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Effect of ripening time on proteolysis, free amino acids, bioactive amines and texture profile of Gorgonzola-type cheese

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ABSTRACT

The effect of ripening time on Gorgonzola-type cheeses at 12 ± 2 °C was investigated. Proteolysis indexes, free amino acids and bioactive amines increased throughout ripening. However, cohesiveness and lactic acid bacteria counts decreased; whereas pH, total protein, total mesophilic aerobic bacteria counts, and other instrumental texture parameters did not change. Valine, lysine, leucine and phenylalanine were the most abundant free amino acids throughout ripening. Glycine, histidine and isoleucine were first detected on the 21st day and aspartic acid and glutamine on the 28th after perforation. The levels of most amino acids increased, except serine that decreased and tyrosine remained constant. Among amines formed during ripening, tyramine and agmatine were first detected on the 21st day, serotonin on the 42nd and histamine and tryptamine on the 49th. On the 21st day, tyramine reached contents which could be of health concern for individuals taking classical monoamine oxidase inhibitor drugs. Principal Component Analysis and Hierarchical Cluster Analysis showed that proteolysis (extend and depth indexes) and free amino acid profile are suitable markers for ripening assessment of Gorgonzola-type cheese. They were also able to assess cheese characteristics, quality and safety.

1. Introduction

Cheese, the fresh or matured product obtained from the coagulation of milk, is widely consumed around the world and plays an important role in human nutrition. It is easily digestible and rich in nutritional components, and therefore, it constitutes an important source of proteins, short-chain fatty acids, vitamins, and minerals (Santiago-Lopes et al., 2018). Although exceptional progress has been made in cheese making, there is still a need to establish parameters to consistently characterize the several different types of cheese (Johnson, 2017).

Gorgonzola is one of the world's oldest blue-veined cheeses. The cheese is mainly produced in Northern Italy, and it generally takes three to four months to attain full ripeness. The cheese has a crumbly and soft texture, nutty aroma and mild to sharp taste. It is extensively used in cooking and gourmet dishes all over the world (Fernández-Salguero, 2004). The production and consumption of Gorgonzola-type and other special cheeses in Brazil increased significantly from 2006 to 2012, which represents a new and promising market to dairy products and also another source of dairy proteins into the diet (Franco, 2013).

Penicillium roqueforti, a saprophytic fungus, grows internally in the fissures of the cheese or in the perforations formed after salting (Cantor, van den Tempel, Hansen, & Ardö, 2004). The desired texture, flavor and aroma results from biochemical changes during cheese production and ripening. Proteolysis is the most intricate event which takes place by the action of natural milk proteases, of added rennet, starter and secondary cultures, and enzymes (Fox, Uniacke-Lowe, McSweeney & O'Mahony, 2015). It is responsible for changes in the protein matrix, modifying its texture and increasing pH (Sousa, Ardö & McSweeney, 2001). It is also responsible for the release of amino acids, which are precursors of bioactive amines. Therefore, cheeses are ideal environments for the formation of amines (Gloria, 2006; Vale & Gloria, 1998). The profile and levels of bioactive amines in cheeses are dependent upon the amount of protein in the raw material, the proteolytic activity of enzymes used during manufacture, microorganisms present in the cheese, the existence of amino acid decarboxylase activity, and the cofactor pyridoxine phosphate (Alvarez & Moreno-Arribas, 2014; Yvon & Rijnen, 2001).

Bioactive amines, at low levels, can impart functional and flavor

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characteristics to cheese. However, some amines can accumulate if there are inadequate hygienic-sanitary conditions during processing. High concentrations of tyramine and histamine are undesirable due to adverse effects to human health, with most common symptoms including headaches, flushing, nausea, cardiac palpitations, and alterations in blood pressure (EFSA, 2011). Therefore, the concentration of biogenic amines may be used as an indicator of the conditions prevailing during manufacture and also of potential risk to health (Alvarez & Moreno-Arribas, 2014; EFSA, 2011; Gloria, 2006).

Several studies are available on the quality of Gorgonzola cheese, mainly focusing on the role of lipolysis and its influence on flavor (Fontecha, Mayo, Toledano, & Juárez, 2006; Calzada, Del Olmo, Picon, Gaya, & Nuñez, 2013; Salvatore, Addis, Pes, Fiori, & Pirisi, 2015). However, scarce information is available on proteolytic activity and its effect on quality, safety and texture throughout ripening, which are important parameters for the standardization and acceptance of Gorgonzola-type cheese, and also from the public health point of view. Therefore, the objective of this study was to investigate the extent of proteolysis, the changes on free amino acid and bioactive amines profiles and levels and on texture during ripening of Gorgonzola-type cheeses for up to 49 days after cheese perforation.

2. Material and methods

2.1. Material

The reagents were of analytical grade, except HPLC solvents which were LC grade. Standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA): putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, agmatine sulfate, cadaverine dihydrochloride, serotonin hydrochloride, histamine dihydrochloride, tyramine hydrochloride, tryptamine, 2-phenylethylamine hydrochloride, aspartic acid, serine, asparagine, glycine, glutamic acid, glutamine, histidine hydrochloride, threonine, arginine hydrochloride, alanine, proline, cystine, tyrosine, valine, methionine, lysine hydrochloride, leucine, isoleucine, phenylalanine and norvaline. AccQ.Fluor™ pre-column derivatization kit was purchased from Waters (Milford, MA, USA).

