Semina: Ciências Agrárias
ISSN: 1676-546X
semina.agrarias@uel.br
Universidade Estadual de Londrina
Brasil

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Seasonal milk contamination by aflatoxin m1, organophosphates and carbamates in Paraná – Brazil
Semina: Ciências Agrárias, vol. 37, núm. 4, julio-agosto, 2016, pp. 2145-2153
Universidade Estadual de Londrina
Londrina, Brasil

DOI: 10.5433/1679-0359.2016v37n4p2145

Seasonal milk contamination by aflatoxin m1, organophosphates and carbamates in Paraná – Brazil

Sazonalidade na contaminação do leite por aflatoxina M1, resíduos de organofosforados e carbamatos no Estado do Paraná

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Abstract

Aiming to evaluate the milk contamination in the dairy production systems (DPS) for mycotoxins and chemical residues of organophosphates and carbamates it was made a study encompassing 96 DPS in three regions of Parana state. There were collected samples of milk, water and food and they were evaluated for chemical residues in all samples and aflatoxin only for food and milk. Mycotoxins in food (aflatoxin B1, B2, G1, G2, zearalenone and ochratoxin) were detected by the method of thin layer chromatography – TLC and for the determination of aflatoxin M1 was used an immunoassay kit competitive ELISA Ridascreen®. The residues of organophosphates and carbamates were performed by colorimetric method qualitatively. There were evaluated the differences between regions, periods and the sources of mycotoxin contamination. Carbamates and organophosphates were screened for their presence in milk and the sources of food and water. Then it was estimated the contributions of each mycotoxin for milk contamination, as well as their respective contaminated food. Differences were found between periods ($p < 0,05$) for milk contamination with aflatoxin M1 – AFM1. For carbamates and organophosphates were found different contamination sources ($p < 0,01$). For the carbamates the source were pesticides used to parasitic herd control and for the organophosphates pesticides used in agriculture. For food sources contamination resulting in the AFM1 contamination it was detected that aflatoxin B1 – AFB1 was the main source. The aflatoxin G1 – AFG1 showed a strong correlation ($p < 0,01$) with AFB1 levels suggesting causal relationship is a function of fungal strains producing both at the same time. It was also found the prevalence of aflatoxin contamination in 70% of contaminated samples and its predominant presence in relation to other mycotoxins in all kinds of foods analyzed. By identifying the checkpoints of contamination can be proposed the inclusion of practical management methods to avoid this.

Key words: Dairy production. Food safety. Mycotoxins. Feedstuffs.

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Recebido para publicação 10/05/15 Aprovado em 26/04/16

Resumo

Objetivando avaliar a contaminação por micotoxinas e resíduos de organofosforados e carbamatos em Sistemas de Produção Leiteira (SPL) foi realizado um estudo em 96 SPL em três regiões do Paraná. Foram colhidas amostras de leite, água e alimentos para a avaliação dos resíduos dos pesticidas, mas para aflatoxinas apenas foram avaliados o leite e os alimentos. As micotoxinas nos alimentos (aflatoxinas B1, B2, G1, G2, Zearalenona e Ocratoxina) foram detectadas por meio da cromatografia de camada delgada. Para a determinação da aflatoxina M1(AFM1) foi utilizado kit de imunoafinidade competitiva (ELISA) Ridascreen®. Os resíduos de organofosforados e carbamatos foram determinados por método colorimétrico qualitativo. Foram avaliadas as diferenças entre as regiões, períodos do ano e fontes de contaminação por micotoxina. A presença de carbamatos e organofosforados foi rastreada no leite, alimentos e água. A contribuição de cada micotoxina para a contaminação do leite foi estimada, bem como a fonte alimentar mais provável. Foram encontradas diferenças ($p< 0,05$) entre os períodos sazonais para a contaminação do leite por AFM1. Para carbamatos e organofosforados foram identificadas fontes de contaminação distintas ($p < 0,01$). As fontes para os carbamatos foram os pesticidas utilizados no controle de parasitos do rebanho, entretanto para os organofosforados foram os pesticidas utilizados na agricultura. A contaminação por AFM1 foi observada no leite em SPL cujos alimentos continham AFB1, portanto esse metabólito foi a maior fonte de contaminação. Foi observada forte correlação ($p < 0,01$) entre as concentrações de AFG1 e AFB1, o que sugere uma relação causal, provavelmente em função da mesma linhagem de fungos produzirem ambos os metabólitos ao mesmo tempo. Foi detectada uma prevalência de aflatoxinas em 70% das amostras contaminadas, em relação às demais micotoxinas, para todos os tipos de alimentos analisados. Pela identificação dos pontos críticos de contaminação por micotoxinas podem ser propostos métodos práticos de manejo para evitá-la nos SPL.

