			195/360		50.5 a 309	[47]
20			52/100		50.2 a 424	[48]
21			188/545		-	[49]
22			26/40		52.5 to 272	
23			35/39		0.004 a 0.023	[50]
24			73/75		-	[51]
25			56/75		0.12	
26			161/210		52.1 a 785.4	[10]
27	Italy	Cheese	6, 83%		50-400	[52]
28			50, 8%		50-100	[53]
29		Artisanal fresh	44/265		50 to 250	[54]
30			11/24		5.0 to 20	[55]
31			12/24		0.05	[56]
32	Japan	Cheese	303, 14%		200-1200	[57]
33			0/41		ND a	[58]
34	Korea	Artisanal fresh	83/108		0.02 to 0.14	[59]
35	Kuwait		32/40		23.8 to 452	[60]

36		Oaxaca fresh	19/30	M1, M2	0.01 to 3.4	[28]
37	Mexico		30/30	B_1,B_2,G_1,G_2, M_1,M_2,P_1, AFL	0.1 to 19.1	[27]
38		Goat cheese	3/20		0 to 0.23	[61]
39		Fresh goat cheese	3/20		0.23	[31]
40	Netherlands	Mature ripened	-/88			[62]
41	North Africa	White soft	20, 75%		110-520	[63]
42	Portugal	Mature cheese	8/128		>0.05	[64]
43	Rumania	Ripened mature	9/50		<50	[65]
44		Cheese	35, 44%		20-200	[66]
45	Spain	Manchego	50%		-	[67]
46		Artisanal fresh cheese	0/72		0	[68]
47		Manchego, requesón	80/82			[69]
48	Slovenia	Raw artisanal cheese	10%		>50 ng/kg	[70]
49		Lor	6, 33%		13-19	
50		Civil	9, 44%		12-18	
51		Tulum	11,63%		11-202	[71]
52		Kashar	14, 42%	M1	7-68	
53		White	23, 39%		11-106	
54		Tulum, white	100, 82%		51->800	
55			49,89%		800	[72]
56		Cream	100, 79%		51-650	
57			200, 4%		100-700	
58		White	200, 5%		100-600	[73]
59		Kashar	200, 6%		120-800	
60	Turkey		53,88%		250	[74]
61		White	91, 91%		250	
62	Turkey	Van otlu (Herb)	60, 86%		160-7260	[75]
63		White Pickle	50, 62%		100-5200	
64		Feta, Kashar liquid	60/60		5 to 650	[76]
65		White & Kashar	123/185	M1	189.5 a 229.8	[77]
66		Urfa	4/64		51 to 100	[78]
67		White & Kashar	28/40		40 to 388	[79]
68		Kashar	109/132		50 to 690	[80]
69		Artisanal	13/64		>250	[81]
70		Brined-Urfa	36/127		70.6 a 770.9	[82]
71		Ripened Konya küflü	0/10		0	[83]
72		Artisanal fresh cheese	70/166		0.02 to 2.1	[84]
73		Mouldy	52/100		10.6 to 702	[85]
74		Swiss cheese				[86]
75		Parmesan & Mozzarella				[87]
76	USA	Cheddar cheese		M_1		[88]
77		Cottage cheese				[89]
78		Cheese	118, 6%		100-1000	[90]

Table 1: Occurrence of Aflatoxins in cheeses worldwide.

Mexico, where raw milk had 0.005 to 0.100 μg/L and pasteurized milk had from 0.005 to 0.637 μg/L, which is 18.6% (25/134) above the maximum tolerable level in the European Community [31].

There are studies on AFM_1 in goat milk cheese in the State of Guanajuato, Mexico, which was found in 15% of the samples (3/20), with a maximum amount of 0.23 μg kg^{-1}. Goat milk exhibited 30% contamination, and 18% had a concentration > 0.05 μg kg^{-1} [32], which is the maximum tolerable level in the European Union.

A report of artisanal Oaxaca fresh cheese from the State of Veracruz, Mexico, examined AFM_1 and AFM_2 in 30 samples, with 16/30 (53%) having AFM_1 contamination (0.01 to 44 μg kg^{-1}). AFM_2 was less frequent, present only in 6/30 (20%), with a concentration range between 0.67 and 3.43 μg kg^{-1} [28].

