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Composition, microstructure and chemical interactions during the production stages of Mozzarella cheese

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ABSTRACT

Changes in chemical composition, microstructure and chemical interactions before and after the stretching stage of mozzarella cheese processing were investigated. The increased acidity and the decreased pH resulted in the solubilisation of total calcium. The protein matrix became more compact and the size of the fat globules decreased with the incorporation of small individual fat globules, aggregates and fat globules of irregular form into the matrix. The predominant bonds in the curd before the stretching stage were hydrophobic interactions, whereas the number of calcium bonds was minimal. After the stretching stage, the primary bonds responsible for maintaining the cheese structure were calcium bridges, electrostatic interactions and hydrogen bridges. These results clarify important aspects of the bonds involved in the production of this type of cheese.

1. Introduction

Worldwide production of mozzarella cheese has greatly increased in the last decade, and this is presently the most produced type of cheese, primarily because of its use as an ingredient in pizza (Jana & Mandal, 2011). The fast growth of the market for mozzarella cheese and competitive pressure has resulted in an impressive increase in industrial production capacities. However, large-scale manufacturing requires a precise control of the production process.

Mozzarella is a cheese of the pasta-filata type. In the stretching process, the cheese curd is subjected to mechanical work in hot water to turn the amorphous structure into an organised, elastic and compact structure (Banville, Chabot, Power, Pouliot, & Britten, 2016; Kindstedt, Carić, & Milanović, 2004). For the stretching process to occur, the calcium content associated with casein (insoluble calcium) must be ideal, and this is usually achieved through calcium solubilisation by acidification (Guinee, 2002). Casein hydration increases when the amount of calcium associated with casein decreases, and this contributes to the stretching process (Mizuno, Matsuda, Lucey, & Ichihashi, 2009). There are two parameters that determine the amount of calcium associated with the casein micelle during the stretching process: the total calcium content in the curd and the total calcium distribution between the soluble and insoluble forms (Kindstedt et al., 2004). Therefore, acidity development during mozzarella cheese manufacturing must be controlled to maintain the desired combination of total calcium content, pH and moisture (Kindstedt, 2007).

Curd heating during the stretching stage causes the calcium dissolved in the aqueous phase to bond again to the casein, resulting in protein interactions through calcium bridges that favour whey removal. In addition, this thermomechanical treatment causes physical-chemical changes that strongly influence the functional properties and the proteolysis of the cheese during its refrigerated storage (Banville et al., 2016).

The impact of different thermomechanical treatments on the characteristics of mozzarella cheese was assessed by Banville et al. (2016), and their results show that the amount of mechanical energy supplied was proportional to the fat loss of the cheese and that the amount of free whey was associated with the intensity of the thermal treatment. The authors concluded that the thermomechanical systems affected the cheese composition, solids loss and microstructure.

Although there have been many studies on mozzarella cheese (Bähler & Hinrichs, 2013; Ma, James, Zhang, & Emanuelsson-Patterson, 2013; Renda, Barbano, Yun, Kindstedt, & Mulvaney,

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1997; Rowney, Roupas, Hickey, & Everett, 2003), the interactions involved in the stretching stage need more studies to be elucidated. To produce pasta-filata cheeses such as mozzarella with specific functional properties, it is necessary to better understand the effect of the thermomechanical processes on the characteristics of the cheese (Banville et al., 2016). Therefore, the objective of this work was to assess the changes in the composition, microstructure and chemical interactions before and after the stretching stage of mozzarella cheese production.