Palavras-chave: Produção leiteira. Segurança alimentar. Micotoxinas. Alimentos.

Introduction

Food contamination is a current and thoroughly discussed issue, involving aspects such as public health (CALDAS; SOUZA, 2000) and animal production (FAGAN et al., 2010). Milk and dairy products have been given special attention, due to the increase in the demand of these products associated with the mounting concern for food safety (FAO, 2008). Milk's main contaminating agents are of microbiological (BRASIL, 2002), biological synthesis – such as mycotoxins (PARAMITHIOTIS et al., 2009) – and chemical nature, the latter represented by pesticides and varied chemical residues (OKADA et al., 1997; FLORES et al., 2004).

Factors related to the source of food contamination are commonly associated with agricultural and animal husbandry production processes, such as: the use of pesticides in crops; parasite control in livestock; food storage (mycotoxins); and processing and transportation of both milk and feeds (IHESHIULOR et al. 2011).

It is widely acknowledged that the results obtained in milk production systems derive from the system's characteristics, specially: handling practices, equipment, installations, workforce and livestock (DEDIEU et al., 2008; HOSTIOU et al., 2006). Bodenmüller Filho et al. (2010) drew said conclusion, in a study in Paraná with a considerable number of Dairy Production Systems (DPS), which demonstrated a diversity of results related to the qualitative and quantitative aspects of milk. Therefore, the characteristics of milk, whether positive or negative, are accrued throughout its production process.

One of the most influencing factors in the quantity (FORBES, 1995), quality (JAHREIS et al., 1997) and distribution of milk production throughout the year, (MOULIN, 2006) is the feed given to livestock.

The intra-annual variation of feeds, whether in quantity or quality, is a practical reality in dairy life, demonstrated by the farmers' efforts to balance livestock demand with feeds supply in the property

(MOULIN, 2006). One of the most widespread strategies employed by farmers is the storage of dry or wet grains and forage: hay and silage. However, when concerning the contamination by mycotoxins, storage can suffer from a few drawbacks, given the proliferating nature of fungi (Genera: *Aspergillus, Penicillium e Fusarium*) within the stored feeds. Not only storage, but also farming and cultivation requirements of grains are determinant for contamination by mycotoxins (WAN NORHASIMA et al., 2009) and pesticides (FLORES et al., 2004).

A correlation between seasonal livestock feeding practices, (MOULIN, 2006),and the regional nature of milk production in Parana, IPARDES (2009), is presumed to exist; both are determining factors in milk contamination.

Taking into account the seasonal character in animal feeding, in addition to grain production, this study aims to determine the effects of region and season on milk contamination by aflatoxin M1, and pesticide and endectocide residues of the carbamate and organophosphate groups.

Material and Methods

The study was performed during the crop year of 2009/2010, in three regions of Paraná's state , Brazil: (1) Northwest region, bounded between: 23º00" and 23º30" south; 51º30" and 52º30" west; Cfa climate and predominantly Ferrasol and Nitosol soils containing 39 DPSs; (2) Southeast region, bounded between the coordinates: 24º00" and 24º30" south; 49º30" and 50º00" west, Cfb climate and predominantly Ferrasol and Cambisol soils containing 32 DPSs and; (3) Southwest region, bounded between: 24º30" and 25º00" south; 53º30" and 54º06" west; Cfa climate and predominantly Ferrasol and Nitosol soils containing 25 DPSs. All regions provided a total of 96 DPSs. When choosing these regions, it was take into consideration how these areas represent the local state production of milk (IPARDES, 2009). During the sampling of DPSs, the most representative factories, in terms of the quantity of milk collected, were selected in each region.

