Fraud with the addition of cow’s milk alters the lipid fraction of buffalo mozzarella

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Abstract
The shortage of milk at certain times of the year leads to adulteration of buffalo mozzarella, and these frauds alter the composition of milk and buffalo derivatives. This study describes the dynamics of the adulteration on the nutritional quality of mozzarella. Mozzarella was produced from buffalo milk incorporated with cow milk at 0, 10, 20, 30, 40 and 50% (v/v). The chemical composition, fatty acids profile and cholesterol content of the cheeses were evaluated. The results showed that the fat and protein contents of buffalo cheeses decreased with the addition of cow milk. Furthermore, C4:0, C16:0, C22:0 and C16:1 fatty acids decreased while C8:0 and C10:0 acids fatty acids increased. The most dramatic observation was the elevation of the cholesterol content when cow milk was added. The altered content of short-chain saturated fatty acids and cholesterol content, due to the addition of cow milk to buffalo milk for mozzarella production, modified the nutritional indices. The addition of cow milk to buffalo milk for mozzarella production altered the content of short-chain SFA and the cholesterol content, thereby modifying the nutritional indices.

Keywords: cholesterol; fatty acid; nutritional indices; seasonality.

Practical Application: Know the effects of the fraud on the buffalo’s mozzarella cheese.

1 Introduction

As of 2018, the volume of cheese traded worldwide had increased, largely due to a rise in consumption prompted by the sensorial and nutritional characteristics of cheese (Food and Agriculture Organization, 2019). Due to its milk origin, cheese provides a broad spectrum of nutrients, such as vitamins, minerals, protein, fatty acids, and other bioactive substances, originating from the composition of milk and the microbiota (Lucey et al., 2017; Ottavian et al., 2012; Santiago-López et al., 2018). Factors such as species, race, stage and number of lactations, reproductive seasonality, feeding, mastitis and genetic polymorphism (Talpur et al., 2008; Nawaz et al., 2009) can influence milk composition. However, the species of origin is the main determinant of milk composition (Lopez et al., 2011; Salman et al., 2014; Boro et al., 2018).

Mozzarella is typically fresh Italian “pasta filata” buffalo milk cheese produced with a Protected Designation Origin (PDO), and it must have the denomination “Mozzarella di Bufala Campana (MBC)” on the seal (European Union 1996; Italy, 2003; Dalmasso et al., 2011; Ilić et al., 2011). It is also a target of fraud, which is still observed despite the rigor of PDO (European Union, 2017; Gonçalves et al., 2017). Factors such as (i) the decrease in buffalo milk production due to the reproductive seasonality of the species (Phogat et al., 2016); (ii) the scarcity of buffalo mozzarella on the market (Locci et al., 2008; Penchev et al., 2016) and (iii) a consequent price increase have motivated fraud through addition of milk from different species to buffalo milk for mozzarella production (Czerwenka et al., 2010; Gunning et al., 2019). PDO mozzarella fraud in Italy is most often perpetrated by including buffalo milk from outside the PDO production zone, followed by adding cow’s milk to mozzarella production (Bontempo et al., 2019), and brings dramatic consequences to allergic people.

The consumption of buffalo mozzarella adulterated with cow’s milk by allergic people can cause them to develop health problems related to the allergenic compounds present in cow’s milk, which is absent in buffalo’s milk (Ramesha et al., 2016; Bontempo et al., 2019).
Cheese ingestion is a sensorial and nutritive experience enriched by these compounds (Santiago-López et al., 2018). Failure as a producer or researcher occurs when adulterated mozzarella is available as an original product.

Aiming to detect falsified buffalo mozzarella, researchers have been using various techniques, such as mass spectrometry (Cozzolino et al., 2002; Poonia et al., 2017; Bontempo et al., 2019), DNA (Feligini et al., 2005), electrophoresis (Pesci et al., 2011), liquid chromatography, coupled or not to mass spectrometry (Enne et al., 2005; Czerwenka et al., 2010; Russo et al., 2012), isoelectric focusing (Sakaridis et al. 2013) and infrared spectroscopy (Hansen & Holroyd, 2019), to detect fraud in milk and cheese. These techniques focus on the milk protein fraction, whereas others focus on the mineral fraction (Bontempo et al., 2019) and vitamins (Dal Bosco et al., 2018) as biomarkers. However, studies analysing the effect of fraudulent mozzarella composition are rare. One paper described the effects of cow's milk inclusion in buffalo milk on some fatty acids (Farag et al., 1984), but the conjugated linoleic acid (CLA) content and nutritional indices were not investigated. To address this, the current work studied the effect of cow milk inclusion on the lipid fraction of buffalo-milk-based mozzarella.

