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Occurrence of Aflatoxin M1 and Risk Assessment in Bovine Milk from the Amazon Region

Luiz C. G. Diógenes[1], Ariane M. Kluczkovski[1], Abrames F. F. Góes[1], Samir de C. B. Pinto[1], Claudio F. Araújo[1] & Augusto Kluczkovski-Junior[2]

[1] Federal University of Amazonas, Av. Gen. Rodrigo Otavio, J. Ramos, 6200, Manaus, AM, Brazil

[2] Amazonas Surveillance Foundation, FVS-AM. Av. Torquato Tapajós, 4010, Manaus, AM, Brazil

Correspondence: Ariane M. Kluczkovski, Federal University of Amazonas, Manaus, AM, Brazil. Tel: 55-92-3305-1480. E-mail: ariane@ufam.edu.br

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Abstract

Aflatoxins (AFL) are fungal metabolites that can contaminate animal feed, causing toxic effects after consumption. Among the AFL, there are reports of the occurrence of aflatoxin M1 (AFM1) in milk, so the objective of this work was to evaluate the AFM1 content in bovine milk samples produced in the State of Amazonas-Brazil. Of the analyzed samples (*n*=30), 20% were found to be contaminated with AFM1 in the range of 0.093-0.320 μg/kg (Limit of detection= 0.0625 μg/mL). No sample exceeded current Brazilian legislation, but all exceeded the maximum European regulatory limit of 0.05 μg/kg. Risk analysis estimated a daily intake of 5.357×10^{-7} mg/kg body weight/day for men and 6.257×10^{-7} mg/kg body weight/day for women. The MOE result did not indicate concern in public health aspects. On the other hand, the occurrence of AFM1 in the state of Amazonas was observed in an unprecedented way and need further investigation including other dairy products.

Keywords: animal feed, mycotoxin, margin of exposure

1. Introduction

Mycotoxins are secondary metabolites produced by fungi that can affect the food production chain, and represent a danger to public health, as they are carcinogenic substances that can cause economic losses (Alshannaq & Yu, 2017; Yang et al., 2020). In this context, studies on the occurrence of mycotoxins are important for defining public policies and mitigation actions. The occurrence of toxigenic fungi and mycotoxin production occurs on a global scale and may be associated with poor practices, inadequate technologies, storage and transport conditions involved in production. Some foods such as tree nuts, fruits, cereals and feed are susceptible to contamination by mycotoxins and in this context aflatoxins (AFL) are intensively studied. AFL are produced by fungi, mainly *Aspergillus flavus* and *Aspergillus parasiticus* species (Agriopoulou et al., 2020; Pankaj et al., 2018). In mammals, ingestion of aflatoxin B1 (AFB1) leads to its hydroxylation and production of the metabolite aflatoxin M1 (AFM1), which can be excreted in the mammary glands of lactating animals or through the urine (Hasninia et al., 2022; Kortei et al., 2022). Milk is an important source of macro and micronutrients and is one of the six most important products in Brazilian agriculture, playing an important role in food, source of income and job creation, with the state of Amazonas-Brazil acquiring 9536 liters in the year 2020 and produced 43,768 thousand liters in 2021 (IBGE, 2022; IBGE, 2020; Gonçalves et al., 2018). Studies evaluating the occurrence of AFM1 in bovine milk in its various forms (whole, skimmed, semi-skimmed, liquid and powder) have been carried out in several countries such as Malaysia and China (Li et al., 2017; Shuib et al., 2017) and also in Brazil, especially in the southern region (de Freitas et al., 2018). Based on the cited studies, the need to assess the occurrence of AFM1 in milk is a global need (Min et al., 2020). According to Sirma et al. (2018) the incidence of contamination of milk and its products by AFM1 is higher in developing countries due to their lower regulatory standards. Considering that in Brazil, especially in the North region, there is little data on contamination by AFM1 in food, the objective of this work is to contribute with data in the analysis of risk of consumption in the population and preventing how much to the occurrence of diseases caused by AFL.

2. Method

2.1 Samples

Thirty samples of bovine milk produced in the state of Amazonas-Brazil were collected, in original packaging from 250 ml to 1L. The samples came from retail and directly from dairy producers in the municipalities of Autazes and Apuí regions in the state of Amazonas and stored until the moment of analysis.

2.2 Reagents and Standards

Mobile phase: ultrapure water obtained from the Simplicity® Water Purification System; acetonitrile and methanol High Performance Liquid Chromatography (HPLC) Honeywell Riedel-de Haën® grade; Toluene, Nuclear®; AFM1 standard, Sigma-Aldrich®.

