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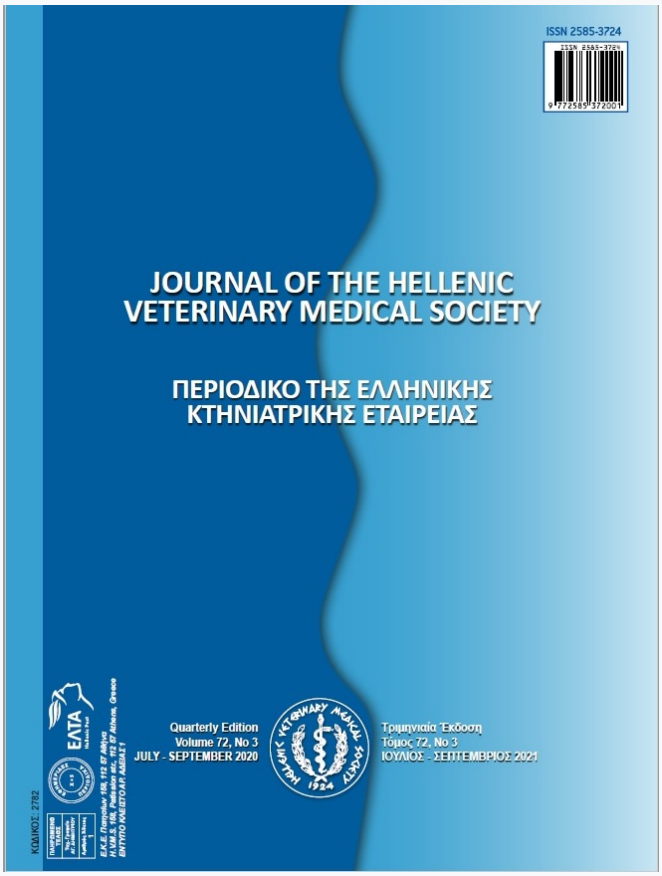

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Molds and Aflatoxins in Traditional Moldy Civil Cheese: Presence and Public Health Concerns

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ABSTRACT: The purpose of this study was to detect the mold biota, the contamination levels of total aflatoxin (AFB1, AFB2, AFG1, AFG2) and aflatoxin M1 (AFM1) in moldy civil cheese. A total of 100 moldy civil cheese were collected from randomly selected retailers. Mold biota was determined with conventional and ITS sequence analysis, and Aflatoxin (AF) analysis was performed usingEnzyme-Linled Immunosorbent Assay (ELISA). In the analyzed samples, *Penicillium roqueforti* (100%) was isolated as the dominant species followed by *P. verrucosum* (83%), *Aspergillus flavus* (17%). Fifteen (15 %) of moldy civil cheese samples contained AF with levels ranging from 12 to 378 ng/kg.Likewise, AFM1 was found in 25 (25 %) of samples (ranging from 5.46 to 141.56 ng/kg), among which 5 (5 %) were above the legal limits. Considering the presence of *A. flavus,* total AF and AFM1 contamination in the analyzed cheese samples it could be emphasized that moldy civil cheese might pose a hazard for public health.

Keywords: AFM_1, ELISA, Moldy civil cheese, Total Aflatoxin

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INTRODUCTION

Moldy civil cheese is a mold-ripened variety of cheese manufactured mainly in eastern Turkey. This cheese is generally made from non-pasteurized and skimmed cow and/or sheep milk using home-made calf rennet at 30-32 ºC without adding starter culture. For moldy civil cheese production, 70-75% civil cheese and 25-30% curd cheese, obtained from whey, are mixed, salted,and pressed into plastic drums or goat skins and ripened for three months or longer in the refrigerator or at chilly conditions(Cambaztepe et al., 2009;Cakmakci et al., 2012, 2015; Celikel et al., 2019). Molds which are naturally growing on the surface of and within the cheese during the ripening are considered important to lipid and protein breakdown that play active roles in the sensorial properties, texture, and flavor of the cheese (Benkerroum, 2016; Fox et al., 2017; Anelli et al. 2019; Ritota and Manzi 2020). However, molds might also be lead to undesirable changes, such as toxic secondary metabolite production, during cheese ripening and the presence of these metabolites in dairy products might result in a considerable hazard on public health (Panelli et al., 2012; Garnier et al., 2017; Anelli et al. 2019).

