

# Potential biogenic amine-producing bacteria in ripened cheeses\*

## Bactérias potencialmente produtoras de amins biogênicas em queijos maturados

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### Abstract

This study aimed to determine which of the eight cheese varieties (Prato, Standard Minas, Gorgonzola-, Moleson-, Raclette-, Gruyère-, Sbrinz- and Reblochon-types) prepared at a dairy processing plant in the state of Rio de Janeiro had higher concentration of biogenic amines (BA) (putrescine, cadaverine, tyramine, histamine, spermidine and spermine), to detect which BA were produced at higher concentrations and to determine if the presence of *Enterobacteriaceae*, biogenic amine producing bacteria (BAPB) or physical-chemical parameters (pH, titratable acidity, fat, moisture, total solids, protein, ash and chloride) would be correlated with BA production in the eight matured cheese varieties. Moleson-type cheese (72.50 mg.Kg<sup>-1</sup>) followed by Standard Minas (107.00 mg.Kg<sup>-1</sup>) showed the lowest levels of biogenic amines. Prato (699.29 mg.Kg<sup>-1</sup>) and Gorgonzola-type (936.37 mg.Kg<sup>-1</sup>) cheeses contained larger amounts of BA. Concentrations of tyramine exceeded the maximum permissible limit in all varieties of cheese. Although the presence of potentially BA-producing bacteria was confirmed in all samples of cheese, there was no correlation with BA content produced in cheeses. Gorgonzola-type cheese showed a positive correlation with the amount of BA in the isolates. Gorgonzola-type, Sbrinz-type and Prato cheeses seem to require greater care in monitoring the presence of biogenic amines, particularly because tyramine reached the highest levels in these varieties. Regardless of the analysed cheese, physical and chemical parameters did not affect the amount of BA produced. An assessment of the capacity to produce biogenic amines should be included as a selection criterion for starter cultures for ripened cheeses.

**Keywords:** biogenic amines, cheese, maturation, starter culture.

### Resumo

Este estudo objetivou determinar qual das oito variedades de queijo (Prato, Minas Padrão, Gorgonzola-, Moleson-, Raclette-, Gruyère-, Sbrinz- e Reblochon-tipos), preparadas em uma fábrica de processamento de laticínios no estado do Rio de Janeiro teve maior concentração de amins biogênicas (AB) (putrescina, cadaverina, tiramina, histamina, espermidina e espermina), detectar quais AB foram produzidas em concentrações mais elevadas e determinar se a presença de *Enterobacteriaceae*, bactérias produtoras de amins biogênicas (BPAB) ou parâmetros físico-químicos (pH, acidez titulável, gordura, umidade, sólidos totais, proteína, cinzas e cloretos) estariam correlacionados com a produção de AB nas oito variedades de queijo maturados. O queijo tipo Moleson (72,50 mg.Kg<sup>-1</sup>) seguido por Minas Padrão (107,00 mg.Kg<sup>-1</sup>) apresentaram os menores níveis de amins biogênicas. Os queijos Prato (699,29 mg.Kg<sup>-1</sup>) e tipo Gorgonzola (936,37 mg.Kg<sup>-1</sup>) continham grandes quantidades de AB. Concentrações de tiramina excederam o limite máximo permitido em todas as variedades de queijo. Embora a presença de bactérias potencialmente produtoras de AB tenha sido confirmada em todas as variedades de queijo, não houve correlação entre essa informação e o teor de AB. Entretanto, o queijo tipo Gorgonzola mostrou uma correlação positiva com a quantidade de AB nos isolados. Queijos tipo Gorgonzola, tipo Sbrinz e Prato parecem exigir um maior cuidado na monitorização da presença de amins biogênicas, particularmente porque a tiramina atingiu os níveis mais elevados nestas variedades. Independentemente do queijo analisado, parâmetros físico-químicos não afetaram a quantidade de AB produzida. A avaliação da capacidade de produção de amins biogênicas deve ser incluída como critério de seleção para as culturas iniciadoras para queijos maturados.

**Palavras-chave:** amins biogênicas, cultura iniciadora, maturação, queijo

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## Introduction

Biogenic amines (BA) are low-molecular-weight organic bases that possess biological activity (Aliakbarlu et al., 2009; Romano et al., 2012). The presence of BA in foods is of interest both because of their possible toxicity and also because they can be used as quality indicators for freshness or spoilage of cheese (Awan et al., 2008; Cunha et al., 2012).

Those amines have been identified in many varieties of cheese, including Cheddar, Ras, Gouda (Ibrahim and Amer, 2010), Spanish traditional cheeses (Roig-Sagués, 2002), Dutch-type hard cheeses (Komprda et al., 2007), Italian Pecorino cheese (Schirone et al., 2012) and others (Cunha et al., 2012). They generally result from enzymatic decarboxylation (Qureshi et al., 2013) of free amino acids, which are primarily released by degradation of cheese proteins (Ibrahim and Amer, 2010).

The most important BA found in cheeses are histamine, tyramine, putrescine and cadaverine (Linares et al., 2012; Qureshi et al., 2013). Various factors that affect BA production have been studied in detail in popular varieties of cheese, including ripening time, ripening temperature, pH, and the presence of microorganisms that can produce BA (Komprda et al., 2012; Linares et al., 2012; Qureshi et al., 2013).