2.2. Production of Gorgonzola-type cheese

Gorgonzola-type cheese was produced in three replicates (three different days). The manufacturing process is described at Fig. 1. Calcium chloride (0.02%), starter culture (R704 - *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*; and CHN19 - *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *cremoris* and *Lactococcus lactis* subsp. *diacetylactis*, Chr. Hansen, Hørsholm, Denmark), adjunct culture (PRN - *Penicillium roqueforti*, Sacco Brazil, Campinas, SP, Brazil), and rennet (Chy-max™, Chr. Hansen, Hørsholm, Denmark) were used to manufacture the cheese.

Periodically, 14, 21, 28, 35, 42 and 49 days, counting from the day of perforation (which represents 17, 24, 31, 38, 45 and 52 days, respectively, after cheese manufacture), samples were analyzed in duplicate. On the first day (14 days after perforation) samples were analyzed for free amino acids, bioactive amines, texture profile, physicochemical and microbiological characteristics. During ripening, free amino acids, bioactive amines, total nitrogen (TN), soluble nitrogen at pH 4.6 ($SN_{pH4.6}$), soluble nitrogen at 12% trichloroacetic acid ($SN_{TCA12\%}$), pH, microbiological counts and texture profile were determined.

2.3. Methods of analysis

2.3.1. Determination of physicochemical characteristics

The samples were analyzed for total solid, fat and chlorides contents according to IDF International Standards (IDF, 1982; 1986; 1988), water activity using a 3TE Aqualab™ analyser (Decagon, Pullman, WA, USA) and pH using a pHmeter (Tec-2 Tecnal, Piracicaba, SP, Brazil). TN was determined by Kjeldahl (AOAC, 2012). $SN_{pH4.6}$ and $SN_{TCA12\%}$ were also determined by Kjeldahl (AOAC, 2012) after the respective extractions (Ubaldo, Carvalho, Fonseca, & Gloria, 2015).

Fat in dry matter (FDM) was calculated as a percentage of the fat content in the total solids. The extension and depth of proteolysis were calculated as % $SN_{pH4.6}$/TN and % $SN_{TCA12\%}$/TN, respectively (Wolfschoon Pombo & Lima, 1989; Ubaldo et al., 2015). Total protein was calculated from TN using the factor 6.38 (AOAC, 2012).

2.3.2. Determination of free amino acids and bioactive amines

Bioactive amines and free amino acids in the cheeses were simultaneously analyzed by liquid chromatography and ultravioleta detection at 249 nm after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) as described by Moreira et al. (2017). Briefly, successive extractions of analytes from 10 g cheese with 1 mol/L hydrochloric acid (Ubaldo et al., 2015) were undertaken; the internal standard norvaline (final concentration of 25 pmol in column) was added; and the volume was brought up to 50 mL in a volumetric flask. The extract was centrifuged at 16,000 g at 4 °C for 10 min, neutralized using an equal volume of 1 mol/L sodium hydroxide and derivatized with AQC (Moreira et al., 2017). A Waters Acquity™ Ultra Performance LC system (Waters, Milford, MA, USA) equipped with an Acquity™ tunable ultraviolet detector was used. The column was a BEH C18 (50 × 2.1 mm i.d., 1.7 µm, Acquity UPLC™). The solvent system consisted of 0.1 mol/L sodium acetate buffer adjusted to pH 4.80 and acetonitrile at gradient elution. The method was validated by Moreira et al. (2017) and it was observed to be fit for the purpose for the analysis of 10 amines, 19 amino acids and the ammonium ion in cheeses.

2.3.3. Determination of texture profile

Analysis of the texture profile – TPA (Sobral et al., 2016) of the cheese samples were carried out using a CT3 Brookfield texturometer (Middleboro, MA, USA). Pre-test, test, and post-test speeds of 1 mm/s, compression distance of 30%, charge cell of 4.5 kg, trigger point of 0.05 N and a cylindrical probe of 50.8 mm diameter were used. Six cube-shaped samples (2 cm^2) were randomly collected from the cheese for each determination. The assessed parameters were hardness (N), cohesiveness, springiness (mm), elasticity, chewiness (J), and adhesiveness (mJ), using a Brookfield Texture Pro CT V1.4 software.

2.3.4. Microbiological analysis

The total mesophilic aerobic bacteria (TMAB) were counted by plating the appropriate dilution of cheese on 3M™ Petrifilm™ AC plates (Merck, Germany) and incubated at 35 °C for 48 h (AOAC, 2012). Lactic acid bacteria (LAB) were counted on Man Rogosa & Sharpe (MRS) agar incubated at 32 °C for 48 h (Frank & Yousef, 2004).

2.3.5. Statistical analysis

The experiment was performed in three replicates, in a block randomized design. The results were subjected to one-way ANOVA followed by Tukey's test ($p = 0.05$). All analysis (including normality tests, heteroscedasticity, regression and multivariate) were performed using XLSTAT software (version 2017.7.48738; Addinsoft, Paris, France).

Two multivariate exploratory techniques, Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA), were applied for the characterization of cheese during ripening. The dataset matrix consisted of 6 rows (times of ripening) and 40 columns comprising the parameters analyzed throughout ripening: pH, contents of individual amines, ammonium ion and individual free amino acids, sum of total bioactive amines, sum of total free amino acids, total protein, proteolysis indexes and texture parameters. Two HCA analyses were made. The first dendrogram was obtained by clustering observations, and the