2 Materials and methods

2.1 Sampling

Morning milk samples were collected from 30 crossbred Jafarabadi/Murrah buffaloes and 30 Holstein/Zebu crossbred cows in the initial lactation phase (45 days on average), on day weekly for three consecutive weeks.

2.2 Experimental design

The design consisted of six treatments composed of cow milk inclusion levels (0, 10, 20, 30, 40 and 50%) in the processing of buffalo milk-based mozzarella cheese, where 0% corresponds to the control treatment (Table 1).

To prepare mozzarella, 30 L of pasteurised milk from each species (buffalo and cow) was used, with three repetitions. The Italian mozzarella cheese-making procedure (Calandrelli, 2007) was changed, and the fat content was corrected to approximately 4%.

2.3 Chemical and physical characteristics

Each method was conducted in triplicate on triplicate samples from every batch (3 batches × 3 repetitions × 3 samples = 27).

Table 1. Experimental design.

| Cheeses          | Milks (%) |
|------------------|-----------|
|                  | Buffalo   | Cow     |
| Mozzarella (0)   | 100       | 0       |
| 10               | 90        | 10      |
| 20               | 80        | 20      |
| 30               | 70        | 30      |
| 40               | 60        | 40      |
| 50               | 50        | 50      |

2.4 Lipid analysis

Total lipid extraction

Lipids were extracted using a chloroform/methanol/water solution, according to Bligh & Dyer (1959).

Fatty acid methyl esters (FAME)

The FAME were prepared from the lipids extracted from the cheese samples by adding 5.0 mL of 0.25 mol.L⁻¹ sodium methoxide solution in methanol/diethyl ether (1:1, v/v) to approximately 150 mg of lipids, with stirring for 3 min. Next, 2.0 mL iso-octane and 10.0 mL of saturated NaCl solution were added, and the tube was agitated again and allowed to stand for phase separation. The supernatant was transferred to duly identified Eppendorf flasks for further chromatographic analysis (Bannon et al., 1982).

The FAME were separated using a Trace-GC-Ultra gas chromatograph (Thermo Finnigan, Milan, Italy), equipped with a fused silica BPX-70 capillary column (120 m, 0.25 mm film thickness; Thermo Finnigan), a flame ionisation detector and an automatic injector (Thermo Finnigan). The gas flows (White Martins, São Paulo, Brazil) were 6.5 mL.min⁻¹ for the entrainment gas (H₂); 30 mL.min⁻¹ for the auxiliary gas (N₂); 30 mL.min⁻¹ for H₂ and 250 mL.min⁻¹ for the synthetic flame air. The sample split ratio was 90:10. The volumes of the injections were 1.2 μL. The peak areas of the FAME were determined using ChromQuest 4.1 software (Thermo Finnigan, Milan, Italy).

The FAME were identified after checking the equivalent chain length of the peaks, evaluating the flame ionisation detector response and comparing the retention times of methyl esters of fatty acids containing cis-9, trans-11 and trans-10, cis-12 linoleic acids (189-19, O-5632 and O-5626, Sigma-Aldrich, Saint Louis, USA).
The fatty acids (mg·g⁻¹ total lipids) were quantified using methyl tricosanoate (C23:0) (Sigma) as the internal standard. Before transesterification of the weighed lipid samples (=150 mg), 1000 μL of the internal standard solution of known concentration (1.00 g·mL⁻¹) was added. For quantification, the theoretical response factors were used, after verifying the agreement of these values with the experimental ones.

**Cholesterol content**

The cholesterol content was analysed by direct saponification and hexane extraction (Bauer et al., 2014) using a high-performance liquid chromatograph model SPD-M20A (Shimadzu, Kyoto, Japan), with a quaternary solvent system, injection valve with 20 μL sampling loop, column furnace and diode arrangement detector. An analytical C18 column, 15 cm × 6 mm × 5 mm (Shimadzu, Kyoto, Japan) was used for the cholesterol quantification, which was done through external standardisation (Golay et al., 2016).