The production of BA in cheeses has often been linked to starter and nonstarter lactic-acid bacteria (LAB) such as *Lactobacillus* and *Enterobacteriaceae*, respectively (Aliakbarlu et al., 2009; Herrero-Fresno et al., 2012) and other species of Gram-negative and positive bacteria (Linares et al., 2012; Schirone et al., 2013). Species of many genera including *Lactobacillus*, *Bacillus*, *Citrobacter*, *Clostridium*, *Escherichia*, *Klebsiella*, *Listeria*, *Photobacterium*, *Proteus*, *Pseudomonas*, *Salmonella*, *Shewanella*, *Shigella*, and *Plesiomonas* are capable of decarboxylating one or more kinds of amino acid (Papavergou et al., 2012).

The main BA producers in cheese are Gram-positive bacteria, with LAB being the main histamine and tyramine producers. The genera *Enterococcus*, *Lactobacillus*, *Leuconostoc* and *Streptococcus* include some strains that have been described as BA producers. These can be present in milk microbiota or introduced through contamination before, during or after the processing of dairy products. BAP-LAB may even form part of the starters or adjunct cultures. Linares et al. (2010) have reported the presence of tyrosine and histamine decarboxylase activity in strains from various starter cultures.

It is therefore important to include the inability to produce BA as an indispensable condition of strains intended to be used as starters (Spano et al., 2010).

Cheese is among the foods that are most commonly implicated in histamine poisoning and tyramine toxicity (EFSA, 2011). Cheeses with high BA contents are associated with pediatric and adolescent migraine (Komprda et al., 2007; Millichap and Yee, 2003). Tyramine-rich foods such as cheese show a close relationship to migraine crises, and the toxic effects have been termed the cheese reaction (Schirone et al., 2013; Rodriguez et al., 2014).

The growing interest in cheeses is partly due to the uniqueness of these products, in which specialized microorganisms can grow and contribute to their organoleptic and qualitative characteristics.

However, these dairy products are often manufactured under poor or uncontrolled hygiene conditions; in addition, they are produced following different protocols, which can vary from one cheesemaker to another. Many cheesemakers use raw milk, considering it essential to produce stronger and more pleasant flavors than pasteurized milk, primarily due to greater proteolysis and lipolysis by the raw-milk microbiota in the cheese (Peter et al., 2016). This microbiota plays a major role in the development of the organoleptic characteristics of cheeses, but it can also be responsible for the accumulation of undesirable substances such as BA (Ladero et al., 2010; Suzzi and Gardini, 2003). In addition, the starter cultures, if not properly selected, may represent a risk of BA formation because the strains have different proteolytic and aminogenic properties (Latorre-Moratalla et al., 2014).

There is no doubt regarding the importance of selecting starter strains unable to synthesize BA. Knowledge of the metabolic pathways involved in BA production and the factors affecting BA accumulation in food may also be useful in suggesting possible means of reducing BA contents. Although BA are present in many different foods and beverages and their concentrations vary widely between and within food types, a shared regulation limiting the amounts of BA in foods is still lacking (except for histamine in fish) (Spano et al., 2010).

Information regarding their presence in foods is also important for the food trade sector (in particular import and export) because recommended upper levels of BA content vary between countries. Therefore, even though information on BA is currently not included in food composition databases, information on their existence, distribution and concentration in fermented foods is crucial and may be useful for the food industry, health professionals and consumers (Spano et al., 2010).

The process of producing cheeses still has limitations in terms of control of these compounds, as well as screening of the bacterial strains used. Therefore, this study aimed to determine which of the eight cheese varieties (Prato, Standard Minas, Gorgonzola-, Moleson-, Raclette-, Gruyère-, Sbrinz- and Reblochon-types) prepared in a dairy processing plant in the state of Rio de Janeiro had the highest concentrations of BA (putrescine, cadaverine, tyramine, histamine, spermidine and spermine), check if the presence of *Enterobacteriaceae*, biogenic amine producing bacteria (BAPB) or physico-chemical parameters (pH, titratable acidity, fat, moisture, total solids, protein, ash and chlorides) would be correlated with BA production in the eight varieties of matured cheese.

## Materials and methods

### Samples

Three samples of each variety of cheese (Gorgonzola-type, Moleson-type, Raclette-type, Gruyère-type, Sbrinz-type, Reblochon-type, Prato, and Standard Minas), from three different batches, were purchased from a dairy processing plant in the mountains in the state of Rio de Janeiro, Brazil. The samples were transported to the laboratory in insulated polystyrene boxes on ice, in their original commercial packages (500g for each package in low-density polyethylene bags). After sharing portions representative sampling of each cheese of 25g and 10g for each of the three batches and each of the 3 repetitions, a total of 315g were used for *Enterobacteriaceae* Count and Biogenic

amine-producing bacteria analysis and the remaining 185g for Physical and Chemical analysis of these same batches and repetitions. The analyzes were performed in the laboratories of Microbiological Control, Physical-Chemical Control and Milk and Dairy Products at Federal Fluminense University.

### Bacteriological Analysis

**Enterobacteria Count.** *Enterobacteriaceae* were counted using Violet Red Bile Glucose Agar, as recommended by APHA (2001).

**Biogenic amine-producing bacteria.** We used the method proposed by Izquierdo et al. (2003). Ten grams of each sample was weighed and homogenized with 90 mL of 0.9% peptone water. From this dilution ( $10^{-1}$ ), serial dilutions to  $10^{-5}$  were seeded in duplicate onto plates containing De Man, Rogosa and Sharpe agar (MRS) using *pour plate* - double-layer agar (to help the microbiota that grows in low oxygen tension as anaerobic or microaerophilic conditions), and incubated at 37 °C, the same temperature used by Izquierdo et al. (2003). Typical colonies were Gram-stained, and the bacterial morphology was observed by means of a stereoscopic microscope using incident and transmitted light. Biochemical tests for oxidase and catalase were also performed.