2.3 AFM1 Assay

The extraction was carried out according to Dragacci et al. (2001), milk samples were heated to 37 ℃ in a water bath and centrifuged at 2000 rpm to separate the upper layer of fat. The lower part of the skimmed milk was filtered through filter paper. The filtrate passed over an immunoaffinity column containing AFM1-specific antibodies (Aflastar-R-M1-Romerlabs®). Other matrix components were washed from the column with water. Elution was performed with acetonitrile HPLC and then the solvent was evaporated under a stream of N2 to dryness. AFM1 was re-dissolved in the mobile phase water: acetonitrile: methanol, in the ratio 65:25:10. The quantification of AFM1 occurred in high performance liquid chromatography (HPLC), Shimadzu LC-20AD XR apparatus coupled to the fluorescence detector, with the parameters as follows: Mobile phase - water, acetonitrile and methanol (65:25:10), column : Waters X-Terra, 150x4.6mm, flow rate 1.0 mL/minute eluting in isocratic mode, with fluorescence detector λex- 365 nm and λem- 435 nm; injection volume 50 µL; 10 minutes running time. For the method validation, linearity was calculated from a calibration curve, in which a stock solution of 17.45 µg/mL of AFM1 in toluene and acetonitrile was used. Solutions were prepared at the following concentrations: 0.125, 0.5, 1.0, 2.5, 5.0 and 10.0 µg/mL, and two injections were performed at each point of the curve (duplicate), obtaining the average of the readings for the construction of the curve of calibration. The injection volume was 50 µL and were made from highest to lowest concentration. For Recovery %, AFM1 negative samples were spiked with a known concentration standard and mycotoxin extraction and quantification performed to assess the recovery rate of the method. Considering that the correlation coefficient (r) must be above 0.990 (BRASIL, 2017), a value of 0.9966 was obtained in the experiment, as recommended. The obtained curve correlated the peak area of the chromatograms with the respective concentrations of AFM1. The recovery rate was 85.52% and the Limit of detection (LOD) was 0.0625 µg/mL.

2.4 Statistical Analysis

For analysis of the results, the statistical tests ANOVA, Test t and Tukey were used, with a confidence level of 95%. Analysis of variance (ANOVA) was used to verify whether there was a significant difference between the means obtained.

2.5 Risk Assessment

In order to estimate the *estimated daily intake* (EDI), the calculation proposed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) was performed with data on the concentration of the substance in the food (ng/kg), the consumption of the food (kg) and individual or population body weight (kg) (JECFA, 2014). The exposure estimate is defined by the equation:

$$EDI = \frac{\text{concentration of the substance } x \textit{ consumption of the food}}{\textit{body weight}} \quad (1)$$

The risk assessment was carried out using the margin of exposure (MOE) proposed by the European Food Safety Authority (EFSA). The MOE was obtained by the relationship between the reference dose level that causes a 10% increase in the incidence of cancer in rodents (benchmark dose – BMDL10) and the estimated daily intake (EFSA, 2005). According to EFSA (2020), the BMDL10 for AFM1 derived from AFB1, whose value is 0.4 µg/kg b.w./day, therefore, BMDL10= 0.9124 µg/kg b.w./day for AFM1, value also assumed in an investigation conducted by Conceçotto et al. (2020). The relationship is defined by the equation:

$$MOE = \frac{BMLD10}{EDI} \quad (2)$$

3. Results and Discussion

3.1 AFM1

Of the 30 milk samples evaluated, six (20%) were considered positive with respect to LOD=0.0625 µg/mL. Table 1 shows the results obtained regarding the occurrence of AFM1 in which none of the positive samples exceeded the maximum limit tolerated by Brazilian legislation (Brasil, 2022).

Table 1. AFM1 occurrence in bovine milk samples

N	Number of positive samples (%)	AFM1 (µg/kg)			MRL (µg/kg)[1]
		Average ± SD	Min.	Max.	
30	6 (20%)	0.238 ± 0.143	0.093	0.320	0.5

[1] Maximum residue level acceptable limit in Brazilian legislation.