Mycotoxins are secondary metabolites naturally produced by certain types of molds including *Aspergillus* spp., *Penicillium* spp. etc. These metabolites especially aflatoxins are known to be potent cause of acute or chronic toxications (teratogenic, carcinogenic, and mutagenic) in humans and animals (Becker-Algeri et al., 2016; Garnier et al., 2017; Kowalska et al., 2017;El-Tawab et al., 2020). Aflatoxins, especially Aflatoxin B1 (AFB1), are immensely toxic secondary metabolites produced by some fungi, mostly by *Aspergillus flavus*, *A. parasiticus* and less often by *A. nomius* (Elkak et al., 2012; Sottili et al., 20117; Peles et al., 2019; Reinholds et al. 2020). In ruminants, ingested AFB1 with contaminated feed is biotransformed in the liver to AFM1, which then passes into the milk of lactating mammals (Bakirci, 2001;Bilandzic et al., 2014; Fontaine et al., 2015; Garnier et al., 2017; Peles et al., 2019). European Commission (EC, 2006) has defined the maximum residue level (MRL)of AFM1 as 50 ng/L in milk. On the other hand, according to the Turkish Food Codex, the MRL of AFM1 is defined as 0.050 µg / kg in raw milk, heat-treated milk, and milk used for milk products (TFC, 2011). Moldy cheese is a commonly produced traditional food in Turkey. However, no regulated standard is available for the production of civil cheese yet (Cambaztepe et al., 2009). In this regard, the aim of this study was to identify mold biota and the total aflatoxins (B1, B2, G1, and G2) and AFM1 in moldy civil cheese obtained from local bazaars in Erzurum/Turkey.

MATERIALS AND METHODS

Samples

A total of 100 moldy civil cheese samples were collected from randomly selected retailers from local bazaars in Erzurum between September and November 2015 with two-week of intervals. The samples were takenaseptically, brought to the laboratory under the cold chain and examined within 1-2 h.

Isolation and characterization of molds from moldy civil cheese

The mold isolation in cheese samples was performed according to the standard ISO 21527-2:2008. Briefly, ten grams of sample was suspended in 90 mL of 0.1% peptone water and mixed in a stomacher for 2 min (BagMixer 400 P, Interscience, France). Serial dilutions were prepared from 10^{-2} to 10^{-5} from each sample and spread onto Dichloran rose Bengal chloramphenicol agar (DRBC, Oxoid, UK). After incubation at 25 °C for 7 days, single colonies were selected based on their morphological properties (shape, size, and color) and examined according to the previous studies of Pitt and Cruickshank (1990) and Samson and Frisvad (2004).

DNA isolation and sequencing of the ITS1-5.8S-ITS2 rDNA region

Genomic DNA was extracted from the isolates using UltraCleanTM microbial DNA isolation kit (Mo Bio Laboratories, Solana Beach, California, USA) with a procedure from that of the manufacturer's. The amplification of the ITS1-5.8S-ITS2 region was carried out using ITS1 (5'-TCCGTAGGTGAACCTGCGG- 3') and ITS4 (5'-TCCTCCGCTTATTGATATGC- 3') primer pairs conducted by previous studies (White et al.,1990; Cardoso et al., 2007;Cakmakci et al., 2012). Ampliconswere purified with a GeneJet PCR purification kit (Thermo Scientific, Waltham, MA) andsubjected to sequence analysis by a commercial company (Atlas Biotechnologies, Co., Ltd., Turkey). The BLAST program was used to align the resulting sequences (BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Detection of Aflatoxin levels ofcheese samples with ELISA

Chemicals and equipment

To determine total aflatoxin (AF) and AFM1 levels in moldy cheese samples, RIDASCREEN® AF Total kit (R-Biopharm, R4701, Germany) and RIDASCREEN® AFM1 test kit (R-Biopharm, R 1101 Germany) were used. Each kit contained standards, 1 x Microtiter plate with 96 wells, wash buffer salt Tween20, conjugate, antibody, substrate/chromogen, and stop solution. Other chemicals (dichloromethane, methanol, and n-heptane) were purchased from Merck (Darmstadt, Germany). Also, the immunoaffinity column (Rida Aflatoxin Column Art. No: R5001/5002) was used for the clean-up of the sample prior to the analysis of total aflatoxin and AFM1 in cheese.

Extraction of Aflatoxin from cheese samples

The extraction of aflatoxin from cheese samples was conducted using dichloromethane according to the procedure applied by Ertas et al (2011).

