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Detection of fraud by addition cow's milk in cheese buffalo and its connection with seasonality*

Detecção de fraude por adição de leite de vaca em queijo de búfala e a relação com a sazonalidade

Gabrielle Virgínia Ferreira Cardoso,** Andrey Carlos do Sacramento de Oliveira,** Andréia Silva da Silva,** Wanessa Shuelen Costa Araújo,*** Vanderson Vasconcelos Dantas,***** Josyane Brasil da Silva,***** Joelson Sousa Lima,** Emília do Socorro Conceição de Lima Nunes,** Ricardo José de Paula Souza e Guimarães,**** Talita Bandeira Roos,** Carina Martins de Moraes**

Abstract

The seasons influence the production of buffalos' milk. Because of this, the producers may produce a mixture of buffalo and bovine milk during cheese production in periods of low production. Therefore, the present work aimed to investigate fraud in buffalo cheese and the relationship between seasonality and different physicochemical properties of buffalo cheeses produced and marketed in eastern Amazonia. We obtained commercial samples of buffalo cheese during two Amazonian climatic periods from commercial points of Marajó-Pará, Brazil. After collection, there were lipid, protein, ash, and humidity analyses. Determination of carbohydrates and energy values was also performed for the nutritional characterization of samples, as well as for mPCR analysis to detect buffalo and/or bovine DNA. DNA extraction protocol of the samples was standardized and two pairs were used for the mPCR reaction, amplifying fragments of approximately 220 bp for *Bubalus bubalis* DNA and 346 bp fragments for *Bos taurus* DNA. Among the samples acquired in the rainy season, we observed that 33% were inadequately labeled, indicating fraud from cow's milk incorporation and fraud from substitution of raw material. From the nine samples obtained in the dry season, all the samples showed cow's milk incorporation fraud. The highest fraud rate coincided with the period of low milk production from buffalo and there was a difference in composition between fraudulent and non-fraudulent cheeses. Therefore, seasonality influences increase in cattle milk for the production of buffalo cheese, and this adulteration may decrease the nutritional content of the product.

Keywords: *Bubalus bubalis*, mPCR, 12S rRNA gene species identification.

Resumo

As estações climáticas influenciam a produção de leite de búfala. Isso pode levar os produtores a misturarem os leites de búfala e bovino durante a produção de queijo em períodos de baixa produção. Portanto, o presente trabalho teve como objetivo verificar fraudes em queijo de búfala, a relação com a sazonalidade e as diferenças físico-químicas de queijos de origem bubalina, produzidos e comercializados no leste da Amazônia. Foram coletadas amostras comerciais de queijo de búfala em dois períodos climáticos da Amazônia em pontos comerciais do Marajó-Pará, Brasil. Após a coleta foram realizadas análises de lipídios, proteínas, cinzas e umidade. A determinação dos carboidratos e do valor energético também foi feita para a caracterização nutricional das amostras, bem como a análise de mPCR para a detecção de DNA de búfalo e/ou bovino. Para isso, padronizou-se um protocolo de extração de DNA das amostras e utilizou-se dois pares na reação mPCR, amplificar fragmentos de aproximadamente 220 pb para o DNA de *Bubalus bubalis* e fragmentos de 346 pb para o *Bos taurus*. Entre as amostras adquiridas na estação chuvosa, observou-se que 33% foram rotuladas inadequadamente, caracterizando fraude por incorporação de leite de vaca e fraude por substituição de matéria-prima. Das 9 amostras coletadas no período seco, todas as amostras apresentaram fraude na incorporação do leite de vaca. Este estudo revelou que a maior taxa de fraude coincide com o período de baixa produção de leite e que há uma diferença na composição entre queijos fraudulentos e não fraudulentos. Portanto, a sazonalidade influencia no acréscimo de leite de bovinos na produção de queijo de búfala e que esta adulteração pode diminuir o conteúdo nutricional do produto.

Palavras-chave: *Bubalus bubalis*, gene 12S rRNA, mPCR, identificação de espécies.

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**Universidade Federal do Pará/Faculdade de Medicina Veterinária, Dept. Higiene e Qualidade de Alimentos e Microbiologia, Castanhal, PA - Brazil.
***Instituto Federal de Educação Ciência e Tecnologia do Pará, Dept. Ciência e Tecnologia, Cametá, PA - Brazil.
****Instituto Evandro Chagas, Lab. Geoprocessamento, Ananindeua, PA - Brazil.
*****Universidade do Estado do Pará – Dept. Tecnologia de Alimentos, Castanhal, PA - Brazil.
Corresponding author: <gabi.virginia.mv@gmail.com>

Introduction

Buffalo milk production has been broadly studied worldwide, representing on average 14% of all milk produced in some Asian countries and part of Italy (FAO, 2018).