**Statistics**

The results were interpreted using analysis of variance and regression analysis. Statistical models were chosen, according to the level of significance and determination coefficients (R²), using the F test and α = 0.05 (R Core Team, 2015).

**3 Results and discussion**

**3.1 Chemical composition**

The DOP standard of MBC was used as a guideline to classify the cheese manufactured in our study (European Union, 1996). The buffalo mozzarella presented fat and moisture contents within the standards established in Italy for MBC (Table 2), indicating that these characteristics are peculiar to buffalo mozzarella.

The crude protein content in the buffalo milk varies between 3.00-3.28% (Feltes et al., 2016; Bondan et al., 2018). Considering these facts, the addition of milk of lower crude protein content to the cheese-making mixture should decrease the cheese crude protein content. The observed decrease in the crude protein content was close to 10.70%. The other factors evaluated, including the moisture, ash and TS, were not affected by the inclusion of bovine milk (P > 0.05), except for palmitoleic acid (C16:1, P = 0.040), which decreased linearly. Some of the C16:0 originates via de novo synthesis in the mammary gland, with β-hydroxybutyrate as the precursor (Bauman & McGuire, 2011). The activity of Δ9-desaturase enzyme is higher in the mammary gland of buffaloes when compared with the mammary gland of cows (Fernandes et al., 2007). Thus, the higher C16:0 concentration in buffalo milk, associated with the higher activity of Δ9-desaturase in the buffalo mammary gland, may explain these results.

The addition of cow milk did not affect (P > 0.05) the PUFA composition of buffalo mozzarella cheese (Table 3). It is possible to find some reports about the effects of ingestion of fatty acids by humans. There is convincing scientific evidence that some PUFA positively affects health (Glick & Fischer, 2013), among them, the rumenic acid (C18:2 cis-9, trans-11), the main

**3.2 Fatty acid composition**

Twenty-four fatty acids were identified and quantified in cheese fat (Table 3). In decreasing order, palmitic (C16:0), oleic (C18:1 n-9cis), myristic (C14:0) and stearic (C18:0) fatty acids were the most concentrated in all treatments, consistent with the trend observed by Romano et al. (2011) when evaluating MBC. Conversely, (Bergamo et al., 2003) and Martini et al. (2016) observed a different order; C16:0 > C18:1 > C18:0 > C14:0. Among the SFA, butyric (C4:0, P = 0.012), palmitic (C16:0, P = 0.014) and behenic acids (C22:0, P = 0.023) decreased linearly with the inclusion of cow milk. In turn, the caprylic acid (C8:0, P = 0.006) and capric acid (C10:0, P = 0.002) contents increased.

Buffaloes present a more significant degradation of the dietary fibre in the rumen compared with cows and, consequently, more of the volatile fatty acids (acetate, butyrate and propionate); however, dietary fiber also generate a higher molar ratio of acetate and butyrate, precursors of the short-chain SFA observed in milk (Terramoccia et al., 2005; Shen et al., 2019), especially C4:0 (starter). These facts may explain the decrease in C4:0 content with the inclusion of bovine milk since studies have indicated a higher content of C4 in buffalo milk than cow milk (Zotos & Bampidis, 2014; Correddu et al., 2017; Pegolo et al., 2017; Teng et al., 2017). Accordingly, the pure mozzarella had a higher C4 content than the others prepared from the blended milk. It is interesting to note that higher levels of C4 in foods are sought because of beneficial effects on the human body, such as antiinflammatory, anti-inflamatorie and apoptotic properties (Mills et al., 2011; Teng et al., 2017). The MUFA were not affected by the inclusion of bovine milk in buffalo milk (P > 0.05), except for palmitoleic acid (C16:1, P = 0.040), which decreased linearly. Some of the C16:0 originates via de novo synthesis in the mammary gland, with β-hydroxybutyrate as the precursor (Bauman & McGuire, 2011). The activity of Δ9-desaturase enzyme is higher in the mammary gland of buffaloes when compared with the mammary gland of cows (Fernandes et al., 2007). Thus, the higher C16:0 concentration in buffalo milk, associated with the higher activity of Δ9-desaturase in the buffalo mammary gland, may explain these results.

