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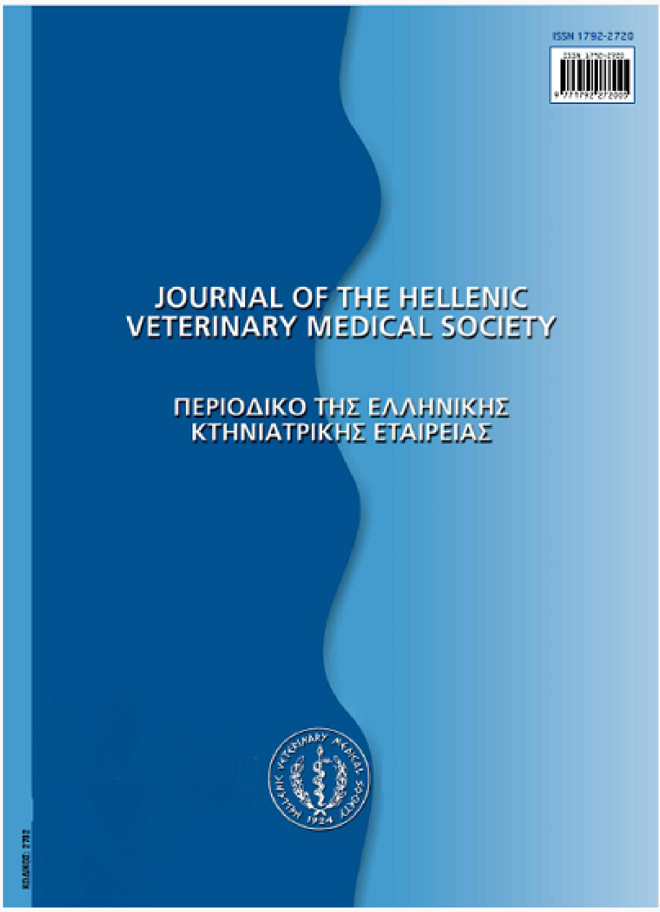

Occurrence of Aflatoxins in compound feeds and feed materials for dairy livestock in Central Greece

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Occurrence of Aflatoxins in compound feeds and feed materials for dairy livestock in Central Greece.

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Παρουσία Αφλατοξινών σε πλήρεις σύνθετες ζωοτροφές και πρώτες ύλες ζωοτροφών για γαλακτοπαραγωγά ζώα στην Κεντρική Ελλάδα.

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ABSTRACT. Aflatoxins (AFs) in feeds are considered as serious hazards for livestock health and additionally they pose a risk for consumers' health, especially when contaminated milk is consumed. Since AFs toxicity and stability during feeds and food processing is well established, this study aimed to determine the level of AFs contamination in compound feeds, feed materials and silage, intended for feeding dairy livestock, in central Greece. Out of 274 feed samples analyzed with Enzyme Linked Immunosorbent Assay (ELISA), in 132 (48.17%) AFs were not detected, while 39 (14.23%) were found above the EU maximum tolerable limit of 10 μg kg^{-1}. The levels of AFs contamination ranged from 0.6 to 45.2 μg kg^{-1}. It was found more possible for corn to be contaminated (p-value<0.001). The relatively high incidence of AFs in feeds and especially in corn was verified in this study, something that is line with the view that Mediterranean countries are in high risk for AFs. As there are several factors affecting the level of AFs contamination, constant monitoring of AFs in feeds is required in order to prevent adverse effects on animals and additionally to act as a precaution measure for the occurrence of AFM1 in milk.

Keywords: *Aflatoxin, corn, compound feed, dairy livestock, feeds, feed materials, risk factors*

ΠΕΡΙΛΗΨΗ. Οι αφλατοξίνες στις ζωοτροφές αποτελούν σοβαρό κίνδυνο για την υγεία των ζώων και επιπλέον θέτουν σε κίνδυνο την υγεία των καταναλωτών από την κατανάλωση γάλακτος επιβαρυμένου με αφλατοξίνη. Δεδομένου ότι η τοξικότητα και η σταθερότητα των αφλατοξινών κατα την επεξεργασία ζωοτροφών και των τροφίμων είναι γνωστή, η παρούσα μελέτη είχε ως σκοπό την αποτύπωση των επιπέδων των αφλατοξινών σε συμπυκνωμένες ζωοτροφές, πρώτες ύλες για ζωοτροφές και σε ενσιρώματα αραβοσίτου, στην κεντρική Ελλάδα. Από τα 274 δείγματα ζωοτροφών που αναλύθηκαν με την ανοσοενζυμική