In order to determine their capacity to produce the amines tyramine, cadaverine, histamine, putrescine, spermine and spermidine, typical bacterial colonies were lifted from the agar and transferred into four different tubes containing MRS broth, 37 mM pyridoxine or vitamin B6, supplemented with 2% of each precursor amino acid separately (first tube: tyrosine; second tube: lysine; third tube: l-histidine; fourth tube: ornithine) and incubated at 35-37 °C for 24 h. The tubes containing MRS broth, vitamin B6 and one of the four amino-acid precursors were tested and selected by the same method of extraction and derivatization for detection and quantification of BA by HPLC.

**Physical and Chemical Analyses.** The following parameters were assessed: pH; titratable acidity (AOAC, 2012); ash (AOAC 2012); fat, determined by the Van Gulik Butyrometer (NEN, 3059); sodium chloride (Legraet; Brulé 1988); total nitrogen content (FIL-IDF, 1993); nitrate; nitrite; and total solids, following the recommendations of the AOAC (2012). Moisture content was measured in a Mettler® LJ 16 infrared moisture analyzer.

### Biogenic amine determination by HPLC

**Preparation of Standards.** Standards of tyramine ( $C_8H_{11}NO$ ), histamine ( $C_5H_9N_3$ ), putrescine ( $C_4H_{12}N_2$ ), cadaverine ( $C_5H_{14}N_2$ ), spermidine ( $C_{14}H_{47}N_6O_{12}P_3$ ), and spermine ( $C_{10}H_{26}N_4$ ) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Stock solutions for each amine ( $40 \mu\text{gL}^{-1}$ ) were prepared in 0.1 N HCl and stored at  $4 \pm 1$  °C. For each phase, stock solutions were diluted with Milli-Q water (Simplicity UV, Millipore, Molsheim, France) and alkalized with 2 N NaOH Sigma-Aldrich® (St. Louis, MO, USA) until pH > 12 was reached. Samples were derivatized using benzoyl chloride (40  $\mu\text{L}$ ) Sigma-Aldrich® (St. Louis, MO, USA), homogenized (vortex, 15 s), and kept at room temperature for 20 min. The mixture was extracted two times with 1,000  $\mu\text{L}$  of diethyl ether Sigma-Aldrich® (St. Louis, MO, USA). The ether layer was aspirated and evaporated to dryness under a stream of nitrogen (Sample Concentrator Techne®, Cambridge, UK). Finally, the residue was dissolved in 1,000  $\mu\text{L}$  of the mobile phase and stored at  $4 \pm 1$  °C (Cunha et al., 2012; Lázaro et al., 2013).

**Sample Preparation.** For BA extraction, 5g of minced cheese was homogenized with 5 mL of 5% perchloric acid Sigma-Aldrich® (St. Louis, MO, USA). The homogenates were kept under refrigeration ( $4 \pm 2$  °C) for 1 h and shaken continuously (Certomat® MV, B. Braun Biotech International, Melsungen, Germany). Then, the mixture was centrifuged at  $503 \times g$  for 10 min at  $4 \pm 1$  °C (Hermle Z 360 K) and filtered through Whatman no. 1 filter paper. The filtrates were neutralized (pH > 6) with 2 N NaOH and placed in an ice bath ( $0 \pm 2$  °C) for approximately 20 min, followed by a second filtration, and addition of NaOH (pH > 12) under the same conditions. The derivatization procedure was carried out in the same way for the standards (Cunha et al., 2012; Lázaro et al., 2013).

**Chromatographic conditions.** The samples of cheese and the tubes containing bacterial strains with possible potential to produce BA were tested by this method. The chromatographic system consisted of a LC/10AS pump coupled to a SPD/10AV UV-Vis detector and a C-R6A chromatopack integrator (Shimadzu, Kyoto, Japan). BA separations were performed on a Teknokroma Tracer Extrasil ODS2 (15×0.46 cm id., 5  $\mu\text{m}$ ) column equipped with a Supelco Ascentis C18 (2×0.40 cm id., 5  $\mu\text{m}$ ) guard column, under isocratic conditions. The mobile phase was prepared by mixing acetonitrile (Tedia®) and Milli-Q water, 42:58 (v/v); the mixture was degassed in an ultrasonic bath (Cleaner USC 2800 A). The flow rate was  $1 \text{ mL} \cdot \text{min}^{-1}$ , the injected volume was 20  $\mu\text{L}$ , the column temperature was 20 °C, and the detector wavelength was set at 198 nm. Injections were performed using a 50  $\mu\text{L}$  syringe (Hamilton Microliter™ 705), and the total run time was 15 min. Between each sample, the HPLC system was flushed with an injection of pure acetonitrile for 10 min. The BA were identified by retention time and were quantified by peak area (Lázaro et al., 2013).

The chromatographic method used resulted in the following retention times (min) for each amine: tyramine (2.7), putrescine (4.6), cadaverine (5.6), spermidine (7.1), spermine (11.7) and histamine (12.9).