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**Table 2.** Chemical composition of buffalo mozzarella frauded with cow milk.

| Variables | 0   | 10  | 20  | 30  | 40  | 50  | L   | Q  | C   | P-value¹ |
|-----------|-----|-----|-----|-----|-----|-----|-----|----|----|-------|
| FDM (%)²  | 61.0| 54.0| 52.2| 54.0| 51.3| 51.4| 0.122| 0.003| 0.097|
| Protein (%)³ | 23.8| 22.3| 22.3| 20.8| 20.8| 20.2| 0.000| 0.383| 0.809|
| Moisture (%) | 46.3| 45.6| 46.4| 48.1| 46.6| 47.5| 0.206| 0.885| 0.527|
| Ash (%) | 2.89| 2.90| 3.04| 2.98| 3.12| 2.85| 0.375| 0.425| 0.212|
| TS (%) | 53.7| 54.4| 53.6| 51.9| 53.4| 52.5| 0.206| 0.885| 0.527|

¹P-value (p<0.05); L. Q. and C: linear. quadratic. and cubic effects. respectively; ²y = 0.0067x² - 0.4705x + 59.578 (R² = 0.86); ³y = -0.0688x + 23.424 (R² = 0.92); FDM = Fat in dry matter; TS = total solids.
Table 3. Fatty acid composition of buffalo mozzarella frauded with cow milk (mg·g⁻¹).

| Fatty acid | Cow milk level (%) | P-Value¹ |
|-----------|--------------------|----------|
|           | 0      | 10     | 20     | 30     | 40     | 50     | L | Q | C |
| *C14:0*²  | 60.57  | 63.48  | 55.92  | 42.67  | 54.51  | 45.77  | 0.012 | 0.647 | 0.501 |
| C6:0      | 27.00  | 26.95  | 26.48  | 21.97  | 28.24  | 26.35  | 0.767 | 0.236 | 0.786 |
| *C8:0*³   | 11.15  | 11.77  | 11.56  | 10.78  | 13.44  | 13.22  | 0.006 | 0.144 | 0.793 |
| *C16:1⁴*  | 19.17  | 19.19  | 21.21  | 20.00  | 24.06  | 25.28  | 0.002 | 0.240 | 0.935 |
| C12:0     | 26.27  | 25.67  | 27.65  | 26.18  | 25.95  | 30.09  | 0.699 | 0.646 | 0.551 |
| C14:0     | 138.79 | 140.39 | 136.35 | 127.31 | 134.13 | 129.16 | 0.080 | 0.989 | 0.832 |
| C15:0     | 17.35  | 16.88  | 17.03  | 15.67  | 17.41  | 16.41  | 0.202 | 0.636 | 0.461 |
| *C16:0*⁵  | 405.08 | 413.63 | 386.61 | 337.10 | 372.16 | 341.28 | 0.014 | 0.783 | 0.538 |
| C17:0     | 10.54  | 11.42  | 10.68  | 9.85   | 10.91  | 7.44   | 0.060 | 0.144 | 0.532 |
| C18:0     | 105.83 | 114.19 | 106.34 | 109.54 | 134.86 | 106.36 | 0.911 | 0.684 | 0.845 |
| C20:0     | 2.00   | 1.63   | 1.79   | 1.90   | 2.15   | 1.67   | 0.805 | 0.957 | 0.063 |
| *C22:0*⁶  | 0.84   | 0.82   | 0.80   | 0.76   | 0.69   | 0.67   | 0.023 | 0.660 | 0.598 |

¹ P-value = p > 0.05; L, Q, and C: linear, quadratic, and cubic effects, respectively.
² ŷ = -0.3262x + 61.974 (R² = 0.56);
³ ŷ = 0.0417x + 10.945 (R² = 0.51);
⁴ ŷ = 0.1256x + 18.346 (R² = 0.81);
⁵ ŷ = -1.4083x + 411.19 (R² = 0.68);
⁶ ŷ = -0.0037x + 0.8548 (R² = 0.95);
²̇ ŷ = -0.084x + 18.118 (R² = 0.95).

conjugated linoleic acid isomer (CLA), playing a significant role (Rodríguez-Alcalá et al., 2014). In this study, rumenic acid varied between 68-71% of the total CLA. Previous work indicated that the C18:2 cis-9 trans-11 content in mozzarella cheese can account for more than 80% of the CLA in milk (Romano et al., 2011), but factors, such as milk origin and processing, may determine variations (Martini et al., 2016; Ruiz et al., 2016).