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μέθοδο Enzyme Linked Immunosorbent Assay (ELISA), στα 132 (48.17%) δεν βρέθηκαν αφλατοξίνες, ενώ στα 39 (14.23%) βρέθηκαν αφλατοξίνες εκτός του ευρωπαϊκά θεσμοθετημένου ορίου των 10 μg kg^{-1}. Τα ποσοστά των αφλατοξινών κυμαίνονταν από 0.6 έως 45.2 μg kg^{-1}. Το καλαμπόκι βρέθηκε να είναι στατιστικά σημαντικά πιο επιβαρυμένο με αφλατοξίνες (p-value<0.001) σε σύγκριση με τις υπόλοιπες ζωοτροφές. Η σχετικά υψηλή παρουσία αφλατοξινών στις ζωοτροφές στην Ελλάδα και ιδιαίτερα στον αραβόσιτο επιβεβαιώθηκε στην παρούσα μελέτη, δεδομένο που συνάδει με την άποψη ότι οι Μεσογειακές χώρες είναι χώρες υψηλού κινδύνου για τις αφλατοξίνες. Δεδομένου ότι υπάρχουν πολλοί εμπλεκόμενοι παράγοντες κινδύνου που επηρεάζουν τα ποσοστά των αφλατοξινών στις ζωοτροφές, απαιτείται συνεχής επιτήρηση των επιπέδων τους στις ζωοτροφές ώστε να προληφθούν οι δυσμενείς επιπτώσεις στην υγεία των ζώων και παράλληλα να λειτουργήσουν ως μέτρο πρόληψης εμφάνισης αφλατοξίνης Μ1 στο γάλα.

Λέξεις ευρετηρίασης: Αφλατοξίνες, γαλακτοπαραγωγά ζώα, ζωοτροφές, αραβόσιτος, συμπυκνωμένες ζωοτροφές, παράγοντες επικινδυνότητας, πρώτες ύλες ζωοτροφών

INTRODUCTION

Milk can become hazardous for humans when contaminated with Aflatoxin M1 (AFM1), which occurs when lactating animals are fed with feed contaminated with Aflatoxin B1 (AFB1). AFB1 is a secondary metabolite produced by *Aspergillus flavus, Aspergillus parasiticus* and less often by other *Aspergillus* species, under specific temperature and humidity conditions and most regularly develops during feed storage (Bennet and Klich, 2003). Monitoring of AFs concentrations in feed may act as a precautionary measure for the occurrence of AFM1 in milk and therefore indirectly safeguard consumer health.

Aspergillus species can grow on livestock crops and aflatoxins can be produced pre- or post –harvest, depending on the climatic conditions, the type of feed, the plant variety resistance, possible insect damage of the crops, the storage conditions, the season, the use of phyto-protective substances, the microbial competition (Hussein and Brasel, 2001; Ruiquian et al., 2005; Kensler et al., 2011; Malissiova et al, 2013). AFs contamination may occur in all kinds of compound feed and feed materials, while corn and silage has been mainly associated with high AFs contamination levels (Bennet and Klich, 2003; Bryden, 2012). When AFs levels in feed exceed the European regulated maximum limit (10μg kg^{-1}) (EC, 2006) adverse health effects may be present in animals, including reduced growth rate, immunodeficiency, infertility (Robens and Richar, 1992; Streit et al., 2013), while there is simultaneously concern for excretion of the AFB1 hydroxylated metabolite, AFM1, in milk in a great proportion (0.20%-6.20% for cows, 0.03-0.25% for sheep and 0.40% for goats) (Rao and Chopra, 2001; Battacone et al., 2003; Ozdemir, 2007; Romero et al., 2010). Toxicity of AFs is well established, affecting animals and humans, ranging from potent carcinogenicity to genotoxicity (Hussein and Brasel, 2001; Marin et al., 2013).

AFs in feeds and milk is an issue of constant concern, especially for South European countries, including Greece, while the incidence of AFs contamination in feeds in Europe is reflected by more than 350 notifications in the Rapid Alert System for Food and Feed for the last decade, including mainly feeds originating from Mediterranean and Middle East countries (RASFF, 2014). As environmental conditions may favor the occurrence of AFs in feed and food, there are specific high risk geographical areas already indicated in the literature, including Greece (Garcia et al., 2009; EFSA, 2012).

