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Evaluation of Aflatoxin M1 contamination and physicochemical adulteration in raw milk from Southeast Brazil

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Abstract

With the increase in Brazil's milk production, it is important to assure the quality and safety of dairy products. Milk being a product of animal origin is susceptible to microbial contamination, as well as toxic products such as mycotoxins or even adulterations, therefore the continued analyzes are needed to ensure that legislation is being enforced, avoiding possible health hazards to the consumers. 62 raw milk samples were collected from tank trucks at dairy processing facilities from Rio de Janeiro, São Paulo and Minas Gerais and were tested for acidity, relative density at 15ºC, fat and cryoscopic index, crude protein, lactose and Aflatoxin M1 according to the official methods. Among the evaluated samples, 11.29% (7) presented some irregularity in their composition and 41.93% (26) presented irregularities in cryoscopy, presenting their results above or below that recommended by the legislation. At the toxicological analyzes, 36.12% (33) presented values were above the allowed for Aflatoxin M1 (AFM1).

Introduction

Livestock has great prominence in Brazil's southeast region, which has the third largest cattle herd in the country with 38,508,537 heads (IBGE, 2014) [1]. The largest dairy basins and the largest industrial concentration of dairy products in the country are located in the southeast region, given its proximity to the largest consumer markets, represented by the large metropolis of São Paulo, Rio Janeiro and Belo Horizonte.

Currently, the major concern regarding food safety is being free of contaminants. Milk is a product of animal origin susceptible to microbial contamination, as well as toxic products such as mycotoxins and adulterations. In Brazil, milk production has grown continuously at a rate of 4% in the last fifteen years, having reached the mark of 32.1 billion liters in 2011. With the expressive increase in production the concern of population with the quality and food security of milk acquires an expressive dimension since Brazil became the fourth largest consumer of fluid milk [2-6].

Aiming to guarantee the standard of identity and quality, as well nutritional values of the raw milk samples collected. The proposal seeks to evaluate possible adulterations, according to the legislation, analyzing the physical chemical quality and contamination of mycotoxins in raw milk produced in the southeast region.

Material and method

Location

Dairy Cooperatives from producing areas were selected based in productions levels (≤150 L/ day). The milk sampling was transported by collection tank trucks, coming from dairy farms from the states of Rio de Janeiro, São Paulo and Minas Gerais. Following the proposed collection routine of Animal Health Defense from the states and regions of the study.

The analyzes were realized at the laboratory of the Food Quality Control Center (CEPQA) of the Agricultural Research Corporation of the State of Rio de Janeiro (PESAGRO-RIO) and in the laboratory of Mycotoxins (LAMICO) of the University Federal Fluminense and University Federal of Minas Gerais (UFMG).

Samples are collected from establishments annually in 2016 and 2017, at two different times, respecting the seasons. Representative samples were collected in triplicate, being sealed directly on site, identified, packed under refrigeration and, thus, maintained throughout the transportation process to CEPQA within twenty-four hours, where they were immediately registered and processed for analysis.

Physical and chemical analysis

The physical chemical characterization was realized according to the *Métodos analíticos oficiais para controle de produtos de origem animal e seus ingredientes:* II – *Métodos físicos e químicos* of Ministry of Agriculture, Livestock and Supply (MAPA) (BRASIL, 2018) [3]. The analysis for acidity was realized (g lactic acid/100 ml of milk), relative

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density at 15ºC (g/mL), fat (g/100 mL of milk) and cryoscopic index (ºC) (BRAZIL, 2018) [3]. For rapid Tests of Crude Protein and Lactose, it was used automatic ultrasound detector (Milkotester-LM2, AKSO, RS, Brazil).

Mycotoxins detection and quantification

Samples were extracted using the modified QuEChERS based extraction method following the methodology described in the Association of Official Analytical Chemists (AOAC) (2007) Official Methods Manual.

All extractions were performed in duplicate. Sample screening was performed using commercial immunoenzymatic kits for AFM1 (Aflatest®, Vicam, Watertown, MA, USA) following manufacturer's instructions. Quantification and analysis were performed using a VICAM® Series-4EX fluorimeter (Watertown, MA, USA). AFM1 standards (5 mg) were purchased from Sigma (St. Louis, MO, USA). The positive samples were diluted with ultrapure water to form a solution of methanol-water (7: 3, v/ v), similar to the HPLC mobile phase. The identification and quantification of AFM1 residues were conducted with the injection of 20 µL of the extracts of the samples in the HPLC system (JASCO LC 2000) of methanol-water (7: 3, v/ v) at a flow rate of 0.8 ml/minute. Under these conditions, the retention time for approximately 3.7 minutes.

The stock solution (50 µg mL^{-1}) and working solutions (2 µg mL^{-1}) were prepared in methanol and their concentrations confirmed by UV light absorption using a Shimadzu UV-1201 spectrophotometer (Kyoto, Japan) (AOAC 2007), stored in amber vials at -10°C for a period of three months. The limits of detection (LOD) and quantification (LOQ) were found by adding, decreasing, concentrations of standard solution and subjected to extraction and quantification to the lowest detectable concentration (LOD) and lowest quantifiable concentration (LOQ) under appropriate conditions. Repeatability (n=5, RSD<15%). The detection and quantification limits found were 0.013 µg kg^{-1} and 0.055 µg kg^{-1}, respectively.