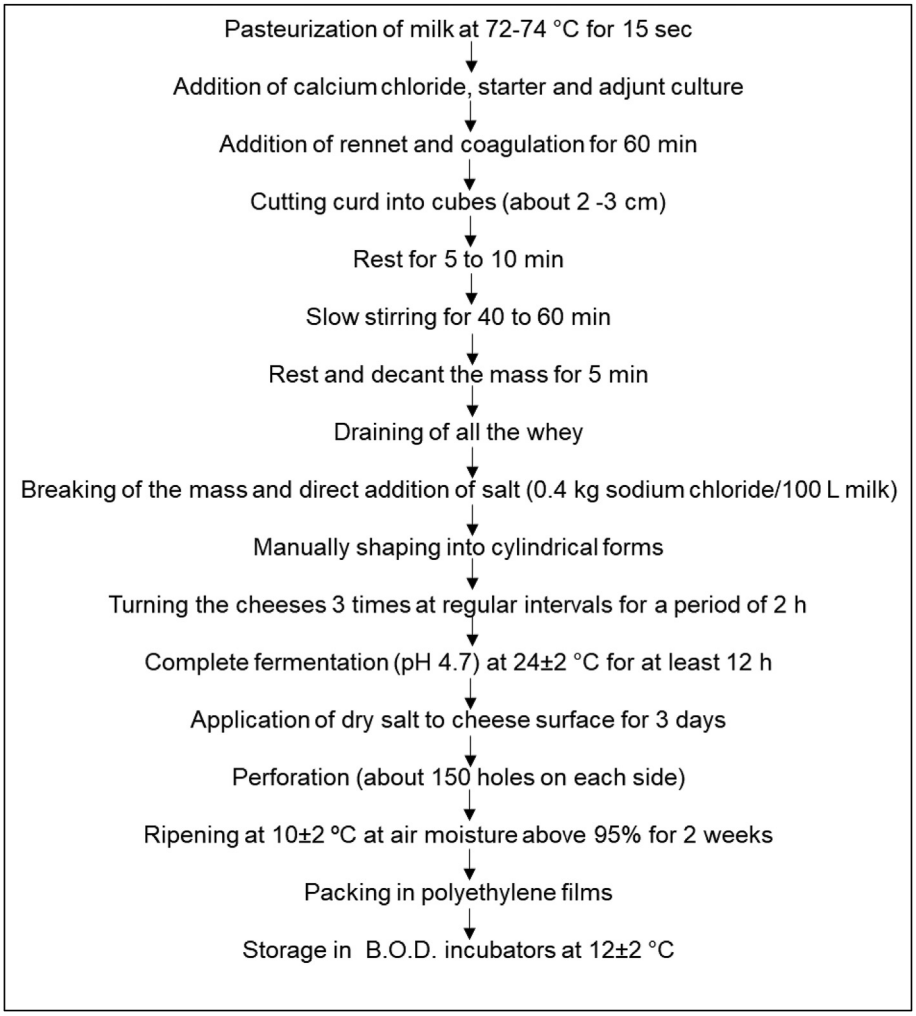

Fig. 1. Flowchart of Gorgonzola-type cheese manufacture.

second by clustering variables (the same used for PCA). Pearson's correlation was used to measure similarity and single linkage in both cases.

3. Results and discussion

3.1. Characteristics of the Gorgonzola–type cheese on the 14th day of ripening

Gorgonzola-type cheeses on the 14th day of ripening after perforation (17th day after cheese production) presented 45.6 g/100 g moisture content, 53.9 g/100 g fat content in dry matter (FDM) and mean pH of 5.10 (Table 1). Similar results were observed in the literature for moisture, FDM and pH in blue cheeses at earlier stages of ripening (Gobbetti, Burzigotti, Smacchi, Corsetti, & De Angelis, 1997; Voigt, Chevalier, Qian, & Kelly, 2010; Zarmpoutis, McSweeney, & Fox, 1997).

The content of salt in moisture was within literature reports, which vary from 3.13% (Furtado, Casagrande, & Freitas, 1984) to 5.2–5.9% (Voigt et al., 2010; Zarmpoutis et al., 1997). Such variation suggests differences in cheese manufacture, mainly concerning salting methods. However, it is important to have good control of this step as salt content in moisture can impact proteolysis because it has a strong influence on mold growth (Seratlić, Miloradović, Radulović, & Maćej, 2011).

Twelve out of the 19 amino acids analyzed were detected. Leucine, lysine and phenylalanine were the predominant ones, with levels higher than 10 mg/100 g each (Table 2), all directly associated with bitter taste (Kabelová, Dvořáková, Čížková, Dostálek, & Melzoch, 2009). No amine was detected, however ammonium ion reached 21.0 mg/100 g and total free amino acids was 79.7 mg/100 g.

Table 1
Physico-chemical characteristics of Gorgonzola-type cheese on 14 days after perforation.

Parameter	Mean values ± SD[a]
Moisture content (g/100 g)	45.61 ± 0.19
Protein (g/100 g)	22.53 ± 3.53
Fat in dry matter (g/100 g)	53.93 ± 2.03
Sodium chloride (g/100 g)	1.69 ± 0.14
Salt in moisture (g/100 g)	3.71 ± 0.30
pH	5.10 ± 0.10
Water activity	0.98 ± 0.01
Extension of proteolysis ($\%SN_{pH4.6}$)	14.25 ± 4.76
Depth of proteolysis ($\%SN_{TCA12\%}$)	9.98 ± 1.95

[a] SD = standard deviation. n = three replicates.

3.2. Changes occurring during cheese ripening

3.2.1. pH and total protein content

pH and total protein content in the Gorgonzola-type cheese remained constant, with equivalent means ($p > 0.05$) throughout ripening (5.06 ± 0.07 and 20.7 ± 1.1 g/100 g, respectively). The lack of change in pH of the Gorgonzola-type cheeses can result from the

Table 2
Free amino acids and bioactive amines in Gorgonzola-type cheeses throughout ripening at 12 ± 2 °C for 49 days after perforation.