Taking into consideration AFs' toxicity and stability during feed and food processing (Hussein and Brasel, 2001; Bryden, 2012), it is necessary to constantly monitor AFs levels in all feeds, in order to safeguard animal health and prevent AFs occurrence in food of animal origin, especially milk. The most accurate quantitative method used for AFs determination is high performance liquid chromatography (HPLC) (Shephard et al., 2012). Thus, this is a costly and complex analytical approach, especially for farmers' and industry's self control. Therefore, enzyme-linked immunosorbent assay (ELISA) has been recently become a popular method for AFs detection (Goraycheva et al., 2009).

As Greece is at high risk for AFs occurrence in feeds and additionally in need of constant surveillance data that may act a precautionary tool, this study aimed to determine the aflatoxins burden in feed for dairy cattle, sheep and goats produced and marketed in Central Greece.

MATERIALS AND METHODS

Area Description – Sampling

The study was carried out in the region of Thessaly, central Greece, in collaboration with 53 dairy farms (sheep, goats and cattle). The study area is a predominantly rural area with substantial livestock population (16 % goats and sheep and 12 % cows of the total Greek livestock) and feed production (45% compound feed and 22% raw feed materials of the total Greek feed production) (Hellenic Ministry for Rural Development and Food, 2014). The inclusion criteria for the farms were: supplying their milk to the biggest dairy industry in Western Thessaly, having a livestock population of at least 50 animals for cattle and 150 for sheep and goat farms and finally receiving their feed exclusively from the two Unions of Agricultural Cooperatives existing in the area. For four consecutive years (2010-2013) a total of 274 samples of feed materials, silage and compound feeds, of 1000g each, were randomly collected, according to the European Feed Sampling Regulations (EC, 2009). The samples inclusion criteria referred to feed only domestically produced and just after harvest or production for silage.

Sample preparation

Five hundred grams' samples were initially ground (IKA-ANALYTICAL MILL A10, STAUFEN GERMANY), so that at least 75% of the ground material passed through a 20 mesh sieve. Fifty grams of ground sample were blended with 250ml of 70% methanol for HPLC (ACROS ORGANICS, NEW JEERSEY, USA) for 2 min in a high-speed blender (Primo 303, SHANGAI, CHINA). The extract was filtered by pouring 5 ml through a Whatman No1 filter and the filtrate was collected for analysis. For silage samples, drying at 105^{o}C (WTB BINDER, TUTTLINGEN, GERMANY) for 24h, preceded the whole sample preparation procedure.

Sample Analysis

All samples were analyzed with ELISA (VERATOX HS, NEOGEN CORPORATION, LANSING, USA), in duplicate, using a microplate photometer (NEOGEN CORPORATION, LANSING, USA) set at 650 nm to determine Total Aflatoxins. Samples were analyzed according to the kit's instructions. The detection limit was 0.5 μg l^{-1}. The quantification limit was 1 μg l^{-1}. AFs levels in samples were calculated with the use of a 5-level calibration graph, covering a 0-8 μg l^{-1} concentration range. For samples quantification above 8 μg l^{-1}, samples were respectively diluted.

Statistical Analysis

Data were analyzed using the statistical package SPSS (v.15). Fisher's exact test and Chi-square test were used for qualitative data analysis. Results were considered statistically significant when the p-value was ≤ 0.05.

RESULTS

Sample characteristics

Out of 274 samples collected, 220 (80.29%) were raw feed materials (including cottonseed, corn sunflower meal, barley, bran, beet pulp, wheat, soybean and alfalfa hay), 27 (9.85%) was compound feeds and 27 (9.85%) was corn silage.

Aflatoxins levels

Out of 274 samples analyzed, in 132 (48.17%), AFs were not detected, while 39 (14.23%) were found above the EU maximum tolerable limit of 10 μg kg^{-1}. The levels of AFs contamination ranged from 0.6 to

Table 1. Aflatoxin contamination frequency and levels in feeds for dairy animals

Years	Feeds for dairy animals			Aflatoxin levels μg kg^{-1}		
	n samples	n samples>10μg kg^{-1}	%	min	max	mean
2010	34	2	5.88	0.6	13.1	6.85
2011	81	9	11.11	0.6	35.8	18.2
2012	102	20	19.61	0.6	33.8	17.2
2013	57	8	14.04	0.8	45.2	23.0
Tota l	274	39	14.23	0.6	45.2	22.9

45.2 μg kg-1. The frequency distribution and the levels of Aflatoxins per year are presented in Table 1.