However, the controversy regarding the effects of SFA is evident due to the complexity of the interaction of fatty acids and other biomolecules present in milk (Gómez-Cortés et al., 2018). In this group, myristic (C14:0), palmitic (C16:0) and lauric (C12:0) acids, which have a hypercholesterolemic action, but C14:0 is the most active (Ulbricht & Southgate, 1991). Among these fatty acids, only C16:0 was affected (P = 0.0014), decreasing with the inclusion of cow milk. In this sense, the origin of the milk (species) can explain the results observed in this work since buffaloes and cows were fed the same diet (Bracharia decumbens and B. ruziziensis pasture).

### 3.3 Nutritional indices of cheeses

The inclusion of cow milk for the production of buffalo mozzarella cheese did not alter the sum of fatty acids and nutritional quality indices (P > 0.05), except for the cholesterol content (Table 4). Linoleic acid (C18:2 n-6) and α-linolenic (C18:3 n-3) are precursors of several metabolites, such as eicosanoids, thromboxanes, prostaoyclins, prostaglandins and leukotrienes, which are associated with immune and inflammatory responses (Riccioi & Fitzgerald, 2012; Samuelsson, 2012; Glick & Fischer, 2013). These fatty acids are known as precursors of important metabolites (Martini et al., 2016). Studies indicate that diets rich in linoleic acid can favour the formation of eicosanoids from arachidonic acid, which, in turn, should favour the synthesis of inflammatory eicosanoids (Glick & Fischer, 2013; Harnack et al., 2009). Contrariwise, the ingestion of foods rich in n-3 fatty acids causes an increase in the formation of docosahexaenoic acid (DHA) and eicosapentaenoic acid, forming anti-inflammatory eicosanoids (Riccioi & Fitzgerald, 2012; Samuelsson, 2012). The scientific community has been studying the relationship between the intake of essential fatty acids and cardiac diseases (Simopoulos, 2008; Harnack et al., 2009; Russo, 2009; Simopoulos, 2016; Sheppard & Cheatham, 2018). In general, the n-6/n-3 ratio intake ranging from 1:1 to 10:1 (Chardigny et al., 2001; Harnack et al., 2009; Russo, 2009; Harnack et al., 2009; Russo, 2009). In an influential article, (Masters (1996) indicated the ideal ratio as 2-3:1, because this ratio preferentially favours the conversion of α-linolenic acid to DHA, which leads to the balanced intake of these fatty acids. Thus, the data observed in this study put the cheeses studied among the foods with adequate n-6/n-3 ratios (Marshall & van der Meij, 2018).
The rumenic acid/ΣCLA ratio did not change with the increase of cow milk in the buffalo milk for the production of mozzarella, remaining around 2 because the respective fatty acids did not change. These are the same for the total trans/total trans fatty acid ratio (Table 3). However, there was an increasing effect (P < 0.05) in the cheese mozzarella with the inclusion of cow milk (Table 3). With the inclusion of cow milk into mozzarella processing, the cholesterol content increased to 32%. Buffalo milk contains less cholesterol compared with cow milk (Pignata et al., 2014; Manuelian et al., 2017), and the fraud of mozzarella with the inclusion of cow milk to the mixture and consequent increase in the content of cholesterol, altered an important nutritional parameter for consumers of animal products. Buffalo milk fraud is being fought on a legal basis in many countries, especially in Europe (European Union, 1996; Russo et al., 2012) and frequently occurs despite advanced food fraud technologies (Zarei et al., 2016). Many techniques exist to detect fraud in milk and buffalo derivatives (Cazzolo et al., 2002; Enne et al., 2005; Feligini et al., 2005; Czerwenka et al., 2010; Pesic et al., 2011; Poonia et al., 2017), but they are expensive, time-consuming and require skilled technicians, and safety when working with reagents, but these techniques are accurate (Roncada et al., 2012). Thus, the use of analytical techniques that are cheaper and precede proper techniques exist to detect fraud in milk and buffalo derivatives advanced food fraud technologies (Zarei et al., 2016). Many techniques exist to detect fraud in milk and buffalo derivatives (Cazzolo et al., 2002; Enne et al., 2005; Feligini et al., 2005; Czerwenka et al., 2010; Pesic et al., 2011; Poonia et al., 2017), but they are expensive, time-consuming and require skilled technicians, and safety when working with reagents, but these techniques are accurate (Roncada et al., 2012). Thus, the use of analytical techniques that are cheaper and precede proper quantitative techniques may help to combat fraud of buffalo milk derivatives, due to the possibility of increasing the number of samples evaluated. The results of the current study showed an increase in the amount of cholesterol in buffalo mozzarella, due to the inclusion of cow milk. Since the cholesterol content is lower in buffalo milk than cow milk (Zotos & Bampidis, 2014; Manuelian et al., 2017), being a species-specific parameter, samples that present a non-standard cholesterol content of the species should be sent for qualitative and confirmatory tests (Roncada et al., 2012; Zarei et al., 2016).