ELISA Test Procedures

The existence and concentrations of total AF and AFM1 in moldy civil cheese samples were determined using ELISA in compliance with the manufacturer guidelines of the commercial kits (R-Biopharm GmbH, Germany). For the AFM1 measurements,100 µL of each the standards and extraction solutions were added per separate wells (in duplicate) of the microtiter plate and incubated at 24 °C in the dark for 60 min. The liquid discarded from wells and washed two times with 250 µL of washing buffer. Then, 100 µL of diluted enzyme conjugate was added to each well and incubated for a further 60 min at 24 °C in dark. The washing procedure was repeated three times and then 100 µL substrate/chromogen was put into each well and incubated for 30 min at room temperature in dark. Finally, the reaction was stopped by adding 100 µL stop solution into the wells. The optical absorbance of each well was read at 450 nm absorbance within 15 minutes in an ELISA plate reader (ELX800, Bio-Tek Instruments, USA).

In the total aflatoxin test protocol, 50 µL of each standard and extraction solutions were separately added to each well. The plates were gently mixed and incubated for 30 min at 24 °C. Following the incubation period, wells were washed with 250 µL of washing solution two times. Then, 100 µL of substrate/chromogen were added to the wells and incubated for 30 min at 24 °C again. Finally, 100 µL stop solution was added and measured absorbance of each well at 450 nm as soon as possible after adding a stop buffer.

Evaluation of the results

RIDA®SOFT Win/RIDA®SOFT Win.net (Art. No. Z9996) software was used in the evaluation of the results obtained from ELISA analysis to determine the total aflatoxin and AFM_1 content of moldy cheese samples. According to the manufacturer guidelines of the RIDASCREEN kit for cheese; the detection limit, recovery rates and the coefficient variation (CV) were 5 ng/kg, 102% and 11%, respectively.

RESULTS AND DISCUSSION

In this study, the mold and yeast count of moldy cheese samples were ranging from 8×10^3 to 1×10^6cfu/g in cheese samples.A total of 175 mold isolates which mainly belonged to two different genera (Penicillium and Aspergillus) were obtained from 100 moldy civil cheese samples. 155 of 175 isolates were determined as *Penicillium* spp., and the rest of the isolates were Aspergillus spp. based on their morphology. ITS sequence analysis revealed that the isolates identified morphologically as Penicillium included *P. roqueforti* (62%) and *P. verrucosum*(27%), while the remaining isolates detected as Aspergillus included only *A. flavus* (11%) (Table 1). Consequently, *P. roqueforti* was isolated from all (100%) samples analyzed. In 83(83%) samples were isolated *P. roqueforti* plus *P. verrucosum*, while *P. roqueforti* plus *A. flavus* isolated from the remaining 17 (17%) samples (Table 2).

In the study, total aflatoxins (B1, B2, G1, G2) were found in 15 (15%) of 100 moldy civil cheese samples, ranging from 687.10 ng/kg to 8273.5 ng /kg (Table 2). AFM1 was found in 25 (25 %) of samples (ranging from 5.46 to 141.56 ng/kg), from which 5 (5 %) were above the limit set by TFC (50 ng/kg) and European Community (50 ng/kg). The contamination level was determined as 54.95 ng/kg, 57.7 ng/kg,74.38 ng/kg, 80.23 ng/kg, and 141.56 ng/kg in these five samples (Table 2).

Table 1. Distrubition Mold isolates obtain from moldy civil cheese samples

Mold species			
Morphological	**Number of isolates (%)**	**ITS sequencing**	**Number of isolates (%)**
Penicillium sp.	155 (89%)	*P. roqueforti*	108 (62%)
		P. verrucosum	47 (27%)
Aspergillus sp.	20 (11%)	*A. flavus*	20 (11%)
Total	175	**Total**	175

Table 2. Mold species and total aflatoxin and AFM1 concentrations in moldy cheese samples

Mold species	Positive number of samples (n=100)	Number of total AF positive samples	Number of AFM1 positive samples	Total Aflatoxin level (ng/kg)			AFM1	Limit according to TFC (> 50 ng/kg)	Range of AFM1 of positive Samples (ng/kg)		
				X±SE	Min	Max			X±SE	Min	Max
P. roqueforti	100 (100%)		-								
P. roqueforti + *P. verrucosum*	83 (83%)	-	16 (16%)	4120.6±699.4	687.1	8273.5	28 (%28)	5(5%)	36.58±1.6	5.46	141.56
P. roqueforti + *A. flavus*	17 (17%)	15 (%15)	12 (12%)								