Amino acids	Bioactive amines and amino acids (mg/100 g) during ripening (days after perforation)					
	14	21	28	35	42	49
Alanine	5.0 ± 2.3	6.4 ± 1.8	8.7 ± 2.8	11.9 ± 3.1	14.4 ± 1.5	16.5 ± 0.3
Asparagine	4.7 ± 1.2	8.4 ± 1.9	9.4 ± 4.6	15.2 ± 2.6	16.5 ± 2.9	21.9 ± 2.4
Aspartic acid	nd	nd	3.2 ± 4.2	11.4 ± 4.3	13.3 ± 6.0	20.6 ± 2.2
Glutamic acid	6.3 ± 4.7	4.7 ± 2.0	10.0 ± 11.8	24.3 ± 14.9	24.6 ± 14.7	22.8 ± 10.2
Glutamine	nd	nd	4.6 ± 4.5	11.9 ± 5.8	17.1 ± 4.3	21.1 ± 1.9
Glycine	nd	1.8 ± 0.7	2.6 ± 0.9	4.3 ± 1.4	6.1 ± 0.9	7.2 ± 0.5
Histidine	nd	4.9 ± 1.7	10.1 ± 3.9	9.7 ± 3.4	14.1 ± 2.5	15.2 ± 2.4
Isoleucine	nd	6.1 ± 2.4	8.6 ± 3.0	15.6 ± 5.3	20.3 ± 3.6	23.5 ± 1.7
Leucine	14.2 ± 4.8	34.6 ± 10.4	44.5 ± 13.0	69.3 ± 12.6	83.4 ± 16.0	96.4 ± 9.8
Lysine	11.6 ± 5.3	21.8 ± 6.7	21.3 ± 10.6	43.8 ± 14.1	51.3 ± 6.4	57.6 ± 4.6
Methionine	4.1 ± 0.7	10.2 ± 2.0	15.3 ± 3.7	17.2 ± 5.1	25.6 ± 3.6	25.8 ± 4.1
Phenylalanine	10.6 ± 3.3	20.2 ± 5.4	30.7 ± 8.2	34.2 ± 7.5	45.6 ± 11.8	49.1 ± 9.4
Proline	2.4 ± 1.3	5.0 ± 1.5	7.5 ± 3.4	13.9 ± 4.9	18.1 ± 3.3	21.7 ± 0.9
Serine	3.4 ± 1.0	3.0 ± 4.1	nd	nd	nd	nd
Threonine	3.2 ± 1.4	5.9 ± 0.8	7.4 ± 2.9	11.2 ± 2.8	15.1 ± 2.2	16.8 ± 1.8
Tyrosine	8.2 ± 3.1	11.3 ± 6.4	12.6 ± 7.1	8.4 ± 5.3	16.3 ± 12.7	9.8 ± 9.3
Valine	6.0 ± 2.0	14.1 ± 3.8	19.4 ± 6.6	32.0 ± 9.9	39.2 ± 5.7	45.8 ± 2.2
Ammonium ion	21.0 ± 13.3	16.2 ± 3.1	13.7 ± 6.1	19.8 ± 4.2	23.2 ± 1.6	24.0 ± 1.0
Amines						
Agmatine	nd	2.7 ± 1.1	3.2 ± 1.1	2.8 ± 0.7	3.0 ± 0.6	3.4 ± 1.4
Histamine	nd	nd	nd	nd	nd	2.4 ± 1.3
Serotonin	nd	nd	nd	nd	4.1 ± 1.8	3.6 ± 1.5
Tryptamine	nd	nd	nd	nd	nd	3.3 ± 0.8
Tyramine	nd	9.6 ± 1.7	12.7 ± 6.6	18.6 ± 8.3	30.0 ± 4.3	33.3 ± 6.9
2-Phenylethylamine	nd	nd	nd	2.5 ± 0.9	3.0 ± 0.4	4.1 ± 0.5
Totals						
Free amino acids	79.7 ± 29.5 ^{c}	158.3 ± 47.0 ^{bc}	216.2 ± 85.2 ^{bc}	334.2 ± 100.1 ^{ab}	421.1 ± 88.0 ^{a}	471.9 ± 31.7 ^{a}
Biogenic amines	nd	9.6 ± 1.0 ^{c}	12.7 ± 6.8 ^{c}	21.2 ± 9.9 ^{bc}	37.1 ± 6.0 ^{ab}	46.7 ± 9.2 ^{a}
Bioactive amines	nd	12.3 ± 0.9 ^{c}	15.8 ± 7.6 ^{c}	24.0 ± 10.5 ^{bc}	40.2 ± 6.2 ^{ab}	50.2 ± 10.8 ^{a}

Means (± standard deviation of three replicates) followed by different letters in each line are significantly different (Tukey test, $p < 0.05$).
nd = not detected.

presence of some salts, such as residual colloidal calcium phosphate, which can play a role in the buffering capacity of cheeses. Furthermore, the production of ammonium ion during ripening can help maintain higher pH values in mold ripened cheeses (Lucey, Johnson, & Horne, 2003). Similar pH and total protein contents were found for other blue cheese (Prieto et al., 1999).

3.2.2. Proteolysis indexes

Cheese proteolysis can be measured in terms of extent and depth indexes (Wolfschoon Pombo & Lima, 1989; Ubaldo et al., 2015). The extent of proteolysis, soluble nitrogen at pH 4.6, is related to the hydrolysis of calcium paracaseinate over time, mainly due to the action of residual chymosin. The depth of proteolysis, soluble nitrogen at 12% TCA, demonstrates how much of the total nitrogen exists as low molecular weight peptides and free amino acids, which is mostly affected by starter bacteria and contaminants (Wolfschoon Pombo & Lima, 1989; McSweeney & Fox, 1997; Bansal, Piraino, & McSweeney, 2010). The cheese showed increased proteolysis during ripening (Fig. 2), as it was expected for Gorgonzola-type cheese, due to the additional enzymes activity from *P. roqueforti*. The linear regression equation estimated for both extent and depth of proteolysis resulted in similar slope coefficients ($p > 0.05$), and, therefore, similar rates. At the end of ripening, the extent of proteolysis reached 30.7%, whereas proteolysis depth reached 24.4%. These results are similar to those in the literature for Gorgonzola cheeses (Zarmpoutis et al., 1997); but more pronounced proteolysis was found in other blue cheeses (Prieto et al., 1999). Although they have in common the ripening by *P. roqueforti*, differences in raw materials and manufacturing technologies can affect ripening pattern.