### Statistical Analysis

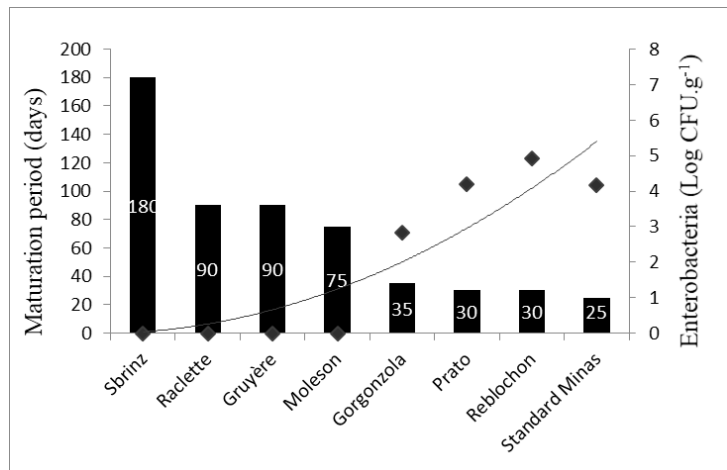
Data were analyzed by one-way ANOVA, using the GraphPad Prism® 5.00 package (GraphPad, 2007) for Windows. The means were compared with a Tukey test ( $p < 0.05$ ) to check for significant differences between the independent and dependent variables. The Pearson product (r) was calculated to assess linear correlations between the physical and chemical parameters, and the different varieties of cheese.

## Results

**Bacteriological Analysis.** Moleson-, Sbrinz-, Raclette- and Gruyère-type cheeses did not show enterobacteria growth. Regarding BAPB, only three varieties of cheese (Raclette-type 2.80 log, Gruyère-type 5.91 log and Prato 2.62 log) showed counts below the mean of 6.73 log. The other BAPB growth results were Sbrinz-type cheese 8.85 log, Reblochon-type cheese 8.41 log, Gorgonzola-type cheese 8.28 log, Standard Minas 8.23 log and Moleson-type cheese 7.49 log. These data suggest that both the starter bacteria and the secondary culture might be causing an increase in BA in all cheeses. The counts of *Enterobacteriaceae* in search of potentially BA-producing bacteria consisted only of isolation and identification of colony morphology (red colonies)



and colony-forming units. It is understood that this constitutes a limitation of the bacterial analysis, since the bacterial strains were not identified. As shown in Figure 1, *Enterobacteriaceae* were detected in cheeses matured for less than 35 days; in particular, Reblochon-type cheese had the highest enterobacteria count (4.9 Log CFU.g<sup>-1</sup>).



**Figure 1:** Relationship between ripening time (days, indicated by bars) and population of *Enterobacteriaceae* at end of ripening period (Log CFU.g<sup>-1</sup>, indicated by diamonds) in eight varieties of cheese. Curved line represents the overall trend for the enterobacteria populations.

**Biogenic Amines.** In the bacterial quantification carried out directly on the samples of cheese, all five BA evaluated were detected in Gorgonzola-type cheese (tyramine, putrescine, cadaverine, spermidine and spermine). In Reblochon, Raclette and Sbrinz-type and Standard Minas cheeses, tyramine, putrescine and cadaverine were detected. In Gruyère-type cheese, tyramine, putrescine, cadaverine and spermidine, and in Moleson-type cheese, only tyramine and putrescine were detected (Table 1). Moleson-type cheese (72.5 mg.Kg<sup>-1</sup>), followed by Standard Minas showed the lowest BA levels (107.0 mg.Kg<sup>-1</sup>). Larger amounts were found in Gorgonzola-type (936.37 mg.Kg<sup>-1</sup>), Prato (699.29 mg.Kg<sup>-1</sup>) and Sbrinz-type (566.51 mg.Kg<sup>-1</sup>).

However, as shown in Table 2, in bacterial plate isolated from Moleson-, Raclette- and Sbrinz-type cheeses, no BA was detected. Isolates from Reblochon-type cheese showed the highest BA content (619.42 mg.Kg<sup>-1</sup>). In isolates from Standard Minas cheese, putrescine, cadaverine and spermidine were detected; in Prato, five BA were found; Reblochon-type contained all BA except spermidine; Gorgonzola-type contained tyramine, cadaverine and spermidine; and Gruyère-type contained only spermidine. Histamine was not detected in any cheese sample (Table 1 and 2).

**Physical and Chemical Analyses.** The chemical composition of the eight varieties of cheeses is shown in Table 3. The cheeses differed significantly ( $p < 0.05$ ) in chemical composition, indicating that they were affected by differences in formulation and processing, as expected. Prato cheese showed the highest pH (6.11) and the lowest titratable acidity (24 °D), already Sbrinz- and Gorgonzola-type showed lower pH (5.35 and 5.92 respectively) and higher titratable acidity (87 °D and 76 °D respectively).

Raclette-type cheese showed the highest fat and protein content (26.97% and 16.04% respectively). Reblochon-type cheese showed the highest water content (52%). Gorgonzola-type cheese showed the highest content of total solids (76.30%). Moleson-, Sbrinz- and Gruyère-type cheeses also showed relatively high NaCl (1.10%, 1.44% and 1.28% respectively) contents and consequently high ash values (3.51%, 3.56% and 3.57% respectively).