X±SE: Mean±Standart Error

In this study,*Penicillium* spp., especially*P. roqueforti,* was the dominant moldstrainin the cheese samples similar to previously other studies (Kivanc, 1992;Gobbetti et al., 1997; Erdogan et al., 2003; Montagna et al., 2004;Hayaloglu andKirbag, 2007;Fernández-Bodega et al.,2009; Cakmakci et al., 2012). Although several species of the genus Penicillium play the main role in the ripening stage of cheese production as a starter culture, these organisms can also cause cheese spoilage resulting in significant economic losses (Kivanc, 1992; Lund et al., 1995; Erdogan et al., 2003; Hayaloglu and Kirbag, 2007).*P. roqueforti* is the major ripening culture in some cheeses such as Roquefort, Stilton, and Gorgonzola (Gripon, 1987; Fox et al., 2017)ensuring the formation of crumbly texture and small air holes in moldy cheese. However, it might also be a spoilage and mycotoxin producer agent, especially for PR toxins and patulin, in some other cheeses (Panelli et al., 2012). Nevertheless, some other earlier studies (Aran and Eke, 1987; Kivanc, 1992; Larsen et al., 2001) have reported *P. verrucosum* as the dominant flora which causes mold growth on the cheese surface and has the ability to produce mycotoxins such as ochratoxin-A and citrine.

Aspergillus flavus contamination, the producer of highly toxic and carcinogenic aflatoxins, has also been reported in several previous studies in cheese (Barrios et al., 1997; De Santi et al., 2010; Baranyi et al., 2015).On the contrary, Aran (1993) reported *A. flavus* or *A. parasiticus*contamination was not found in tulum or civil cheeses.Mycotoxin producing fungi are regarded to be among the most important contaminants in foods from the point of public health, food safety, and the economy, as mycotoxins are resistant to industrial processes (Pitt, 2000; Abdel-All et al., 2008; Zhang et al., 2013).

Compared to our results, Ozgoren (2012) reported higher values with the mean contamination level of total AFas 6896.73ng / kg, ranging from 3148.11 to 13603 ng /kg in moldy cheese samples. Moreover, in a study presented by Abdel-All et al. (2008) the content of AF B1-B2, G1 and G2 of 126 cheese samples were 3100-13000 ng/kg, 2000-12000 ng/kg and 2300-12000 ng/kg, respectively. Abd Alla et al. (2000) found AFB_1 and G_1 in only one cheese sample at levels of 10000 ng/kg and 4000 ng/kg, respectively. On the contrary, Guley et al. (2013) reported no total AF in the tested moldy cheese samples. In this study, it is remarkable that *A. flavus* was isolated from total AF-contaminated cheeses.The contamination of total AF might be associated with the growth of *A. flavus* on the surface and inside the cheese during the ripening period.No limit values have been determined for total AF in cheeses in TFC. However, the results of this study and other previous studies (Abd Alla et al., 2000; Abdel-All et al., 2008; Ozgoren, 2012) show that consuming these cheeses, contaminated with AF, may pose a potential public health concern. Therefore, it is important to standardize the moldy cheese production process using non-toxic strains as starter

cultures to prevent contamination of unwanted mold species and the formation of mycotoxins in moldy cheese.

Very limited data are available on the AFM1 contamination of moldy cheese. In this study, the analyzed AFM1 incidence of moldy cheese samples were lower than that reported by Ozgoren and Seckin (2016) who detected AFM1 in 52 % of moldy cheese samples with concentrations ranging from 10.61 to701.54 ng/kg. In contrast, Guley et al. (2013), Kokkonen et al. (2005) and Fontaine et al. (2015) found no AFM1 in the analyzed moldy cheese samples. The difference in AFM1 concentrations in cheese in other previous studies may be related to various reasons including contamination levels in milk, cheese manufacturing procedures, type of cheese, condition of cheese ripening, different geographical regions, sampling methods and the analytical methods employed (Galvano et al., 1996; Bakirci,2001; Nakajima et al., 2004).

CONCLUSION

In this study,the dominant mold species in moldy civil cheese samples analyzed was *P.roqueforti* followed by *P. verrucosum* and *A. flavus*.Considering the mycotoxin producing capability of these species and the presence of *A. flavus*in aflatoxin determined cheeses, it could be concluded that moldy cheese might pose a public health concern as these cheeses are produced and ripened under uncontrolled conditions without using any pure culture in Turkey. Thus, to protect consumer health, further studies areneeded to determine whether these mold isolates are toxic or nontoxic. Moreover, considering the preventive measures be taken for AFM1 in cheeses, the best way to protect the consumer against AFM1 induced health hazards is the monitoring andavoiding AFB1 contaminationin feed due totheclose relationship between the AFM1 concentration in milk products and AFB1 concentration in the feed.

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CONFLICT OF INTEREST STATEMENT

The authors declared that have no conflicts of interest with anyone

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