3.2.3. Texture properties

Texture properties of Gorgonzola-type cheese are described in Table 3. Cohesiveness, which expresses the degree to which the chewed mass sticks together in the mouth (Diezhandino et al., 2016), was the only parameter that changed throughout ripening, decreasing significantly. Cohesiveness correlated significantly with total free amino acids (-0.979, $p < 0.05$), extension (-0.948, $p < 0.05$) and depth of proteolysis (-0.930, $p < 0.05$), evidencing that the hydrolysis of the protein matrix of the cheese affected cohesiveness. As the interactions within the protein matrix decrease, the interactions between protein and water increase and, consequently, the cheese becomes less cohesive.

3.2.4. Free amino acids and bioactive amines

The total levels of free amino acids and bioactive amines in Gorgonzola-type cheeses increased over ripening time, with significantly different mean total values ($p < 0.05$) ranging from 100.7 to 495.9 mg/100 g and 'not detected' to 50.2 mg/100 g, respectively, from the 14th to the 49th day after perforation (Table 2). These data shows the same behavior of proteolysis indexes, which increased as ripening occurred, and corroborate with other studies demonstrating that proteolysis during ripening impacts the production and accumulation of amines in cheese (Linares et al., 2012; Fiechter, Sivec, & Mayer, 2013).

During ripening, up to the 49th day, 17 out of the 19 amino acids analyzed were found in the Gorgonzola-type cheeses, five more compared to the profile observed on the 14th day after perforation: aspartic

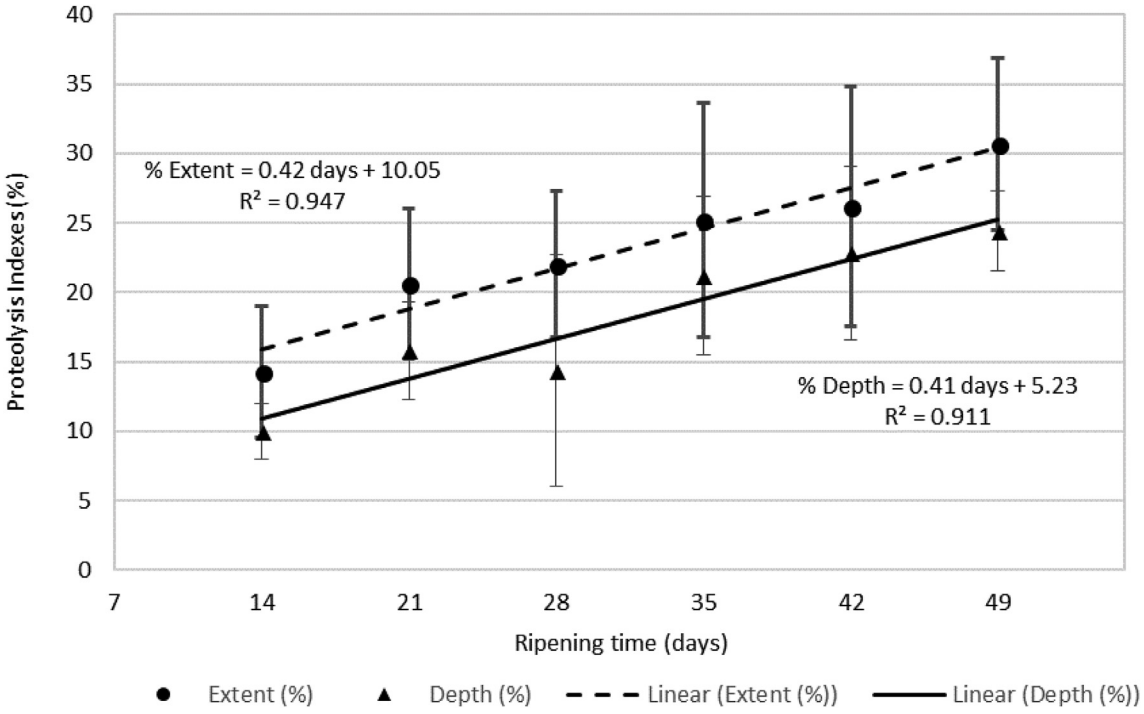

Fig. 2. Regression lines for the extent (soluble nitrogen at pH 4.6) and depth (soluble nitrogen at 12% trichloroacetic acid) of proteolysis of Gorgonzola-type cheese during ripening at 12 ± 2 °C for 49 days after perforation.

Table 3
Texture properties of Gorgonzola-type cheese throughout ripening at 12 ± 2 °C for up to 49 days after perforation.