Sampling of concentrates, fodder, conserved grains, water and milk was performed between May and August 2009 and, subsequently, between October 2009 and April 2010. Interviews with dairy farmers were undertaken between May 2009 and September 2010, according to their availability. Samples of feed supplied to livestock were refrigerated immediately once collected, and then kept in a freezer at –20ºC until analyzed. Milk and water samples were collected and stored in the same manner. Subsequently, milk samples were subject to plasma extraction for aflatoxin M1 reading in the Food and Animal Nutrition Laboratory at the State University of Maringá – UEM.

For the detection of carbamates and organophosphates in milk, feeds and water, we used thin layer chromatography (TLC) with rhodamine and p-nitroaniline staining, a qualitative method described in AOAC (2003). Analyses were performed in the Veterinarian Toxicology Laboratory at the State University of Londrina.

For the detection of mycotoxins in feeds, we used a TLC technique described by Soares and Rodriguez-Amaya (1989) and adapted by Gimeno (1983). The reference standards and concentrations used for AFB1, B2, G1, G2, Ochratoxin and Zearalenone (Sigma Inc. – USA), were: 2.55; 2.62; 2.45; 4.55; 10.0 and 100.0 $\mu g.mL^{-1}$, respectively. Detection limits for this method were 2.0; 5.0 and 260.0 $\mu g.Kg^{-1}$ for aflatoxins, ochratoxin and zearalenone, respectively.

For plasma extraction, milk samples were defrosted in a water bath at 40ºC for 30 minutes and immediately centrifuged for 10 minutes at 6700 rpm in a refrigerated centrifuge at 10ºC. Subsequently, aliquots were obtained from the intermediate phase. The fat content present in the supernatant was removed, and transferred with a pipette to eppendorf tubes, later identified and stored at 8ºC for further analysis.

Milk samples were analyzed with R-biopharm®'s RIDASCREEN® FAST Aflatoxin M1 immunoenzymatic kit. Said kit comprises a microplatecontaining wells, covered with anti-IgG antibodies; 5 standard solutions of AFM1 (0; 250; 500; 1000 and 2000 ηg.L^{-1}), containing the IgG polyclonal anti-AFM1 conjugated antibody; enzymes and the blocking solution.

Reading was performed on a spectrophotometer at a wavelength (λ) of 450 ηm, and results were expressed by the mean of values observed for each duplicate sample. Absorbances were calculated as follows:

$$A_\lambda = \left(\frac{A_i}{A_{0\,ppt}}\right) * 100$$

Where:

A = absorbance at λ = 450 ηm;

A_{0ppt} = absorbance for standard solution "0" (100% absorbance, 0 ppt of AFM1);

A_i = sample absorbance (from *i* to *n*).

Absorbance results for each reading were converted to concentration (μg.L^{-1}) values through a standard curve, plotted for each assay, and supplied by the Softmax-pro® software, version 5.4. The analysis protocol was designed for competitive immunoassays – ELISA, read at each reaction endpoint, based on a melamine protocol (Softmax-pro 5.4).

Data analysis was performed with the aid of the R software, version 2.12.0 (R, 2011). Data was analyzed according to the following statistic model:

$$Y_{ijk} = \mu + R_i + P_j + RP_{ij} + e_{ijkl}$$

Where:

Y = observations associated to Region *i* for Period *j;*

μ= general constant, representing the mean of observations;

R = region, where *i* varies from 1 to 3;

P = seasonal period, where *j* varies between dry and rain;