Due to seasonality in milk production, fraud from addition of milk from other species with higher availability and/or lower cost is frequently reported worldwide (Drummond et al., 2013). This illegal act has been reported in the northern part of Brazil, a region known for having the largest buffalo herd (Silva et al., 2015).

One factor that may be causing cheese production fraud is the direct effect of season on dairy production of animals that is directly influenced by the seasons, and this may be one of the reasons growers use buffalo and bovine milk during the production of cheeses (Seixas et al., 2015).

According to Köppen-Geiger climatic classification, known as the global classification system, the eastern Amazon has a climate Af or equatorial climate, which is characterized by periods of intense rainfall, alternated by well-defined dry periods. In the world, the regions under this classification are the deserts, polar, and some tropical regions, such as the Sahara in Africa, Saudi Arabia, central Australia, northern Canada and Russia, and Brazilian Amazon (Peel et al., 2007). In Amazon region, the rainy season is between Nov and Mar and the dry season is between May and Sept. Between Apr and Oct, the transition from one season to another occurs (Maeda et al., 2014; Lopes et al., 2016).

Although some authors have reported the effect of season on the physical-chemical and microbiological characteristics of buffalo cheese (Seixas et al., 2015), there are few studies on the association between season and increase in bovine milk use during cheese production. Although buffalo cheese fraud is a worldwide reality (Gupta et al., 2011), few authors have reported their effect on the quality of the produced derivatives.

According to Cardoso et al. (2019), adulterated buffalo cheese has lower protein compared to original buffalo cheese. This is because buffalo milk used as a raw material for cheese contains 48% more protein than cow milk. Therefore, it is important to employ methods that can be used to detect fraud. Different polymerase chain reaction (PCR) methods such as traditional PCR, mPCR, and real time PCR may be feasible for detecting adulterated dairy product (Mane et al, 2012). Compared to other PCRs, multiplex PCR (mPCR) effectively reduces the use of reagents and the number of operating steps by simultaneously detecting 2 or more target genes in a single analysis (Freitas et al., 2010).

Therefore, the present study aimed to verify if fraud detected by mPCR assay of buffalo cheeses, containing cow milk and seasonality have a connection with possible differences in the physicochemical properties of buffalo cheeses produced and marketed in eastern Amazonia.

Materials and methods

Based on the climatic conditions of the region, we established collection periods of trade samples. We collected 18 samples of exclusively buffalo cheese, the Marajó Cheese. Nine samples were collected in May (rainy season) and nine samples in July (dry season), identified from 1 to 9, per period, according to manufacturer's instruction.

Samples were collected in two seasons of the year (dry and rainy), in different commercial points of the cities: Salvaterra (00° 45' 10" S 48° 31' 01" W, elevation: 5 m), Soure (00º 43' 00"S 48º 31' 24" W, elevation: 10 m), Joanes (0° 52' 39.8" S 48° 30' 38.4" W, elevation: 5 m), and Camará (0° 56' 13.6" S 48° 36' 26.9" W elevation: 5 m), in the Marajó Island-Pará, Brazil.

The places of collection were georeferenced at the time of first collection, through AndroidTS Test®, and the obtained data were processed in the software ArcGIS 10, for spatial distribution of the results, using IBGE databases (http://www.ibge.gov.br) and Prodes Project (http://www.obt.inpe.br/OBT/assuntos/programas/amazonia/prodes).

After collection, the samples were kept under refrigeration in Castanhal (01° 17' 49" S 47° 55' 19" W, elevation: 45 m), for the determination of centesimal composition and detection of fraud through mPCR. Samples of fresh cheese produced using bovine raw material and bubaline raw material were also produced and were used as standard for each species in analyses.

Were manufactured two types of cheeses, and was used as positive control in DNA extraction and mPCR reaction. One cheese was manufactured exclusively from buffalo's milk and the other was produced exclusively from cow's milk. For detection of fraud through mPCR, DNA sample was extracted according to the protocol previously proposed by López-Calleja et al. (2005) with modifications.