Texture properties	Values during ripening (days after perforation)					
	14	21	28	35	42	49
Hardness (first bite) (N)	33.08 ± 12.26 [a]	30.91 ± 10.50 [a]	32.51 ± 10.28 [a]	31.57 ± 4.32 [a]	31.48 ± 4.80 [a]	31.23 ± 5.28 [a]
Hardness (second bite) (N)	28.18 ± 9.81 [a]	24.77 ± 7.06 [a]	25.84 ± 7.05 [a]	24.96 ± 3.53 [a]	24.11 ± 3.36 [a]	23.17 ± 4.14 [a]
Gumminess (N)	21.47 ± 7.24 [a]	18.75 ± 5.48 [a]	18.80 ± 5.05 [a]	18.09 ± 2.70 [a]	15.76 ± 2.40 [a]	15.33 ± 2.87 [a]
Chewiness (J)	0.105 ± 0.032 [a]	0.092 ± 0.031 [a]	0.090 ± 0.030 [a]	0.086 ± 0.015 [a]	0.069 ± 0.016 [a]	0.070 ± 0.014 [a]
Cohesiveness	0.66 ± 0.04 [a]	0.62 ± 0.06 [ab]	0.58 ± 0.04 [abc]	0.57 ± 0.03 [abc]	0.50 ± 0.06 [bc]	0.48 ± 0.03 [c]
Springiness (mm)	4.89 ± 0.22 [a]	4.84 ± 0.26 [a]	4.73 ± 0.33 [a]	4.71 ± 0.23 [a]	4.33 ± 0.37 [a]	4.46 ± 0.17 [a]
Elasticity	0.82 ± 0.04 [a]	0.81 ± 0.04 [a]	0.79 ± 0.06 [a]	0.79 ± 0.04 [a]	0.72 ± 0.06 [a]	0.75 ± 0.03 [a]
Adhesiveness (mJ)	0.76 ± 0.22 [a]	1.01 ± 0.06 [a]	0.83 ± 0.26 [a]	0.70 ± 0.30 [a]	0.85 ± 0.13 [a]	1.21 ± 0.51 [a]

N = six analyses of three replicates.
Means (± standard deviation) followed by different letters in the same line are significantly different (Tukey test, $p < 0.05$).

acid, glutamine, glycine, histidine and isoleucine. Valine, lysine, leucine and phenylalanine were the most abundant amino acids throughout ripening. Lysine, valine and phenylalanine are important in Gorgonzola-type cheese, as they are precursors of relevant flavor compounds (Yvon & Rijnen, 2001). Korös, Varga, and Molnár-Perl (2008) also reported the predominance of phenylalanine and lysine in blue cheeses.

On the 21st day, glycine, histidine and isoleucine were also detected; whereas on the 28th all of the amino acids were detected except for serine, which was no longer detected. There was a significant increase ($p < 0.05$) on the levels of the majority of the amino acids; however, the levels of serine decreased and the levels of tyrosine did not change during ripening. As the precursor amino acid for tyramine formation (Gloria, 2006), possibly, tyrosine levels did not increase because of the formation of the corresponding amine – tyramine, which was the most abundant amine in the cheese.

During ripening, some amines, which were not initially present, were formed. Agmatine and tyramine were first detected on the 21st day, 2-phenylethylamine on the 35th, serotonin on the 42nd day, and tryptamine and histamine only on the 49th day. Spermidine, spermine, putrescine and cadaverine were not detected at all. Agmatine was found at low levels after the 21st day of ripening. The beneficial effects of agmatine for human health, as neurotransmitter, neuromodulator, stimulator of insulin release and tumor suppressor agent are well known (Galgano, Caruso, Condelli, & Favati, 2012). The neuroactive amines serotonin, tryptamine and 2-phenylethylamine were detected from the 35th day on. They are involved in the regulation of important functions such as sleep, hunger and mood (Gloria, 2006). Therefore, some of the amines formed can add interesting functional properties to the cheese.

Regarding adverse health effects to human health associated with biogenic amines, tyramine and histamine are the most worrisome. Histamine was detected at the end of ripening at low levels, which would not cause any harm to normal individuals (EFSA, 2011). Tyramine was the prevalent amine reaching 33.3 mg/100 g at the 49th day of ripening. Considering the no adverse health effects limit of 6 mg of tyramine per person per meal of an individual taking classical MAOI drugs, Gorgonzola-type of cheese should be avoided by these individuals.

There are some reports on the occurrence and levels of biogenic amines in blue cheeses from the consumers market. Vale and Gloria (1998) found, in Gorgonzola-type cheeses, histamine, tyramine and spermidine at levels up to 2.99, 1.07, and 3.23 mg/100 g, respectively.

Table 4
Mean counts (log CFU/g ± standard deviation) of lactic acid bacteria (LAB) and total mesophilic aerobic bacteria (TMAB) of Gorgonzola-type cheese throughout ripening at 12 ± 2 °C for up to 49 days after perforation.

Ripening time (days after perforation)	Mean counts (log CFU/g)	
	LAB	TMAB
14	9.42 ± 0.38 [a]	9.64 ± 0.21 [a]
21	9.31 ± 0.42 [a]	9.56 ± 0.48 [ab]
28	9.37 ± 0.27 [a]	9.54 ± 0.15 [ab]
35	9.34 ± 0.37 [a]	9.41 ± 0.24 [ab]
42	9.23 ± 0.41 [a]	9.15 ± 0.44 [ab]
49	8.56 ± 0.11 [a]	8.44 ± 0.75 [b]

Means (± standard deviation of six analyses of three replicates) followed by different letters in each column are significantly different (Tukey test, $p < 0.05$).

Tyramine was detected in 67% of the samples. Novella-Rodríguez, Veciana-Nogués, Izquierdo-Pulido and Vidal-Carou (2003) analyzed 20 blue cheeses and observed that tyramine, cadaverine, putrescine and histamine were the prevalent amines. Tyramine was present at high levels – 158.5 mg/100 g. In another work, tyramine in blue cheeses reached 22.8 mg/100 g (Ladero, Fernández, Cuesta, & Alvarez, 2010). Based on these results, despite the differences concerning types and levels of amines found in blue cheeses, tyramine is always present at high amounts. Therefore, tyramine should be considered the target amine when undertaking mitigation measures to improve the quality of Gorgonzola-type cheese and to reduce the risk of intoxication.