Aflatoxins levels and associated risk factors

Several associated risk factors, namely type of feed, feed commodity, seasonality and year were evaluated in relation to the levels of aflatoxins contamination (Table 2). Comparing the type of feed in the 39 samples found to exceed the regulated limit, it was apparent that raw material 37/39 (94.87%) were the most heavily contaminated (p-value=0.053), while only one compound feed sample and one sample of corn silage were found above the tolerable EU limit. Out of the 37 samples of raw feed material found above the EU tolerable limit, 36 were corn (97.29%) and 1 was cottonseed (2.71%). Comparing AFs occurrence in corn to other feed commodities there was significant statistical difference found (p-value<0.001). Twenty-six of the total samples found to exceed the maximum level (39) were collected during summer months (66.66%), while 13 were collected during winter months (33.33%) without this difference being statistically significant (p-value=0.202). As presented in Table 1 there was a variation in the incidence of AFs over the maximum limit during the 4 years of this survey, without though a statistically significant difference (p-value=0.171). Nevertheless, during 2012 the highest incidence was note (19.61%).

DISCUSSION

AFs occurrence in feeds in Europe is a critical issue for livestock health and for food safety as reported in official data and research surveys (Streit et al., 2012; RASFF, 2014). In this study, Total Aflatoxins in compound feeds, feed materials and silage, over the 10**μg** kg^{-1} EU regulatory limit, was found at 14.23% of the samples analysed, results that exceed previous published data for Greece, referring to 3.80% in 2004 and 7.04% in 2010 (Vlachou et al., 2004; Streit et al., 2012), but are closely related to those recently published by Tsiplakou et al. (2014). This difference could be attributed to a series of reasons such as the year/season that the survey was conducted, something that is is closely related to the climatic conditions, the number of samples tested, the representativeness of the sampling, the inclusion of different feed commodities, the sampling procedures and also the type of the instrumental analysis conducted for the AFs determination. Nevertheless, it is not expected to reveal similar incidence per year, as the levels of AFs vary based on many associated risk factors such as the climatic conditions, the season, the crop variety used, the storage practices followed (Hussein and Brasel, 2001; Ruiquian et al., 2005; Malissiova et al, 2013). There are several reports for AFs occurrence in various feeds, obtained by using ELISA, revealing a wide range of contamination levels; indicatively 60% in Turkey, 40% in Brazil, 13.1% in India, and 38% in Romania (Tabuc et al., 2011; Ratnavathi et al., 2012; Kocassari et al., 2013; Queiroz et al., 2013). The range in occurrence levels reported may be due to the different years of surveys that are closely related to different climatic conditions (Hussein and Brasel, 2001).The fact that 37.6% of the samples tested were found with AF over the limit of detection, just after harvest, but below the EU maximum level implies that is closely related to feeds that might become hazardous especially during future storage. The timely detection and quantification of the AFs levels in feeds may act as an alert for farmers, in order to prevent increase over the tolerable limit. In any case, feed official control system in Greece reported a relatively high compliance to EU limits in the majority of samples tested in 2010 (5/71) (Streit et al., 2012). In this study, corn was found to be significantly associated to AFs contamination, while Vlachou et al. (2004) found maize grain and cottonseed meal to be contaminated over the tolerable limit and Tsiplakou et al. (2014) report that AFs were mainly found in cottonseed cake, maize and alfalfa hay. Additionally, in the last decade there were 4 notifications in RASSF for AFs in feeds (3 in cottonseed and 1 in maize) originating from Greece (RASFF, 2014). There is variability in feeds being mostly affected over the years, possibly due to differences in varieties, cultivating and pre and post harvest practices. Nevertheless, the necessity for constant precautionary monitoring is apparent based additionally on global surveys that present a general peak of AFs in feeds during 2012-2013 with 59% prevelance in Asia and 55% in Southern Europe (BIOMIN, 2014), that is closely related to the results of the present study.