## Discussion

**Bacteriological Analysis.** During the steps of bacterial isolation, the bacteria were subjected to subculturing with specific conditions of medium and incubation. However, these bacteria were isolated from cheese, which showed different concentrations of free amino-acid precursors. Moreover, changes in culture media, variations in temperature (ambient temperature and incubation) and pH harm or promote sometimes sublethal damage in the bacteria. These factors may explain the failure to detect some BA in isolates from the BAPB colony-forming units tested. However, in the cheese samples, the strains characteristic of each cheese remain present during the entire maturation process and have a higher chance of producing BA, and they were detected by the HPLC method (Cunha et al., 2012; Lázaro et al., 2013). There may have been selection pressure during the preparation of the cheese, and the predominant remaining bacterial strains may have been capable of producing only one or a

few kinds of amines. In summary, the same bacteria involved in cheese production may be used *in vitro*, but the BA production results may be totally different because of secondary aspects like bacterial injury, cofactors vitamins, temperature and pH variation, concentration and kind of amino-acids; this can explain the quantification of BA in samples of cheese and in the isolates were not similar.

Enterobacteria were not found in cheese ripened over 75 days. Similarly, Psoni et al. (2003) and Macedo et al. (2004) found high numbers of enterobacteria in different cheeses produced in the Mediterranean, prepared from raw goat's and sheep's milk after 30 days of ripening; and Serio et al. (2006) found 10<sup>3</sup> CFU.g<sup>-1</sup> and 10<sup>5</sup> CFU.g<sup>-1</sup> (summer and winter, respectively) after 15 days of curing, and 10<sup>2</sup> CFU.g<sup>-1</sup> after 60 days of ripening for Pecorino Abruzzese cheese. Although there was no correlation between the levels of titratable acidity and pH of the cheeses (Table 3) and the bacterial growth (Figure 1), it appears that the maturation period affected this growth. Prolonged maturation periods decreased the risk of the presence of enterobacteria.

*Enterobacteriaceae* are sensitive to derivatives from the fermentation of lactic-acid bacteria, such as acids, alcohols, aldehydes, ketones, bacteriocins, hydrogen peroxide, carbon dioxide, acetaldehyde and diacetyl (Onilude et al., 2002) and by other stress factors such as NaCl concentration and low water content. Consequently, are not normally detected after long periods of maturation of cheese (Dahl et al., 2000).

The different results may be accounted for by changes in the composition of the milk, which can occur due to the season, animal nutritional status and health, and stage of lactation (Heck et al., 2009; Golinelli et al., 2011).

**Table 1:** Contents of biogenic amines (mg.Kg<sup>-1</sup>) in eight varieties of cheese, in order of increasing ripening time (from left to right)

BA (mg.Kg <sup>-1</sup> )	Standard Minas	Prato	Reblochon	Gorgonzola	Moleson	Gruyère	Raclete	Sbrinz	Total of each BA
<b>Maturation Period (days)</b>	<b>25</b>	<b>30</b>	<b>30</b>	<b>35</b>	<b>75</b>	<b>90</b>	<b>90</b>	<b>180</b>	
<b>TYR</b>	92.51 <sup>a</sup> ±0.03 (92.48-92.54)	246.62 <sup>ab</sup> ±0.08 (246.53-246.70)	266.82 <sup>ab</sup> ±22.86 (243.96-289.68)	875.72 <sup>b</sup> ±210.30 (665.42-1086.00)	70.99 <sup>a</sup> ±0.07 (70.92-71.06)	245.61 <sup>a</sup> ±0.73 (244.87-246.34)	328.87 <sup>ab</sup> ±0.00 (328.87-328.87)	525.10 <sup>ab</sup> ±0.01 (525.08-525.11)	<b>2652.24</b>
<b>PUT</b>	9.76 <sup>b</sup> ±0.00 (9.76-9.77)	200.47 <sup>h</sup> ±0.04 (200.42-200.51)	149.81 <sup>g</sup> ±0.03 (149.77-149.84)	20.66 <sup>c</sup> ±0.26 (20.40-20.93)	1.51 <sup>a</sup> ±0.01 (1.50-1.53)	15.25 <sup>d</sup> ±0.04 (15.21-15.30)	70.07 <sup>f</sup> ±0.09 (69.98-70.17)	11.44 <sup>e</sup> ±0.01 (11.44-11.45)	<b>478.97</b>
<b>CAD</b>	4.73 <sup>b</sup> ±0.01 (4.72-4.74)	241.91 <sup>f</sup> ±0.20 (241.71-242.11)	8.19 <sup>c</sup> ±0.01 (8.18-8.20)	31.84 <sup>g</sup> ±1.02 (30.82-32.87)	n.d.	5.37 <sup>a</sup> ±0.11 (5.26-5.49)	3.02 <sup>g</sup> ±0.07 (2.95-3.09)	29.98 <sup>d</sup> ±0.01 (29.98-29.99)	<b>325.04</b>
<b>ESD</b>	n.d.	10.31 <sup>e</sup> ±0.01 (10.30-10.32)	n.d.	6.50 <sup>a</sup> ±0.10 (6.40-6.61)	n.d.	6.73 <sup>b</sup> ±0.01 (6.73-6.74)	n.d.	n.d.	<b>23.54</b>
<b>ESM</b>	n.d.	n.d.	n.d.	1.66 <sup>g</sup> ±0.04 (1.62-1.71)	n.d.	n.d.	n.d.	n.d.	<b>1.66</b>
<b>Total of BA for cheese</b>	<b>107.00</b>	<b>699.29</b>	<b>424.81</b>	<b>936.37</b>	<b>72.50</b>	<b>272.95</b>	<b>401.96</b>	<b>566.51</b>	<b>3481.45</b>

Means of determinations with standard deviation of cheeses Standard Minas, Prato, Reblochon, Gorgonzola, Moleson, Gruyère, Raclette and Sbrinz-type cheeses respectively. Values in the same row with different letters are significantly different ( $p < 0.05$ ). TYR: tyramine; PUT: putrescine; CAD: cadaverine; ESD: spermidine; ESM: spermine and n.d.: not detected.