3.2.5. Microbiological analyses

The total counts of lactic acid bacteria (LAB) and total mesophilic aerobic bacteria (TMAB) were performed during Gorgonzola-type cheese ripening (Table 4) and it was observed that there was no change with time of the log of colony forming units per gram of cheese (CFU/g) for LAB ($p < 0.05$) and that a decrease in the log CFU/g of TMAB occurred only at the last ripening time evaluated. Flórez, Ruas-Madiedo, Alonso, and Mayo (2006) found similar results in blue cheeses. The presence of these microorganisms in the cheese allows the formation of biogenic amines through the action of amino acid decarboxylation enzymes on the free amino acids (Gloria, 2006).

3.3. Multivariate analysis

Multivariate statistical techniques developed for analytical chemistry has been adopted widely in food science and technology, and it is useful to bridge the gaps in multidisciplinary data needed for solid scientific conclusions (Granato et al., 2018).

In the PCA, the first two main components (PC1 and PC2) explained 86.7% of the total variance and allowed a good separation of ripening times, as the cheeses analyzed at 14, 21 and 28 days after cheese perforation were placed on the left side of the graph, whereas the 35, 42 and 49 days were on the right side (Fig. 3b). PC1 contributed with 78.6% of the total variance and it correlated with almost all free amino acids and amines, proteolysis indexes (positive correlations), and texture parameters (negative correlations), all of them around 0.9. PC2 contributed with 8.1% of the total variance, and its highest positive correlation was found with pH (0.785), whereas negative correlation was observed with tyrosine (−0.760) (Fig. 3a). HCA of the observations revealed that three groups could be discriminated during ripening: (i) 14 days; (ii) 21 and 28 days; and (iii) 35, 42 and 49 days (Fig. 3c). This arrangement suggests that ripening time affects cheese characteristics mostly in the beginning of ripening and, as the time passes by, the cheese becomes more homogeneous and suffers less differentiation.

HCA of the variables showed three clusters of analysis grouped by similarity (Fig. 4). The first included all texture parameters (except adhesiveness), total protein and the amino-acid serine. This cluster is of special interest as it can help explain the maintenance of almost all texture parameters significantly unchanged during ripening and proteolysis. The organic phosphate inside the caseins is esterified to the protein by means of the hydroxyl group from serine. Calcium binds to it

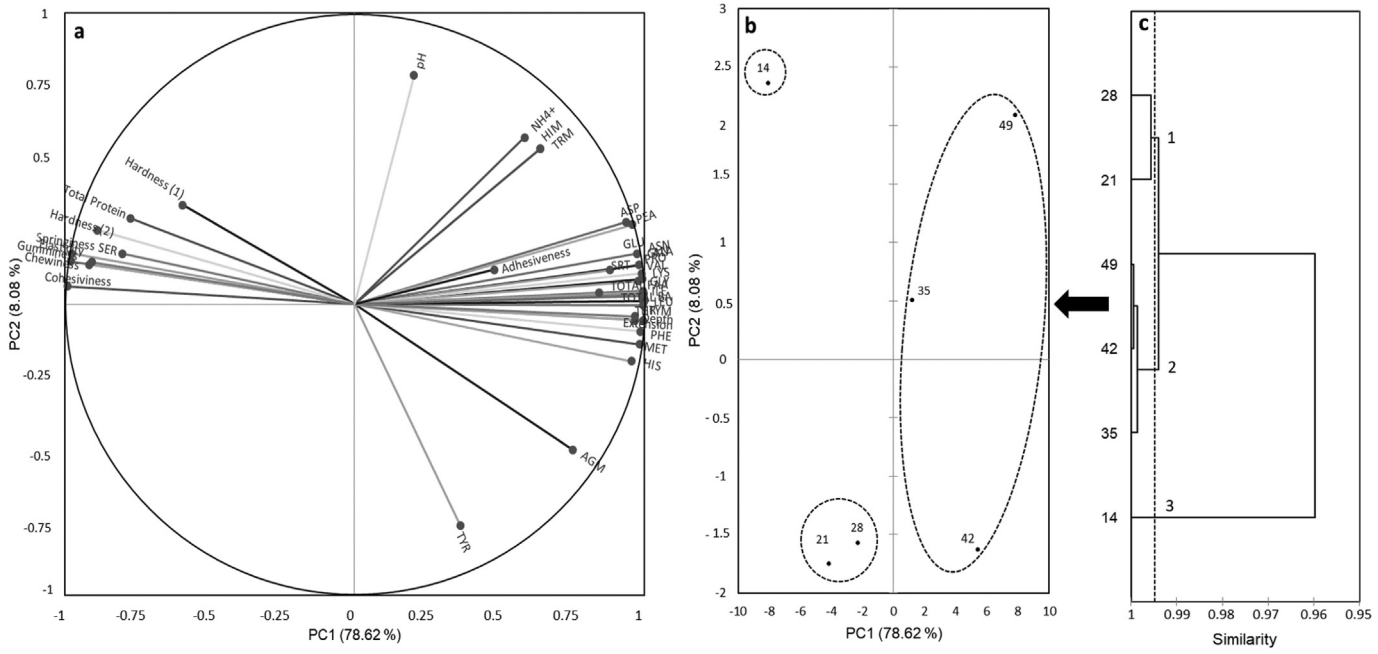

Fig. 3. Score (a) and loading (b) plots, obtained by Principal Component Analyses, for the means of texture parameters, proteolysis indexes, pH, total and individual bioactive amines and free amino acids* throughout ripening of Gorgonzola-type cheeses at 12 ± 2 °C for 49 days after perforation; (c) Dendrogram from Hierarchical Component Analysis for observations obtained at each ripening time.
*FAA-free amino acids, BA-bioactive amines, AGM- agmatine, ALA-alanine, ASN-asparagine, ASP-aspartic acid, GLN-glutamine, GLU- glutamic acid, GLY-glycine, HIM-histamine, HIS-histidine, ILE-isoleucine, LEU-leucine, LYS-lysine, MET-methionine, NH_4^+-ammonium ion, PEA-2-phenylethyl amine, PHE-phenylalanine, PRO-proline, SER-serine, SRT- serotonin, THR-threonine, TRM-tryptamine, TYM-tyramine, TYR-tyrosine, VAL-valine.