Statistical analysis

All data collected integrated a database in order to generate statistical data and determining frequency and average absolute values among the data established in the analysis. A comparison was made between the proportions of contaminants obtained throughout the study. Analysis of variance (ANOVA) were be applied to the data in the elaboration of the data on a logarithmic scale. The definitive statistical evaluation was only be established after detailed exploratory analyzes of the collected data (STEEL; TORRIE, 1985; AGRESTI, 1990; Statistics for Windows 5.0 SAS System for Windows®).

Result and discussion

Physical chemical and toxicological analyzes were carried out on 62 samples from the states of Rio de Janeiro, Minas Gerais and São Paulo. The numerical results were expressed in the Table 1 as a percentage per gram of sample.

The decree No. 9,013 of March 29, 2017, having not been subsequently amended, establishes parameters for raw milk. According to it, raw milk must have at least 3.0% of fat, this value being determined as a way of standardizing whole milk. It is known that the average value of fat in milk from the southeast region is 3.85% [7], thus adjusting the fat content of whole milk to the minimum parameters stipulated by the Ministry of Agriculture, Livestock and Supply (MAPA), are a practice performed by many processing industries. From the evaluated samples, averages of 3.37% ± 0.6% were obtained, a value that is in accordance with the legislation. However, some samples were in the minimum indices of 2.2% ± 0.6%. It is known that linked to the fatty fraction of milk are Vitamins A and D, the reduction of this fraction, by either skimming or dilution contributes to a poorer food from the nutritional point of view. These data can assist in the detection of possible fraud. Among the main forms of fraud found in the country is the decrease in the amount of fat in the product, either by adding water, whey or even by skimming (ABRANTES, 2014) [1].

The evaluation of acidity, expressed in Dornic degrees, according to the legislation must remain between 14º and 18º D (0.14 and 0.18 g/100 mL Lactic acid). As observed in Table 1, the average acidity was 21.8 ± 0.85, which indicates a milk with greater acidity than that recommended by MAPA. The increase in acidity is directly related to the presence of lactic acid-producing microorganisms [8] being an indicator of the quality of raw milk and the delay in post-collection transport and a problem in the various later processing steps. (Fonseca; Santos). Poor microbiological milk can be harmful to the consumer, since that bacteria such as *Salmonella* spp.*, Leptospira, Listeria Monocytogenes, Clostridium or Brucella* spp. can cause foodborne diseases [9].

Solids Non-fat (SNF) is required with at least 8.4% by law. From the data found, they presented an average of 9.44 ± 0.73%, indicating satisfactory nutritional quality of the analyzed products. However, some samples presented indexes below the recommended, with a minimum of 7.6%; the reduction in SNG values and density in milk may be related to the addition of water and skim [10].

For the evaluation of cryoscopy indices, temperatures should be between -0.512ºC and -0.536ºC. The evaluated cryoscopy averages showed a value below that recommended by the legislation (-0.584º ± 0.06ºC), which represents a non-compliance with the regulation. The presence of water can be perceived by interpreting the values obtained in cryoscopy. Possibly related to the acidity content, indicating the addition of serum as a potential dilution by liquid. The water used in milk adulteration is often obtained from an unsafe and inexpensive source, and can be contaminated with pesticides, heavy metal or microorganisms, being a possible health hazard for the consumers [11] The highest cryoscopy approaches the freezing point of water, Abrantes *et al.* [1] while the lowest temperature, may indicate fraud by adding solute or cryoscopy constituents.

Table 1. Results found in physical chemical analyzes of 62 samples of the parameters of solids not-fat, total proteins, mineral matter, cryoscopy, acidity and fat

Values	SNF[1]	Protein[2]	Minerals[3]	Crioscopy[4]	Acidity[5]	Fat[6]
Maximum	10,45%±0,73%	3,85%±0,26%	0,9%±0,09%	-0,476±0,06	50±0,85	4,0%±0,67%
Minimum	7,60%±0,73%	2,81%±0,26%	0,58%±0,09%	-0,695±0,06	15,2±0,85	2,2%±0,67%
Average	9,44%±0,73%	3,48%±0,26%	0,78%±0,09%	-0,584±0,06	21,8±0,85	3,37%±0,67%
Median	9.58%	3.53%	0.81%	-0.608	19.8	3.59%

1. SNF: Solids not-fat; 2. Protein: Total caseins, Whey proteins and nitrogenous solids of non-protein origin; 3. Residual mineral salts 4. Freezing point 5. Acidity: expressed in Dornic degrees

As for the evaluation of total proteins, MAPA recommends a minimum of 2.9% ± 0.26%, during the evaluation averages of 3.48% ± 0.26% and a minimum close to the recommended of 2.81% ± 0.26%.