**Table 2:** Representation of mean content of biogenic amines from bacterial plate isolated from cheeses Gorgonzola-, Gruyère-, Standard Minas, Moleson-, Prato, Raclette-, Reblochon- and Sbrinz-type cheeses respectively

AB	Standard Minas	Prato	Reblochon	Gorgonzola	Moleson	Gruyère	Raclette	Sbrinz
<b>TYR</b>	n.d.	17.32	484.24	179.99	n.d.	n.d.	n.d.	n.d.
<b>PUT</b>	6.57	21.28	10.13	n.d.	n.d.	n.d.	n.d.	n.d.
<b>CAD</b>	2.14	89.84	109.57	3.80	n.d.	n.d.	n.d.	n.d.
<b>ESD</b>	69.98	21.40	n.d.	7.60	n.d.	9.99	n.d.	n.d.
<b>ESM</b>	n.d.	43.99	15.48	n.d.	n.d.	n.d.	n.d.	n.d.
<b>Total of BA by isolate of colony forming unit</b>	<b>78.69</b>	<b>193.83</b>	<b>619.42</b>	<b>191.39</b>	<b>n.d.</b>	<b>9.99</b>	<b>n.d.</b>	<b>n.d.</b>

Means of determinations of bacterial plate isolated from cheeses Standard Minas, Prato, Reblochon-, Gorgonzola-, Moleson-, Gruyère-, Raclette- and Sbrinz-type cheeses respectively. Values in the same row with different letters are significantly different ( $p < 0.05$ ). TYR: tyramine; PUT: putrescine; CAD: cadaverine; ESD: spermidine; ESM: spermine and n.d.: not detected.

**Table 3:** Composition of dry base of eight varieties of cheeses, arranged in order of increasing ripening time (left to right)

Chemical Composition	Standard Minas	Prato	Reblochon	Gorgonzola	Moleson	Gruyère	Raclette	Sbrinz
<b>pH</b>	5.55 <sup>b</sup> ±0.04	6.11 <sup>d</sup> ±0.02	5.34 <sup>a</sup> ±0.02	5.92 <sup>c</sup> ±0.05	5.41 <sup>ab</sup> ±0.04	5.80 <sup>c</sup> ±0.03	5.55 <sup>b</sup> ±0.09	5.35 <sup>a</sup> ±0.01
<b>Titrateable Acidity (°D)</b>	30.00 <sup>c</sup> ±0.02	24.00 <sup>b</sup> ±0.17	74.00 <sup>g</sup> ±0.03	76.00 <sup>a</sup> ±0.04	48.00 <sup>e</sup> ±0.07	40.00 <sup>d</sup> ±0.05	56.00 <sup>f</sup> ±0.11	87.00 <sup>b</sup> ±0.05
<b>Fat (%)</b>	15.87 <sup>ab</sup> ±0.79	18.10 <sup>ab</sup> ±0.40	25.76 <sup>ab</sup> ±0.78	22.34 <sup>a</sup> ±0.45	25.41 <sup>ab</sup> ±0.31	24.15 <sup>ab</sup> ±0.57	26.97 <sup>b</sup> ±0.75	21.20 <sup>ab</sup> ±0.50
<b>Moisture (%)</b>	46.70 <sup>a</sup> ±0.50	39.05 <sup>a</sup> ±0.45	52.09 <sup>b</sup> ±0.29	46.55 <sup>a</sup> ±0.60	40.25 <sup>ab</sup> ±0.15	31.73 <sup>a</sup> ±0.60	40.55 <sup>ab</sup> ±0.33	30.40 <sup>a</sup> ±0.10
<b>Total Solids (%)</b>	70.30 <sup>c</sup> ±0.50	60.95 <sup>c</sup> ±0.45	47.90 <sup>a</sup> ±0.29	76.30 <sup>f</sup> ±0.60	59.74 <sup>b</sup> ±0.15	68.30 <sup>d</sup> ±0.60	59.44 <sup>b</sup> ±0.33	69.59 <sup>e</sup> ±0.10
<b>Protein (%)</b>	11.71 <sup>b</sup> ±0.15	11.78 <sup>b</sup> ±0.05	11.47 <sup>b</sup> ±0.01	9.21 <sup>a</sup> ±0.02	15.51 <sup>d</sup> ±0.14	15.30 <sup>d</sup> ±0.03	16.04 <sup>e</sup> ±0.16	14.42 <sup>c</sup> ±0.37
<b>Ash (%)</b>	1.36 <sup>a</sup> ±0.06	2.74 <sup>abc</sup> ±0.10	3.18 <sup>bc</sup> ±1.38	1.89 <sup>ab</sup> ±0.06	3.51 <sup>c</sup> ±0.13	3.57 <sup>c</sup> ±0.12	2.79 <sup>bc</sup> ±0.03	3.56 <sup>c</sup> ±0.20
<b>Chlorides (%)</b>	0.42 <sup>a</sup> ±0.01	0.86 <sup>c</sup> ±0.01	0.98 <sup>cd</sup> ±0.02	0.88 <sup>c</sup> ±0.01	1.10 <sup>d</sup> ±0.01	1.28 <sup>e</sup> ±0.01	0.57 <sup>b</sup> ±0.10	1.44 <sup>f</sup> ±0.10

Means of determinations with standard deviation. Values in the same rows with different letters are significantly different ( $p < 0.05$ ).