Portions of 0.4 g cheese were moistened and macerated with 600 µL lysis STES buffer (0.2 M Tris base, 0.5 M sodium chloride, 0.1% sodium dodecyl sulfate, and 0.01 M acid Ethylenediamine tetraacetic acid). Thereafter, the obtained material was homogenized in an orbital shaker and incubated with 10 µL of proteinase K (20 mg mL^{-1}), in a water bath at 65 °C for 16 h. The digested samples remained for 5 min under refrigeration with 700 µL of phenol-chloroform (1:1), and they were subsequently centrifuged at 14000 *g* for 10 min. Afterwards, 700 µL of isopropanol was added to the clear and aqueous supernatant obtained after centrifugation (400 µL) and centrifugation was performed at 14000 *g*, for 1 min. Finally, the obtained supernatant was discarded and 600 µL of 70% ethanol was added. After further centrifugation, the remaining precipitate was kept in an oven for 2 h at 37 °C for evaporation of ethanol.

The obtained DNA was then eluted in 100 µL of enzyme-free water and subjected to 1.5% agarose gel electrophoresis run in Tris Borate EDTA (TBE) buffer. The gel was stained with non-mutagenic the Safer™ dye (1 µL dye / 5 µL sample. The analysis of electrophoresis results was performed using photo documentation equipment on ultraviolet light Gel Documentation System. After determining DNA presence, its concentration and purity verification were determined by spectrophotometry at 230 nm, 260 nm, and 280 nm.

The DNAs of the species *Bos taurus* and *Bubalus bubalis* (25 $ng.\mu^{-1}$), extracted from standard samples were subjected to conventional PCR to confirm the species, and were subjected to sequencing to confirm the efficiency of the used primers. Sequencing of the samples was performed using automatic sequencer (AB 3500, Genetic Analyzer, Applied Biosystems, USA), assembled with 50 cm capillaries and POP7 polymer.

The DNA templates were labeled using 2.5 pmol of each primer as previously described by López-Calleja et al. (2005). 12SBT-REV2 primer (reverse 5'-AAATAGGGTTAGATGCACTGAATCCAT-3') was specific for *Bos taurus*, 12SBUF-REV2 primer (reverse 5'-TTCATAATAACTTTCGTGTTGGGTGT-3') was specific for *Bubalus buballis*, and 12SM-FW primer (forward

5'-CTAGAGGAGCCTGTTCTATAATCGATAA-3') primer was common to both species.

The sequencing data were collected through a specific software (Data Collection 2, Applied Biosystems, USA) with the parameters, Dye Set "Z"; Mobility File "KB_3500_POP7_BDTv3.mob"; BioLIMS Project "3500_Project1"; Run Module 1 "FastSeq50_POP7_50cm_cfv_100"; and Analysis Module 1 "BC3500SR_Seq_FASTA.saz. For local alignment of the sequences, BLAST (Basic Local Alignment Search Tool) software tools, available on the NCBI (National Center for Biotechnology Information) website were used.

The same primers described in the sequencing step were used to perform the proposed mPCR. These primers amplify fragments of approximately 220 base pairs (bp) for *Bubalus bubalis* DNA and 346 bp fragments for *Bos taurus* DNA. Oligonucleotides were prepared in Tris EDTA buffer (10 mM tris-HCl, 1 mM EDTA, pH 8.0) to the concentration of 5 pmol μL^{-1} according to the manufacturer's instruction.

To determine the centesimal composition, the Goldfisch method was used to measure total lipids. Calcination method in muffle at 550 ºC was used for the determination of ash (AOAC, 2005). Kjeldahl method was used to measure the protein contents, and oven drying method at 105 ºC until constant weight was used to determine humidity and total volatiles (AOAC, 2005). Carbohydrate content and energy value were determined indirectly as recommended by MAPA (1996).

The data were tabulated and subjected to statistical analysis to evaluate the presence of significant difference (ANOVA) and the association (Tukey) between the cheeses in two collection seasons, and these were performed through BioEstat® software version 5.3.

Results

For the alignments obtained by sequencing the PCR products for the identification of *B. taurus* and *B. bubalis* species from 12S rRNA gene, resulting in 346 bp and 220 bp products, respectively, 96% compatibility was found with bovine genome (MF925711. 1) and 95% compatibility with the target region of buffalo genome (KX758295.1).

Figure 1 demonstrates the occurrence of fraud and its distribution map in nine sampling points in each study period and Figure 2 shows mPCR results of the samples sold with buffalo cheese labeling.

Among the 9 samples acquired in the rainy season, we observed that three (33%) were inadequately labeled, since bovine DNA (*B. taurus*) and buffalo DNA (*B. bubalis*) were simultaneously detected in 2 of the samples (22%), indicating fraud by cow's milk incorporation. Further, only bovine DNA (*B. taurus*) was detected in one (11%) sample, indicating fraud by substitution of raw material. From the 9 samples collected in the dry period, all the samples showed the presence of both bovine and buffalo DNA fragments simultaneously, indicating cow's milk incorporation fraud, thus totaling 100% fraud in this period of the year.