RP = interaction between region *i* and period *j;*

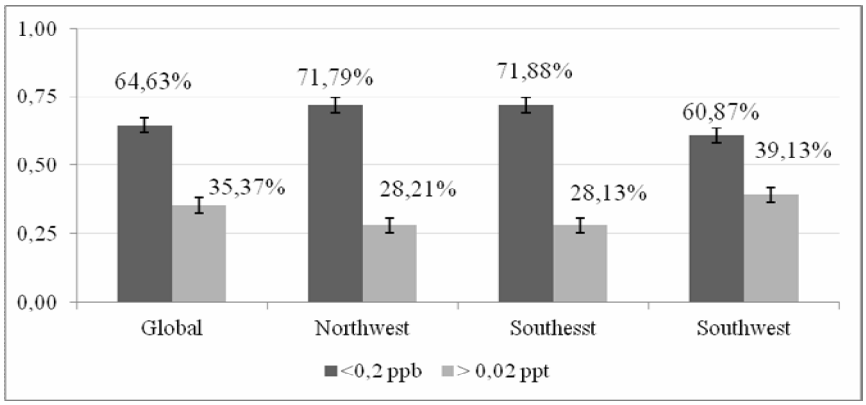
e = random error associated with the observations.

For the analysis, we used a general linear model (GLM) with a gamma distribution and *car* package for the R software, version 2.12.0. The variables of carbamate and organophosphate values found in milk and feeds were analyzed by GLM with a binomial distribution and with nonparametric methods based in contingency tables. Mean tests for AFM1 were performed by multiple comparisons ($p < 0,05$) with the assistance of the *multcomp* package (R, 2.12.0).

Results and Discussion

AFM1 milk contamination data from DPSs was compiled in Figure 1

Figure 1. Prevalence of AFM1 contamination in milk in Parana state.



Contamination values higher than the assay detection limit (around 0.2 ppb) were found in 35,37% of samples. Samples exceeding the national legislation limit (0,5 ppb) established by ANVISA (2006) – the National Health Surveillance Agency – comprised 4,84% of all samples in this study. Considering the limits adopted by countries such as Canada and the European Union, where the limit is 0,05 ppb (FAO, 2004), at least 64,63% of samples were above the tolerance level. Results for the independent variables tested on Aflatoxin M1 and their statistics are collated in Table 1.

Table 1. Estimates for aflatoxin M1 concentration for the seasonal periods and geographical region of Parana state using Generalized Linear Models (GLM).

	Mean of Factors				
n = 95	Northwest	Southeast	Southwest	PER1	PER2
μ	0,1673	0,1869	0,2164	0,2594[a]	0,1171[b]
Max	0,5650	0,4420	1,2370	1,2370	0,3480
Min	0,0180	0,0230	0,0100	0,0910	0,0100
Sd	0,1101	0,1115	0,2124	0,1650	0,0767
GLM	*Ns*	*ns*	*ns*	**	**

** Significant for p< 0,001. Different letters on the same row differ by Tukey's test (p < 0,001). Periods (PER1 and PER2) that describe seasonal feeding phases of the livestock, varying between dry (fall/winter) and wet (spring/summer). Sample number of Dairy Production Systems (*n*).

AFM1 levels did not present significant variation (p > 0,05) among the DPSs in the different regions, although the difference is significant between seasonal livestock feeding phases.

Considering international standards for AFM1 tolerance in dairy products, the studied regions fell short of international requirements from potential buyers such as the European Union. Considering market expansion expectations, Farina et al. (2005), milk exporting and the national intention to increase its market share in this area, AFM1 milk contamination is still an obstacle to be overcome.

Upon considering different seasonal periods, can be also considered different feeding periods, (MOULIN, 2006), where one period has a higher intake of concentrate feeds and conserved grains than the other. Results suggest contamination is a consequence of animal feeding planning in the DPSs.

Fagan et al. (2010) found differences between DPSs (from 0,180 to 0,628 ppb), although their approach did not involve sampling, rather, a case study. In this study, it is possible to understand individual variations that were not noticed between dairy farmer groups, demonstrated by a coefficient of variation higher than 60%.