The Mexican legislation on fresh, mature and industrialized cheese has no explicit maximum tolerance level for AFM_1, although the milk used to make these cheeses must meet the sanitary criteria specified for pasteurized milk [32].

Of all the countries that reported AFM_1 contamination in cheese, there were approximately 1,479 (48.30%) positive for this mycotoxin. The most applied quantitative technique was ELISA with 60% frequency, followed by HPLC, with 38% frequency. AFM_1 was the most detected AF (92%), and only three studies from Mexico reported more AFs in cheese (Table 1) [27,32].

The Middle East published 19 studies, with Turkey writing 8 manuscripts with 634 samples, of which 334 (52%) showed AFM1. Meanwhile, the American continent has reported 9 studies related to the detection of AFs in cheeses, of which 3 are from Mexico and contain not only AFM_1 but also other AFs and hydroxylates that increase the ingestion and risk to humans. Very few studies have described AFB_1 and AFG_1 in cheese, and they were stable in Swiss cheese [91]. Only in Mexican reports were eight aflatoxins as well as hydroxylates, such as aflatoxicol (AFL) and aflatoxin P_1 (AFP_1), quantified in natural non-spiked cheese [27]. Other mycotoxins such as sterigmatocystin (STC), which is an aflatoxin precursor, have been detected in cheese [92]. Mycotoxin cheese contamination can occur indirectly via milk contamination or directly by mycotoxins producing spoilage or filamentous fungi [93].

The percentage of transference from cattle feed with AFB_1 to elaborated dairy foods with AFM_1 are as follows: cheese, 40 to 60%; skim milk, 10%; and butter, <2% [94].

Several studies have detected AFM_1 in cheese worldwide, and its presence is a public health concern. Several reviews of mycotoxins in cheese [95,96] have enriched the knowledge about the subject. Iran and Turkey had the most numerous studies of AFM_1 in cheese (Table 1).

The AFM_1 stability in milk and dairy sub-products was reported [94], with no significant changes in the AFM_1 concentration after pasteurization, boiling or ultra-pasteurization. There were no significant AFM_1 concentration changes in cheese after 3 months of storage [97]. However, the binding ability of AFM_1 by lactic acid bacteria such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus* [98] effective in reducing the extent of free AFM_1 content in liquid culture medium and during yogurt processing [98,99]. The stability of AFM_1 during processing and storage makes it dangerous. As was expressed before, AFM_1 from milk can be passed to cheese (Table 1).

For cheese-related *Aspergillus* spp., the AF biosynthetic pathway was reported to contain 25 genes clustered in a 70 kb region and involves at least 23 enzymatic reactions [100,101]. Aflatoxins are by far the most abundant and important mycotoxins in dairy product food and feed. Lethal dose (LD50) values range from 0.5 to 10 mg kg^{-1} [102]. Aflatoxins are secondary metabolites that confer a selective advantage to the fungal producers within complex ecosystems, and they act as chemical signals to communicate; act as a competitive weapon to defend habitat by inhibiting the growth and reproduction of competitors of the same trophic niche competing with other organisms [103,104]; and play a role in survival functions [105].

Conclusion

AFM_1 was the most reported contaminant of the AF hydroxylated metabolites, in cheese worldwide. ELISA and HPLC were the main techniques employed. There are few reports on other AFs in cheese, and in Mexico, eight different unreported AFs were published. Other AFs, mainly AFB_1 and AFL were found in artisanal and industrialized cheeses from Mexico because during processing, they incorporated maize flour that increases the AFs. The addition of all AFs (AFt) must be considered in cheeses because their amount of increase represents a higher risk for human health.

Acknowledgments

The authors express thanks to Posgrado en Ciencias Biomédicas, Universidad Nacional Autónoma de México [Posgraduate in Biomedical Sciences of the National Autonomous University of Mexico].

The authors thank the Instituto de Biología, Universidad Nacional Autónoma de México (IBUNAM), for the data of this work. The authors also thank IBUNAM´s personnel: Noemí Chávez from the Secretaría Técnica, Joel Villavicencio, Jorge López, Alfredo Wong, Celina Bernal, Diana Martínez and Julio César Montero. Additionally, we thank Georgina Ortega Leite and Gerardo Arévalo for library information.

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