**Biogenic Amines.** As seen in Table 1, the percentages of BA in the cheeses, with different maturation period, differed significantly ( $p < 0.001$ ).

In contrast, Cunha et al. (2012) found no correlation between maturation time and BA production in cheeses. This difference can be explained by differences in the strains used as starter

cultures in each variety of cheese, and in the products generated during the fermentation process.

A significant finding was the high concentration of tyramine in all the cheeses, especially Gorgonzola-, Raclette- and Sbrinz-type cheeses (Table 1). Cheeses containing 10-80 mg of tyramine can cause a "cheese reaction"; 6 mg would be sufficient if the patient is being treated with monoamine oxidase (MAO). This reaction is a hypertensive crisis accompanied by severe headache, following intake of foods high in tyramine (Chang et al., 1985).

The concentration of tyramine present in the cheeses (75.82%) was significantly ( $p < 0.001$ ) higher than those of the other BA. Buňková et al. (2010) reported that tyramine is the most commonly detected BA in fermented dairy products, since many lactic acid bacteria can produce microbial tyrosine decarboxylase, which explains the high values of tyramine found in this study.

Cunha et al. (2012) found lower BA contents in Gouda, Mozzarella, Prato and Standard Minas cheeses, using the same analytical method. In Prato cheese, they identified and quantified putrescine ( $3.29 \text{ mg.Kg}^{-1}$ ), cadaverine ( $16.18 \text{ mg.Kg}^{-1}$ ), tyramine ( $152.91 \text{ mg.Kg}^{-1}$ ), histamine ( $14.82 \text{ mg.Kg}^{-1}$ ), and spermidine ( $0.13 \text{ mg.Kg}^{-1}$ ), totaling  $187.35 \text{ mg.Kg}^{-1}$ . In the present study, the levels found for Prato were putrescine:  $200.47 \text{ mg.Kg}^{-1}$ ; cadaverine:  $241.91 \text{ mg.Kg}^{-1}$ ; tyramine:  $246.62 \text{ mg.Kg}^{-1}$ ; spermidine:  $10.31 \text{ mg.Kg}^{-1}$  and spermine: not detected, totaling  $699.29 \text{ mg.Kg}^{-1}$ . The levels probably differ because of factors such as variations in the composition of the milk, different manufacturing processes, with and without initial standardization of milk, the use of different bacterial strains, type of salting, maturation time, presence of contaminants during marketing, and the time on the market shelf, i.e., nearness to the "sell by" date.

Ibrahim and Amer (2010) studied tyramine, histamine and cadaverine in Cheddar, Ras and Gouda sample cheeses. Cheddar had the highest histamine level of  $22.12 \pm 1.35$  and  $10.36 \pm 0.78 \text{ mg.100 g}^{-1}$ , respectively. While, the highest histamine level of  $17.43 \pm 1.05$  was found in processed cheese samples. In conclusion, comparing the obtained results with processed cheeses consumed in Egypt exceeded the permissible value (10 mg%) which seemed to pose a threat to public health. Nearly, similar results were recorded by Fernandez-García et al. (2000). While, higher results were obtained by Jarisch (2004) in Gouda cheese samples. In contrast lower concentrations of histamine and cadaverine in Ras processed cheese were reported by Amer (2015). But, in all cases, the results were lower than the results found in the present study.

The maximum permissible limit of tyramine, histamine and cadaverine is  $10 \text{ mg.100 g}^{-1}$  stipulated by FDA (2001). EOS (1996) recommended a value of  $20 \text{ mg.100 g}^{-1}$  to be the safe permissible value of BA in food. Consequently, all values of processed cheeses exceeded such limit either of tyramine, histamine or cadaverine respectively. The other BA cited in this article has no limit stipulated by the literature (Tamime, 2017).

Ingestion of foods containing high levels of biogenic amines, such as tyramine and histamine, may be deleterious, since these amines have vasoactive, psychoactive, and toxicological properties. In addition, putrescine and cadaverine may potentiate the toxicity of these biogenic amines causing financial losses and health damage (Flick et al., 2001). The presence and accumulation of these substances are influenced

by numerous factors, such as the composition and availability of free amino acids, water activity, temperature, the pH of the medium, and especially the presence of decarboxylase-positive microorganisms (Schirone et al., 2012).

In Table 2, the high level of BA agree with high bacterial plate isolated from Reblochon-type cheese  $4.91 \text{ Log CFU.g}^{-1}$ , Prato  $4.2 \text{ Log CFU.g}^{-1}$ , Standard Minas  $4.17 \text{ Log CFU.g}^{-1}$ , and Gorgonzola-type cheese  $2.84 \text{ Log CFU.g}^{-1}$ . Although Reblochon-type cheese have higher level of BA, it was detected five BA in bacterial plate isolated from Prato cheese, followed by Reblochon-type cheese with four and Gorgonzola-type cheese and Santandard Minas with three BA. Bacterial plate isolated from Gruyère-type cheese showed spermidine production probably from to their strain used as starter culture because there were no growth of enterobacteria in its plate count. The types and contents of biogenic amines present in fermented dairy products vary with the feedstock, product type, ripening/fermentation time, culture starter strains, proteolytic activity, and manufacturing conditions (Andic et al., 2010; Priyadarshani and Rakshit, 2011).