The variation found between seasonal periods can be explained by two factors: cultivation treatments of grains (WAN NORHASIMA et al., 2009); and grain storage practices (IHESHIULOR et al., 2011). Both factors are closely related to annual feeding plans, in other words, the supply and demand management of feeds (DAMASCENO et al., 2008).

Oliveira et al. (2010), when studying DPSs in the region of São Carlos, SP, found higher levels of AFM1 for DPSs combining rations concentrates (based on corn and soy) with other oleaginous compounds (0,322 ppm) or with silage and citrus pulp (0,121 ppb) than in combination with silage alone (0,010 ppb). In the present study, particularly the southeast and southwest regions demonstrated combinations of oleaginous compounds with higher levels of concentrates, in which the southwest region indicated a higher variation between the

upper and lower limits for AFM1 levels, 0,100 and 1,2370 ppb, respectively.

The probability of feed contamination with organophosphates was higher in milk ($p < 0,05$), and no variation was observed for any other feed. Concerning carbamates, no variation was found among the studied feeds. The first dry period showed an increase in the presence of organophosphates when compared to the wet period. In Table 2 we present the interaction between feed, seasonal feeding periods and regions. The northwest and southwest regions were found to be more prone to milk contamination by organophosphate residues, which is not true for the periods, suggesting there are local causes for this type of contamination. In the case of carbamates, milk from periods 1 and 2 showed a higher propensity for contamination, particularly for period 1, having a 25% probability. Ration concentrates demonstrated to be influenced by period on organophosphate contamination. In order to determine the correlations between the most probable contamination sources and origins, we used nonparametric methods, depicted in Table 3. In milk, the presence of carbamates was not associated to the presence of organophosphates ($p<0,01$), indicating these come from various sources. Carbamates, according to Nero et al. (2007) are the basis of the available endectocides. Hence, in this study, their presence is likely related to the inadequate and misguided parasitic treatment given to the livestock. A variety of factors can contribute to this issue, including the disregard for withdrawal times and recommended dosages CALDAS; SOUZA, 2000). The organophosphate transference to milk through feeding is positively correlated ($\tau \leq 0,05$), although this hypothesis was not supported by the χ^2 test ($p> 0,05$).

Table 2. Probability of finding contaminants according to the binomial *probit* GLM model, for feed interaction between region (of Parana state) and feed period.

Feed	**Organophosphates**			**Carbamates**		
	Northwest	Southeast	Southwest	Northwest	Southeast	Southwest
Water	0,07[a]	< 0,01 [a]	0,03[a]	< 0,01 [a]	< 0,01 [a]	< 0,01 [a]
Concentrate	< 0,01 [a]	< 0,01 [a]	< 0,01 [a]	0,19[a]	0,21[a]	0,21[a]
Milk	0,35[b]	< 0,01 [a]	0,42[b]	< 0,01 [a]	< 0,01 [a]	< 0,01 [a]
Silage	0,03[a]	0,13[a]	0,06[a]	0,07[a]	0,34[a]	0,15[a]
	Per 1	Per 2	Per 3	Per 1	Per 2	Per 3
Water	< 0,01 [a]	< 0,01 [a]	< 0,01 [a]	< 00,1 [a]	< 00,1 [a]	< 00,1 [a]
Concentrate	0,17[b]	< 0,01 [a]	0,06[b]	0,22[a]	0,21[a]	0,19[a]
Milk	0,54[a]	0,35[a]	0,56[a]	0,25[b]	0,08[b]	< 0,01 [a]
Forage	0,04[a]	0,10[a]	-	0,22[a]	0,11[a]	-

Period (PER1, 2 and 3) are seasonal livestock feeding phases, varying between dry (Per 1 and 2 = fall/winter) and rain (Per 3 = spring/summer). Different letters on the same row differ by the χ^2 test with Bonferroni correction ($p < 0,05$).

Table 3. Effect of contamination sources by organophosphates (ORG) and carbamates (CARB) on the presence, or absence, of contaminants in milk.