2. Materials and methods

2.1. Processing of mozzarella cheese

To produce the cheese, 130 L of raw milk was used in each batch. The cheese processing was repeated twice on different days to ensure that similar results would be obtained from different milk batches of similar composition. A sample of the raw milk was taken from each batch to measure the fat content using the Gerber method (AOAC, 2006a) and the casein content using the formaldehyde method (Lourenço & Wolfschoon-Pombo, 1982) for the standardisation calculations. The milk was skimmed and standardised to a casein and fat ratio of 1.00. Then, the standardisation was checked by measurement of the casein and fat contents using the Kjeldahl (AOAC, 2006b) and Mojonnier (AOAC, 2006c) methods, respectively, and a value of 0.93 ± 0.05 was obtained. The standardised milk was subjected to a slow pasteurisation in a 110 L electrical, automatic pasteurisation tank made of stainless steel, with control of agitation and heating and cooling temperatures (Inadal, Mixmatic 110, Osasco, Brazil). After pasteurisation, the milk was cooled to 7 °C, placed in 50 L cans and stored in a cold chamber below 5 °C for one night. On the following day, the milk was transferred to an automatic cheese fabrication tank of 210 L capacity with a cutting lyre and adjustable agitation (Biasinox, Lambari, Brazil). A total of 1% lactic thermophilic bacteria, consisting of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (TCC 20; Chr. Hansen Ind. e Com. Ltda, Valinhos, Brazil) and 0.25 mL L^{-1} 50% calcium chloride (ECIBRA®; Ind. e Com. de produtos químicos Ltda, Santo Amaro, Brazil) were added to the milk at 37 °C. Pure chymosin obtained by fermentation (CHY-MAX® 100% Chymosin; Chr. Hansen Ind. e Com. Ltda) was used to coagulate the milk, and it was added at a sufficient amount to coagulate 130 L of milk in 35 min. After coagulation, the curd was cut in cubes with 1.5 cm edges and then slowly agitated for 25 min. The curd was treated by indirect heating until reaching 42 °C. One third of the whey amount was removed and the curd rested until the stretching point. Once the stretching pH was reached, the curd was salted in a 2% (w/w) proportion (Refinaria Nacional de Sal S/A, Cisne®; Cabo Frio, Brazil) and then cooled in an ice bath. The stretching process was adapted from Costa et al. (2017). After cooling, the curd was chopped into cubes of the same size and then underwent a manual stretching stage with water at 85 °C (62.0 ± 0.0 °C in the centre of the curd). The volume water and time spent in each cheese were controlled to standardise the stretching conditions. The volume of water used in the stretching stage of the cheese was approximately 3.25 ± 0.24 L, and the time spent for each piece of cheese was 2.73 ± 0.57 min. After stretching stage, the cheeses were shaped, cooled in an ice bath for 1 h, followed by drying at 12 °C for 24 h and vacuum packed in polyethylene bags.

2.2. Sampling during the processing

Samples of pasteurised milk (PM), curd at the cutting point (CC), curd after partial whey removal (CR), whey from the partial removal (WR), the curds during the acidification stage ($C_{pH5.8}$, $C_{pH5.6}$, $C_{pH5.4}$, $C_{pH5.2}$, $C_{pH5.1}$), whey at the stretching pH ($W_{pH5.2}$) and stretching water (SW) were taken for analyses. Several studies indicate that the stretching stage of mozzarella cheese occurs at a pH between 5.2 and 5.4 (Jana & Mandal, 2011; Lucey, Johnson, & Horne, 2003). The curd was also analysed at a pH of 5.1 to understand what happens to its composition at a pH below 5.2. The stages and the sampling performed during the processing are shown in Fig. 1.

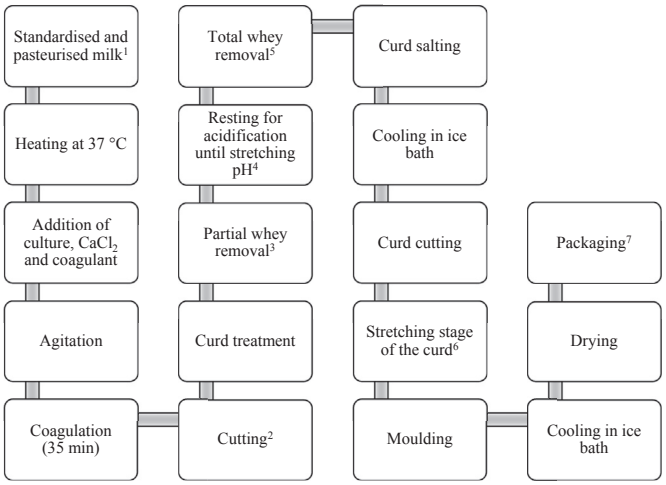

Fig. 1. Sampling stages of the standardised and pasteurised milk (PM)[1], curd after cutting (CC)[2], curd after partial whey removal and whey from the partial removal (CR and WR, respectively)[3] and curd samples during acidification ($C_{pH5.8}$, $C_{pH5.6}$, $C_{pH5.4}$, $C_{pH5.2}$, $C_{pH5.1}$)[4], whey after total removal when the curd reached pH ($W_{pH5.2}$)[5], stretching water (SW)[6] and cheese (C)[7].