Other authors have identified the occurrence of AFM1 in bovine milk in countries such as Nigeria, United Arab Emirates, Mexico and in derived products in Greece, China and West Asian countries (Mukhtar et al., 2022; Mohamadin et al., 2022; Álvarez- Días, 2022; Panara et al., 2022; Xiong et al., 2022). However, the lack of investigations into the occurrence of AFM1 in bovine milk in northern Brazil makes it difficult to monitor contamination in the state of Amazonas, despite reports of the presence of the mycotoxin in other Brazilian regions. In studies carried out in the states of Paraná and Minas Gerais, respectively, 87.5% and 30.7% of the samples were found to be contaminated with AFM1, with the latter being milk samples processed at ultra-high temperature (Silva et al., 2015; Oliveira et al., 2013). In the municipality of Ribeirão Preto in the state of São Paulo, studies were carried out on milk samples and 20.9% were positive for AFM1; in investigations with derivative products in the same municipality, the mycotoxin was detected in 84% of the cheese samples and 95% of the yogurt and dairy drink samples (Garrido et al., 2003; Iha et al., 2011). Thus, it is noticeable that mycotoxin contamination is a reality all over the world and in certain Brazilian states. The data obtained in this work demonstrate that the same applies to the state of Amazonas, even though dairy farming in the Amazon region is considered unrepresentative in the national scenario (Salman & Pfeifer, 2020). Because it is a worldwide problem, countries adopt different regulatory limits for AFM1 levels. In Brazil and the United States, the regulatory limit is equal to 0.50 µg/kg, while the European Union adopts the value of 0.05 µg/kg (Brazil, 2022; EC, 2006). According to Brazilian guidelines, none of the positive samples is above the recommended by law. However, when European legislation is adopted as a reference, all of them exceed the regulatory limit, even though the detection limit of the method is higher than the described value. Furthermore, all positive samples were from raw milk while those negative included both pasteurized and raw milk. According to Hassan et al. (2018) AFM1 is relatively stable to routine pasteurization and ultra-high temperature treatment and, if present, could be detected in pasteurized milk samples, as well as in a study conducted by Nejad et al. (2019) in which AFM1 was detected both in samples of pasteurized milk and in milk processed by ultra-high temperature. Another important factor in the occurrence of AFM1 in milk are the agricultural practices that can be adopted by producers and differ from one Brazilian region to another. According to Santos et al. (2017) dairy production in the Amazon is heterogeneous regarding the production system and technological level, cattle feeding is almost exclusively based on native or cultivated pastures. Thus, it is understood that the use of feed or grains as feed for cattle is infrequent in the region, but it still occurs, which is corroborated by the results obtained that demonstrate the ingestion and conversion of AFB1 present in the grains or feed to AFM1, excreted in the animal's milk and which was detected in our work. This practice contrasts with the practice in the state of Minas Gerais, for example, where the relationship between AFM1 levels and the amount of feed is notable when observing higher concentrations of AFM1 in the dry period, when cows are fed with greater amounts feed possibly contaminated with AFB1 (Picinin et al., 2013). In the state of Amazonas, in turn, livestock occur mostly in an unconfined area, thus differentiating the environmental conditions that would provide contamination.

3.2 Risk Assessment

For the estimation of daily intake, data provided by Brazilian government, POF - IBGE (2020) were used. The chosen age group was 30-34 years old, in which the median weight of men and women in Amazonas is, respectively, 74.2 and 62.0 kg. For daily consumption of whole milk, consumption of 16.7 g/day for men and 16.3 g/day for women in Brazil was used. From the *EDI*, the MOE can be obtained through the relationship with BMDL10 of AFM1 (0.9124 µg/kg b.w/day). In table 2 the MOE values for adults did not indicate concern in public health aspects, as it was >10,0000.

Table 2. AFM1 Estimated daily intake and Margin of Exposure

	Weight (kg)	Consumption (kg)	AFM1 (mg/kg)	EDI (mg/kg b.w./d)	MOE
Man	74.2	0.167	2.38×10^{-4}	5.357×10^{-7}	1.703×10^{6}
Woman	62.0	0.163	2.38×10^{-4}	6.257×10^{-7}	1.458×10^{6}

The investigation of *EDI* is also of interest in other countries, as well as the occurrence of AFM1. In countries like Pakistan, the highest estimated value was in winter with 0.55 ng/kg b.w./day for men and 0.44 ng/kg b.w./day for women, all individuals over 16 years of age (Ismail et al., 2016). In Serbia, in individuals aged 25-55, the estimate was 0.49 and 0.56 ng/kg b.w./day for men and women, respectively (Kos et al., 2014). The values obtained for the estimated daily intake in Amazonas are lower than the results cited above and other studies in Brazil, such as in the municipality of Londrina, where for adolescents, adults and the elderly the estimate was 0.468, 0.384 and 0.559 ng/kg p.c./day, respectively (Sifuentes dos Santos et al., 2015). As for the MOE, the values obtained were greater than 10,000 for both sexes, a value that indicates less concern from the point of view of public health, according to the EFSA Scientific Committee regarding genotoxic and carcinogenic substances (EFSA, 2005). However, caution is needed regarding exposure versus consumption when dealing with children, in which the consumption of milk and dairy products may be higher than that of adults.

4. Conclusion

Bovine milk samples were evaluated for the occurrence of AFM1 and risk calculation. The results obtained in this analysis demonstrate that milk produced in the state of Amazonas, Brazil contains AFM1. Although milk production in the region is not as expressive as in others and the average quantified value is not higher than what is recommended by Brazilian legislation, the occurrence of AFM1 is indicative of practices in dairy cattle breeding. the MOE result did not indicate concern in public health aspects. Even so, we suggest that public policy actions be addressed regarding the sanitary condition of the food supplied to the herd, as well as the monitoring of dairy products produced in the state of Amazonas.

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