Among the evaluated samples, 11.29% (7) presented some irregularity in their composition and 41.93% (26) presented irregularities in cryoscopy, presenting their results above or below that recommended by the legislation. Of the possible adulterations that may have been carried out, it is mainly suspected of adding water, whey, and cryoscopy reconstitutes. These being considered the most common frauds found in Brazil due to their easy execution and low cost. These frauds generate a final product of less nutritional value to the consumer, in addition to a lower quality product for the processing industries, other possible frauds carried out include the addition of density reconstitutes together with the addition of water or serum; these can be identified in the evaluation of non-fat solids and density. Another type of fraud commonly found is the addition of components that regulate acidity or that have bacteriostatic action; such as bicarbonate and formaldehyde, which can damage consumer's health [6].

From the toxicological analyzes to detect mycotoxins, the results of the evaluation of Aflatoxin M1 (AFM1), can be found in Tables 2 and 3.

At the toxicological analyzes, 36.12% (33) presented values above the allowed for Aflatoxin M1 (AFM1), which indicates a poor quality of the food provided to the animals and a low sanitary quality of the product. The RDC Nº 138, of February 8, 2017, which approves the maximum limit of mycotoxins in food, indicates that the maximum allowed for AFM1 in fluid milk must be 0.5 µg/kg; as shown in the table above, values ranging from 0.004 µg kg^{-1} to 63 µg kg^{-1} were found, and an average of 4.07 µg kg^{-1}, which is much higher than recommended in current legislation and indicates that corrective measures still needs to be taken, however, when analyzing the median of 0.08 µg kg^{-1}, it can be concluded that most of the samples analyzed obtained a satisfactory result regarding contamination by AFM1. Any difference was observed when comparing the different samples collected by seasons or from different states ($p>0.05$). These results show of southwest region have the same type of microclimate regions conditions and animal breeding protocols.

Table 2. Quantification by mass spectrometry of Aflatoxin M1 in whole fluid milk

Values	Quantification of Aflatoxin M1 (µg/kg)[1]
Maximum	63
Minimum	0,004
Average	4.07
Median	0,08

1: expressed in µg kg^{-1}; LOD: 0.001 and LOQ: 0.004

Table 3. Incidence range of M1 in farms of Rio de Janeiro, São Paulo and Minas Gerais states

Samples by States	Season	Compared Aflatoxin Level Range (µg.Kg^{-1}/µg.L^{-1})			
		AFM1 (not processed milk)		AFM1 (processed milk)	
		%	Mean±SD	%	Mean±SD
Rio de Janeiro (n=30)	Su	22	0.039±0.01	10	0.03±0.01
	Au	23	0.041±0.01	11	0.01±0.01
	Wi	26	0.042±0.01	10	0.02±0.01
	Sp	24	0.031±0.01	14	0.01±0.01
São Paulo (n=2)	Su	24	0.28±0.12	19	0.02±0.01
	Au	26	0.31±0.13	17	0.04±0.01
	Wi	30	0.52±0.21	19	0.04±0.02
	Sp	28	0.41±0.21	17	0.03±0.01
Minas Gerais (n=30)	Su	24	0.29±0.22	14	0.03±0.01
	Au	22	0.41±0.10	12	0.04±0.01
	Wi	29	0.62±0.13	11	0.06±0.01
	Sp	27	0.41±0.21	12	0.04±0.02

* SD: Standard deviation; limits expressed in µg kg^{-1}; LOD: 0.001 and LOQ: 0.004

These results also indicate a concern of the producers with the quality of their production, food provided and storage, since the presence of AFM1 in milk occurs through the transport of it to the milk after hepatic metabolism of Aflatoxin B1 [12], this commonly present in feed intended for animal consumption. All samples collected are from products commonly distributed and sold in the state of Rio de Janeiro.

When comparing the results found with legislation such as that of the European Union, where the maximum tolerated limit is 0.05 µg/kg, we can see that there are still several quality controls measures to be taken aiming to bring national production closer to sanitary requirements of first world countries.

The exposure of AFM1 can lead to both acute and chronic aflatoxicosis, due to its high hepatotoxicity, carcinogenicity and cytotoxicity it is considered a health risk even in small quantities in milk, especially to the most frequent consumer groups of the product [5]. Milk and products consumption are usually associated with infant and elderly people. The Aflatoxin B1 and M1, when present are classified as carcinogenic associated to liver and bile duct cancer by the International Agency for Research on Cancer [2,13-20].

Conclusion

It can be concluded with the analyzes carried out that adulterations such as the addition of water or whey still are a practice found in the dairy industries in the southeastern region, harming the consumer and decreasing the nutritional quality of the food sold.

As for the detection of Aflatoxin M1 at levels higher than permitted in the legislation, it is concluded that the feed provided to animals is not of ideal quality, generating residues with toxic and carcinogenic potential in milk, decreasing its quality and putting at risk mainly the groups that eat the most of this product such as children and the elderly.

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