The centesimal composition and energy value of the cheese did not vary significantly ($p < 0.05$) between the seasons. However, Table 1, which shows the results of centesimal composition and energy value of the eighteen commercial cheeses samples distributed into two groups according to fraud detection (fraudulent and non-fraudulent), revealed that there was variation between cheeses fraudulent and non-fraudulent ($p < 0.05$).

Figure 1: Seasonal distribution of fraud in Marajó cheeses. Positive result for fraud is represented in red; Negative result for fraud is represented in yellow; Points that are completely filled by red color represent fraud detection in the dry and rainy season; Points that are filled with red and yellow color represent fraud detection only in dry season.

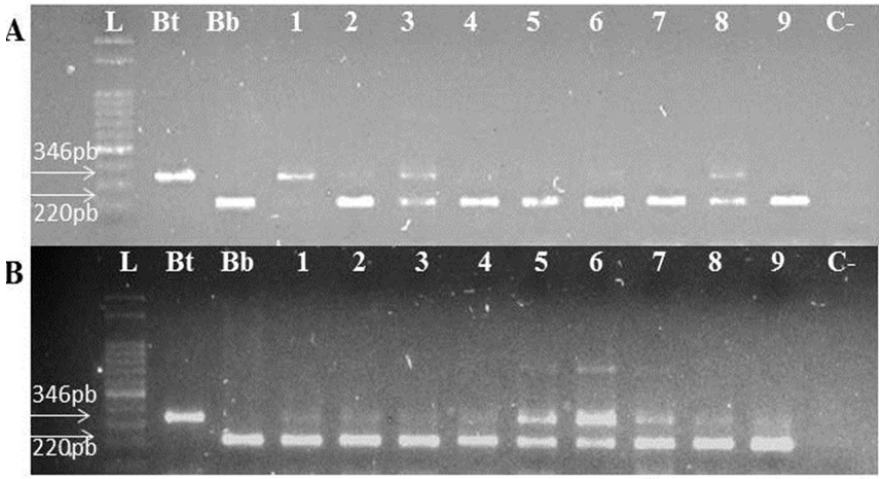

Figure 2: Agarose gel of 1.5% demonstrating the presence of bovine (346 bp) and buffalo (220 bp) DNA fragments, obtained through mPCR. A: samples of cheese collected in the rainy season, represented by 1 to 9, demonstrate fraud detection in samples 1, 3, and 8. B: samples of cheese collected in the dry period represented from 1 to 9, demonstrate the presence of fraud in all samples. L: molecular marker 100 bp; Bt: bovine control (346 bp); Bb: buffalo control (220 bp); C-: Negative control.

Table 1: Analysis of centesimal composition and energy value of buffalo cheese samples according to fraud detection

Fraud	Lipids (%)	Humidity (%)	Ashes (%)	Proteins (%)	Carbohydrates (%)	Energetic Value (Kcal/100 g)
Frauded	26±[9]a	41±11[a]	1.8±0.6[a]	13±0.5[a]	8±4[a]	191.43±43[a]
Non frauded	30±8[b]	38±10[b]	1.4±0.2[b]	25±0.7[b]	10±5[a]	342.34±45[b]

* Equal letters ("a" and "b") in the same column indicate that there is no significant difference at the 5% probability level.

Discussion

Several industries use buffalo milk in cheese production due to the superiority of its component raw materials compared to cow milk (Bittencourt et al., 2013). However, consumption of food products from this production chain is informal due to lack of regulation of standards for the identity and quality of buffalo milk and its derivatives, which complicates the control and inspection measures (Pereira Júnior et al., 2009).

The practice of fraud in cheese and other foods of animal origin has been an increasing concern among the inspection services that have been mobilized to detect such frauds and fight against them. Therefore, it is important to understand the motivation behind this practice and propose tests that can identify fake and / or adulterated foods. Thus, our work sought to identify accurately possible frauds, as well as to generate relevant information to the consumer about these irregularities.

The results from the sequencing showed that the primers used in mPCR to analyze the field samples have a high degree of complementarity above 90%, with the genomic DNA of each species. This guarantees greater reliability of the results obtained, since it presents, in addition to the size of the fragment, the exact sequence of the amplified product and its compatibility with the gene of the primers (Simões et al., 2013).

Detected fraud in the samples through mPCR is by substitution and addition of bovine raw in buffalo cheeses without this being described in label. This is according to the Regulation of the Industrial and Sanitary Inspection of Products of Animal Origin (RIISPOA) characterizing this as fraud by adulteration of the product (Brasil, 2017).