Kendall rank correlation coefficient τ (Kendall's tau)					χ^2Test	*p* value
Statistics		ORG_{milk}	$CARB_{milk}$	ORG_{feed}		
$CARB_{milk}$	t	-0,320**	-	-	7,43**	0,0064
	n	66	-	-		
ORG_{feed}	t	0,234*	-0,03	-	2,09ns	0,1486
	n	64	64	-		
$CARB_{feed}$	t	0,058	-0,149	0,086	Ns	Ns
	n	63	63	63		
ORG_{water}	t	0,011	0,106	0,240*	18,39***	0,0000
	n	66	66	64		

*, **, *** significant at p <0,05; <0,01; <0,001, for Kendall's tau (t) and χ^2 (McNemar's chi-squared test),[a]Carbamates as a water contamination source were absent in all samples.

The effect of different periods on contamination may be associated to the rates of rainfall or evaporation of these compounds into the atmosphere, as observed by Rodrigues (2006). However, in this case, it is likely to be further associated to more intensive cultivation treatments caused by larger infestations by weeds and pests during the summer, as observed by Waquil et al. (2004), when studying seasonal periods and most frequent pests in corn crops.

The increase on milk contamination in regions with more intense agricultural activity, as in the northwest and southwest regions, suggests there is a connection between this activity and the contamination of the livestock feed sources.

According to Nero et al. (2007), who studied the influence of organophosphates and carbamates on milk contamination in four Brazilian states (MG, SP, PR and RS), in a total of 209 samples, 75,12% of samples were contaminated with organophosphates and around 74% with carbamates. Only around 6% DPSs were free of contaminants. Among our samples, 46,97% DPSs contained organophosphates and 24,2% DPSs contained carbamates, however the decontaminated DPSs presented a higher proportion, 33,3% milk samples.

In any case, two-thirds of contaminated DPSs is still a worrying finding for public health. Caldas and Souza (2000) conducted a study demonstrating many of the food sources ingested by man and animals have higher levels of contaminants, in many cases reaching 100% of the acceptable daily intake within only one type of food. Therefore, according to these authors, the combined consumption of various food sources, including milk, represents a risk to human health, leading to the chronic and cumulative ingestion of said contaminants. To further increase the discussion concerning the risk of milk ingestion, further studies are necessary to quantify these and other pesticide residues.

The data found in this study is consistent with the literature and ANVISA's (2006) estimates showing Brazil as one of the world's biggest consumer of pesticides, a worrying public health as well as environmental matter (AZEVEDO, 2011).

The increase on milk contamination in regions with more intense agricultural activity, as in the northwest and southwest regions, suggests there is a connection between this activity and the contamination of the livestock feed sources.

In the Netherlands, Valeeva et al. (2005) classified risks to food safety related to the chemical contamination of milk. The production process within the dairy farms was the most important factor (among 18 factors), accounting for 6,84% of the impact on milk safety, followed by the quality

of feeds (6,74%), determined through a survey with those involved in all sectors of the milk production chain. Efforts like these demonstrate there are feasible solutions for the implementation of food safety protocols, once numerous efforts have been made in the sense of promoting studies to elucidate the contamination problem.

Both contamination by chemical compounds (carbamates and organophosphates) and biotoxins (specifically in this study, the aflatoxins) cause endemic problems to public health and can lead to expressive economic losses, when considering international restrictions on importing Brazilian dairy products, and their correspondent safety (FAO, 2004).

Conclusion

Milk contamination by aflatoxin M1 varied according to seasonal feeding practices, related to the larger or smaller supply of concentrates and conserved grains, such as hay and silage. The presence of organophosphates in milk was associated with conserved grains and forage, attesting its origin to the pesticides used in crops. In the case of carbamates, they did not demonstrate the same origin, and could be related to products used for parasite control within the livestock. It is, therefore, necessary to quantify chemical residues in order to predict the real risks to human health, and their impact in the health of livestock in the DPSs.

Acknowledgments

The authors wish to thank the sponsors of this studyin publication CNPq/MAPA/SDA Nº 64/2008 –thematic line 2.

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