Taking into consideration that Greece is a country at risk for Aflatoxins (Magan et al., 2011; EFSA, 2012), it was considered valuable to assess the level of AFs in feeds produced and marketed within the country. As AFs occurrence in feeds is closely related with the excretion of AFM1 in milk, it is noteworthy that during the specific sampling period, there were reports in Thessaly for 1.7% (n=4/324) of goat and sheep milk samples tested to present AFM1 above the tolerable limit (Malissiova et al., 2013), while the official reports from 2010 to 2013 refer to 0 to 4.1% AFM1 contamination of milk in Greece (EC, 2014). Thus, by the end of 2011 there was a yoghurt recall for AFM1 in the region of Thessaly (EFET, 2011), implying that probably there was a peak in AFs contamination during that period, something that is reflected by our results as well. The variability in the occurrence of AFs in feeds and consequently in milk is expected, as there are several associated factors with the presence of AFs.

In this study certain risk factors (year, season, feed commodity and the feed type) for the AFs presence in feeds, over the tolerable limit were investigated with univariate analysis. Corn, barley, wheat, oat, rye and cottonseed are considered as feed commodities of high risk for AFs (Creppy; 2002; EFSA, 2012; Streit et al., 2012), while our results presented statistically significant difference only for corn

Table 2. Aflatoxins levels in feeds and associated risk factors

		<10 μg kg-1		>= 10 μg kg-1		p-value
Risk Factor	**Parameter**	**n**	**%**	**n**	**%**	13
Year	2010	32	94.1	2	5.9	0.171*
	2011	72	88.9	9	11.1	
	2012	82	80.4	20	19.6	
	2013	49	86.0	8	14.0	
Season	Summer	131	83.4	26	16.6	0.202**
	Winter	104	88.9	13	11	
Feed Commodity	Corn	146	80.2	36	19.8	**<0.001****
	Other	89	96.7	3	3.3	
Feed Type	Raw material	183	83.2	37	16.8	0.053**
	Compound feed	26	96.3	1	3.7	
	Silage	26	96.3	1	3.7	

** Chi-square Test*

*** Fisher Exact Test*

(p-value<0.001). In relation to the type of feed (feed material, compound feed and silage) there was a marginal non significant statistically significant difference (p-value=0.053) noted for feed material, that have been previously reported as of high risk for AFs occurrence in comparison to other feed types (Streit et al., 2012). When comparing seasonality of AF occurrence over the tolerable limit, there was no significant statistical difference found, while it was noted that during summer months there was excess in AFs occurrence (66.66%) that could be attributed to the high average temperatures (28^{o}C) and very low rainfall (less than 1 cm) during April to October in Greece (Weather and Climate, 2014). Comparing the results during the 4 years of the study, there was no statistical difference noted, while during 2012 the highest incidence was note (19.61%) and this could be due that 2012 in Greece, was a year with high temperatures during summer months, extremely law rainfall and extended dry periods (National Observatory of Athens, 2014), parameters that support the higher incidence of AFs in livestock crops during the specific year. Thus, there are several reports relating AFs occurrence to the climatic conditions, seasons and years (Thompson and Henke, 2000; Cotty and Jaime-Garcia, 2007), as these are considered to significantly affect the production of AFs on livestock crops.

With reference to the method used for the AFs determination, ELISA is considered as an efficient method for screening purposes (Shephard et al., 2012; Coda-Cerva, 2013). The specific ELISA kit used in this survey has been recently comparatively assessed and reported to posses the selectivity and sensitivity to adequately screen feed samples for AFs (Coda-Cerva, 2013).

A possible limitation to be noted regarding this study is the restricted geographical area that the study was conducted that may not be necessarily representative for other areas. Nevertheless, this area is considered appropriate for assessing AFs levels initially due to the significant livestock and feed proportion it presents but also because of the inclusion criteria used in this study to select the sample. With regards to the sample size, the appropriate statistical tests have been used to minimize the possible impact of the relatively small sample size. Future studies could possibly address the specific limitations and focus on a systematic risk assessment of AFs in feeds, especially by the use of prediction modelling, taking into consideration all relevant risk factors.

CONCLUDING REMARKS

This study assessed the AFs level in compound feeds, feed materials and silage, intended for feeding dairy livestock, in central Greece. Out of 274 feed samples analyzed 39 (14.23%) were found above the EU maximum tolerable limit of 10 μg kg^{-1}. The levels of AFs contamination ranged from 0.6 to 45.2 μg kg^{-1}. It was found more possible for corn to be contaminated in comparison to other feeds. As there are several factors affecting the level of AFs contamination, such as climatic conditions, seed quality/resistance, pre, post harvest and storing conditions constant monitoring of AFs in feeds is required in order to prevent adverse effects on animals and additionally to act as a precaution measure for the occurrence of AFM1 in milk.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

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