Spermidine and spermine were found in low concentrations in all cheeses (Table 1). These amines are not commonly associated with adverse health effects, but do indicate loss of quality and degradation of precursor amino acids, indicators of deterioration or maturation (Doeun et al., 2017). Another aspect is the presence of four of the five BA analyzed in Prato cheese (Table 1), three of them in concentrations in excess of  $200 \text{ mg.Kg}^{-1}$  although this cheese has a short ripening time of approximately 30 days. The optimal period for ripening Prato cheese is approximately 40 days. However, Brazilian legislation allows this cheese to be sold after 25 days of storage (Brazil, 1997).

Based in Table 1 and Table 2, a possible explanation for the difference in BA detection of the isolates and samples of cheese can be explained by the matrix characteristics from medium and cheese. Moreover, growth conditions and enzyme synthesis are different in each condition. In the controlled systems used for bacterial analyses *in vitro* such as the methods of Izquierdo et al. (2003), often the bacterial behavior differs from systems in biological matrices such as food.

**Physical and Chemical Analyses.** The highest pH of Prato cheese is probably due mainly to proteolysis, resulting in the formation of soluble nitrogen compounds and consequent elevation of pH (Hickey et al., 2017). The highest fat and protein contents in Raclette-type cheese is because dry milk and cream are added at the beginning of processing. This cheese needs additional fat to allow it to melt properly. Due to its short maturation time (30 days) and thin rind, which helps to retain moisture, Reblochon-type cheese showed the highest water content. The highest content of total solids found in Gorgonzola-type cheese is because of it is standardized to contain a higher concentration of solids. The highest level of ash in Gruyère-type cheese is because the processing involves cutting the curds into small pieces to increase syneresis and release the whey, which makes this cheese more compact with higher residual dry weight. Probably because of its long maturation period, the highest NaCl content was found in Sbrinz-type cheese. In Moleson-, Sbrinz- and Gruyère type cheeses, probably because of the NaCl, this salt may have helped to reduce the population of this bacterial group.

Diezhandino et al. (2015) found values of 8.92g NaCl.100 g<sup>-1</sup> to relation chlorides /moisture, and 61.8g.100g<sup>-1</sup> to total solids in Spanish blue while in this study, Gorgonzola-type cheese showed a relation chlorides/moisture very low, the total solids value was higher (76.3 Prato cheese; Reblochon-, Moleson- and Raclette-type cheeses showed total solids values lowest than Spanish blue cheese. Standard Minas cheese, Gorgonzola-, Gruyère- and Sbrinz-type cheeses showed total solids values highest than the cheese studied by Diezhandino et al. (2015).

Comparing present results with Choi et al. (2015), it is possible to say that moisture and ash values were very similar, protein values were higher in eight cheeses studied and all fat values were lower, and all eight pH values were lower than 6.0 while Gouda's pH varied between 5.24 and 8.20.

Narimatsu et al. (2003) found the following values in your experiment with Prato cheese: pH 5.41, titratable acidity 0.57, fat 24.89, moisture 48.4, total solids 51.6, protein 18.16, ash 3.73, chlorides 1.63. Three items studied titratable acidity, pH, and total solids were higher than those of the cited article.

**Correlation between parameters.** The Gorgonzola-type cheese showed a strong positive correlation ( $r = 0.9987$ ) with the amounts of BA found in samples of this cheese (Table 1) and in isolates of colony-forming units of BAPB from the same cheese (Table 2). Reblochon-type cheese showed a moderately positive correlation ( $r = 0.7980$ ) with the amount of BA in isolates of colony-forming units of BAPB. The other cheeses varieties showed no significant correlations among these parameters.

Reblochon-type cheese showed highest growth enterobacteria and high BAPB growth. Considering physical-chemical parameters, this cheese showed highest ( $p < 0.05$ ) content of moisture, which seemed contribute to bacterial growth, even

showing to low pH and high titratable acidity. Although the literature (Sangaletti et al., 2009; Carrijo et al., 2011) indicate enterobacteria growth inhibition by high titratable acidity and low pH, these data were not confirmed by the present study. Besides the Reblochon-type cheese, Gorgonzola- type cheese showed high titratable acidity and low pH, and also enterobacteria growth. It seems that the factors that negatively had interfered with enterobacteria growth was the high salt content found in Moleson-, Gruyère-, Sbrinz- and Raclette- type cheeses and maturation period.

On the other hand, cheeses that showed high counts of BAPB (Sbrinz-, Reblochon-, and Gorgonzola-type cheeses) were those which showed high titratable acidity and low pH. In this case, it seems that BAPB growth was influenced by cultures added during the cheeses preparation, which may also have affected the production of BA. However, there was no relation between BAPB and enterobacteria counts with BA production.

## Conclusions

Among ripened cheeses, Gorgonzola-type cheese showed the highest content of total BA (936.37 mg.Kg<sup>-1</sup>) and tyramine appeared in the highest concentration in all analyzed cheeses (2652.24 mg.Kg<sup>-1</sup>), which creates a concern due to their high toxic potential.

However, although large counts of BAPB have been verified in some cheeses there was no correlation between this factor and BA production. Except to NaCl contents and maturation period, other physical and chemical parameters had any interference in BA amount, and the care in the choice of the bacteria culture added by dairy is a determining factor in the quality thereof in relation to BA production.

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