### Table 4. Effect of the inclusion of cow milk to buffalo milk on the nutritional quality of buffalo mozzarella.

| Fatty acid | Cow milk level (%) | P-Value<sup>1</sup> |
|------------|--------------------|---------------------|
|            | 0                  | 10                  | 20                  | 30                  | 40                  | 50                  | L       | Q       | C       |
| Sum (mg.g<sup>-1</sup>) | 824.92             | 846.27              | 802.65              | 718.06              | 799.66              | 735.12              | 0.058   | 0.832   | 0.664   |
| Σ SFA<sup>2</sup>     | 230.58             | 252.31              | 238.95              | 229.79              | 273.31              | 216.59              | 0.907   | 0.299   | 0.429   |
| Σ MUFA<sup>3</sup>    | 18.78              | 20.39               | 17.79               | 17.15               | 19.94               | 17.28               | 0.475   | 0.879   | 0.814   |
| Σ PUFA<sup>4</sup>    | 10.32              | 9.59                | 9.19                | 8.90                | 9.97                | 8.88                | 0.492   | 0.730   | 0.534   |
| Σ CLA<sup>5</sup>     | 31.54              | 29.45               | 27.95               | 25.19               | 29.86               | 26.49               | 0.207   | 0.178   | 0.245   |
| Σ Trans<sup>6</sup>   | 4.97               | 6.47                | 5.75                | 4.85                | 6.25                | 5.60                | 0.564   | 0.566   | 0.169   |
| Σ n-6<sup>7</sup>     | 3.75               | 4.32                | 2.85                | 3.40                | 3.72                | 2.79                | 0.197   | 0.689   | 0.526   |
| Σ n-3<sup>8</sup>     | 76.1               | 74.6                | 77.1                | 82.1                | 82.6                | 88.4                | 0.014   | 0.154   | 0.216   |

<sup>1</sup>P-Value = p > 0.05; L, Q and C: linear, quadratic = and cubic effects, respectively; <sup>2</sup>Σ of the saturated fatty acid; <sup>3</sup>Σ of the monounsaturated fatty acid; <sup>4</sup>Σ of the polyunsaturated fatty acid; <sup>5</sup>Σ of the conjugated linoleic acid; <sup>6</sup>Σ of the trans fatty acid; <sup>7</sup>Σ of the ømega-6; <sup>8</sup>Σ of the ømega-3; <sup>9</sup>Polysaturated fatty acid/Saturated fatty acid ratio; <sup>10</sup>Ømega-6 and ømega-3 ratio; <sup>11</sup>Desirable fatty acid; <sup>12</sup>Rumenic acid and total conjugated fatty acid ratio; <sup>13</sup>Vaccenic fatty acid and total trans fatty acid ratio; <sup>14</sup>𝑦 = 0.3871x + 68.95 (R<sup>2</sup> = 0.95).

### 4 Conclusion

The addition of cow milk to buffalo milk for mozzarella production altered the content of short-chain SFA and the cholesterol content, thereby modifying the nutritional indices.

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