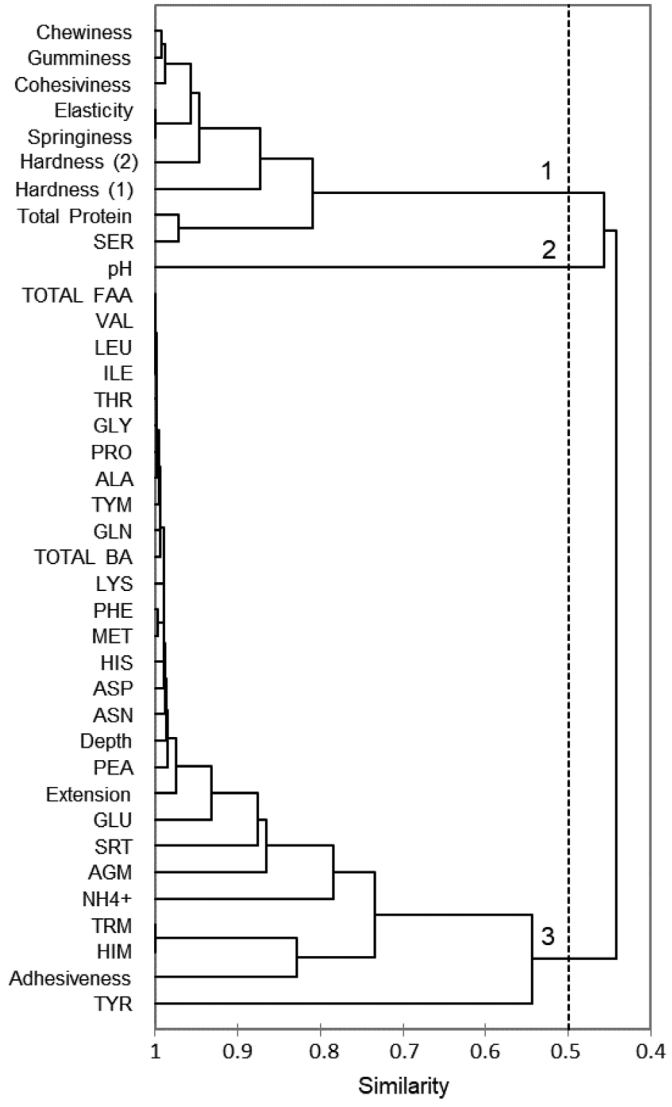

Fig. 4. Dendrogram from Hierarchical Component Analysis for the means of texture parameters, proteolysis indexes, pH, total and individual bioactive amines and free amino acids* throughout ripening of Gorgonzola-type cheeses at 12 ± 2 °C for 49 days after perforation.
*FAA-free amino acids, BA-bioactive amines, AGM- agmatine, ALA-alanine, ASN-asparagine, ASP-aspartic acid, GLN-glutamine, GLU- glutamic acid, GLY-glycine, HIM-histamine, HIS-histidine, ILE-isoleucine, LEU-leucine, LYS-lysine, MET-methionine, NH_4^+-ammonium ion, PEA-2-phenylethyl amine, PHE-phenylalanine, PRO-proline, SER-serine, SRT- serotonin, THR-threonine, TRM-tryptamine, TYM-tyramine, TYR-tyrosine, VAL-valine.

forming colloidal calcium phosphate clusters, which are very important to maintain the casein micelle structure, together with other hydrophobic interactions (Fox, Uniacke-Lowe, McSweeney, & O'Mahon, 2015). As serine was not detected during ripening from the 28th day after perforation on, it may be involved in the proteins structure, preventing texture changes throughout ripening. pH appears as an isolated parameter in the second cluster, whereas free amino acids, bioactive amines, extension and depth of proteolysis, and adhesiveness are joint together in the third cluster.

Further determinations may increase the knowledge about parameters interactions, acceptance, quality and safety throughout ripening of Gorgonzola-type cheese, such as sensory tests using consumers (Oliveira et al., 2017). Furthermore, mitigation measures to improve the quality of Gorgonzola-type cheese should focus on tyramine to prevent public health concerns.

4. Conclusion

Several changes are prevalent during ripening of Gorgonzola-type cheese at 12 ± 2 °C and ripening time is determinant of its characteristics. Proteolysis increased but texture kept unchanged, except for cohesiveness. There was no change on LAB counts during ripening and TMAB counts decreased. Amino acids were released and accumulated, with predominance of lysine, valine and phenylalanine. Tyrosine levels kept unchanged whereas serine levels decreased. No amine was detected on the 14th day after perforation. However, after that time, amines were formed, some with health promoting properties but also others with adverse health effects to individuals taking classical MAOI drugs.

Multivariate analysis summarized the interactions and helped explain some of the observed behaviors. PCA showed that proteolysis, expressed by extend and depth indexes, and free amino acid profile, are good markers for ripening assessment of Gorgonzola-type cheese.

Declarations of interest

None.

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