It was noted that in the period of low milk production, which corresponds to dry season, there was a higher index of frauds, suggesting that fraud is related to the season of milk production and that one of the factors that may be influencing is variation in the region. Therefore, there is a need for planning dairy production so that the season of dairy production does not affect productivity and profitability (Meneghini et al., 2016).

It has been known that buffalo cheese production takes place mainly because buffalo milk offers nutritional benefits, related to higher levels of total solids, fat, protein, calcium, and phosphorus (Arora and Khetra, 2017). In this study, it was shown that the nutritional composition of the cheeses did not vary significantly in relation to the periods of the year analyzed. However, when evaluating the correlation between total fraudulent and non-fraudulent samples and nutritional composition, the results showed that fraud cheese had a lower nutritional quality than buffalo cheese, since it had lower values of protein, lipids, and energy. For the variation observed in the ash content, it may be

due to the addition of sodium chloride (NaCl) in the preparation of the cheese, which is possibly related to the failure to standardize the processing of these products. It is important to note that even if there were no nutritional variation between samples, adulteration would still be illegal.

Sheehan and Phipatanakul (2009), when evaluating the effects of the use of bovine milk in goat's cheese, verified that the values of humidity in the analyzed products increased as bovine milk in the composition increased. The results in this experiment are similar to those found by the authors in the present study demonstrating that adulterated cheese may have higher humidity and total volatiles contents than unadulterated cheeses. This fact demonstrates that an increase in humidity caused by the addition of bovine milk in buffalo cheese may compromise the final quality of the product.

Higher moisture content in cheese may influence and hamper standardization of this product and increase the risk of microbial contamination due to increased water activity, which favors the growth of pathogenic microorganisms. In addition to general health risk and the nutritional losses caused by fraud, changes in sensory parameters such as texture and color of cheese can also be determined, since buffalo milk has a higher amount of lactose, calcium, and vitamins A and C. Moreover, there is presence of β-carotene in bovine milk, which is absent in buffalo milk, and for this reason, bovine cheese has a more yellowish hue, while buffalo cheese has white coloration. In addition, buffalo milk has a higher concentration of protein than bovine milk, which gives buffalo cheese a firmer texture (ABD El-Salam and El-Shibiny, 2011; Medhammar et al., 2012; Seixas et al., 2015). All these factors can influence the commercialization of the product, which ends up not having a sensorial pattern, and therefore, not pleasing to some consumers.

The addition of bovine milk in buffalo cheese during production has been adopted to maintain production in periods of low buffalo milk production in the need to complement the volume of the matter, with increase of bovine milk in the production of derivatives (Simões et al., 2013). However, fraud was also detected in the period of high buffalo milk production. According to geo-referencing data obtained during sampling, there is more intense commercialization of adulterated samples on the access points of the island, where there is a greater circulation of visitors. This suggests that the irregularity may intentionally occur, aiming at higher profits as previously described by Mane et al. (2012).

Simões et al. (2013), when evaluating the effect of bovine milk addition to buffalo milk on different characteristics of cream type Marajó buffalo cheese, showed that if this product is made with the addition of up to 40% bovine milk, there is a change in its final quality. Thus, the inclusion of bovine milk percentages in the production of buffalo cheese could be studied, so that values that do not alter the quality of this dairy could be determined for later use, which would possibly inhibit the frequency of fraud. It should be indicated that regardless of the added quantity of milk from another species, if this is not described on the label and/or does not meet the standards of hygiene and product quality, a fraud is configured.

To guarantee consumer's right, the detection of fraud in cheese has great relevance, and mPCR can be used as an instrument to verify the authenticity of this product, since this is a technique capable of detecting bovine DNA in buffalo cheese sample (López-Calleja et al., 2007; Seixas et al., 2015). Therefore, the present data suggest that surveillance for the identification of fraud in buffalo cheese should be performed mainly in places with greater concentration of commercial spots and where there are a greater number of consumers. The surveillance should also be intensified in the period from June to December where there is greater fraud index.

Conclusions

The mPCR is a useful tool for detecting fraud in commercial samples of buffalo cheese, and when it is used with other methods of analysis such as georeferencing and analysis of centesimal composition, may bring more information that is complete to the consumer. The assessment indicated that the seasonality of buffalo milk production is a factor that influences fraud resulting in addition of bovine milk in the production of buffalo cheese and this adulteration reduces the nutritional content of the product, and the final consumer is harmed both from economic and nutritional point of view.

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