All samples were placed in a 100-mL flask and immediately cooled in an ice bath. After cooling, the PM, WR, $W_{pH5.2}$ and SW samples were stored at 15 °C for analysis. The samples of CC, CR and the curds during acidification ($C_{pH5.8}$, $C_{pH5.6}$, $C_{pH5.4}$, $C_{pH5.2}$, $C_{pH5.1}$) were dispersed using a hand disperser (Ultra-Turrax, Polytron PT 1200E; Kinematica AG, Luzern, Switzerland) and stored at 15 °C for analysis.

2.3. Chemical composition

All samples were analysed for their casein content (AOAC, 2006b); fat content using the Mojonnier method (AOAC, 2006c); acidity by titration according to the official procedure (AOAC, 2006d); pH using the potentiometric method (Digimed DM20 potentiometer; Digicrom Analítica Ltd, Santo Amaro, SP, Brazil); total calcium (TC) using the dry digestion method (AOAC, 2006e) followed by titration with ethylenediaminetetraacetic acid (EDTA) in the presence of murexide (Taras, 1995); insoluble calcium (NC) calculated from the difference between the TC and soluble calcium contents, as described by Metzger, Barbano, Rudan, and Kindstedt (2000); and moisture (AOAC, 2006f). The salt (NaCl) content of the cheese was also analysed using the Volhard method (Richardson, 1985). All characterisation analyses were performed in triplicate.

2.4. Assessment of the fat particle size

The sample was prepared according to Lopez, Carmier, and Gassi (2007). One millilitre was removed from the milk sample (PM), 10 mL of a solution of 1% (w/w) sodium dodecylsulphate (SDS; Synth, Diadema, SP, Brazil) and 35 mmol L^{-1} of EDTA (Vetec Química Fina Ltda, Duque de Caxias, RJ, Brazil) were added to it, and the pH was then adjusted to 7.0. To determine the fat particle size in the curds (CC, CR, $C_{pH5.8}$, $C_{pH5.2}$) and cheese, 1 g of the sample was dissociated with 5 mL

of dissociation buffer: 6 mol L^{-1} urea (Synth, Diadema, SP, Brazil), 100 mmol L^{-1} EDTA (Vetec Química Fina Ltda, Duque de Caxias, RJ, Brazil), 20 mmol L^{-1} imidazole buffer (Sigma, Saint Louis, USA), pH 6.6. Next, the samples were slowly stirred at 8 rpm (mixer model AP 22; Phoenix, Araraquara, Brazil) for 30 min, and the particle size was measured immediately afterwards using laser diffraction in a Mastersizer instrument (Malvern Instruments Ltd., Worcester, United Kingdom). The average diameter based on volume d[4,3] was calculated using Eq. (1), where η_i is the number of particles of diameter equal to d_i. The value of d[4,3] is highly influenced by large fat and protein particles (Lopez-Sanchez, Svelander, Bialek, Schumm, & Langton, 2011).

$$d[4,3] = \sum n_i d_i^4 \Big/ \sum n_i d_i^3 \quad (1)$$

2.5. Apparent zeta (ζ) potential

The samples of milk (PM), the curds (CC; CR; $C_{pH5.8}$; $C_{pH5.2}$) and cheese (C) were prepared according to Michalski, Michel, Sainmont, and Briard (2001). The zeta potentials of the samples taken during processing, and of the cheese, were measured in a ZetaSizer (Malvern Instruments Ltd). The samples (1 mL of PM and 1 g of CC, CR, $C_{pH5.8}$, $C_{pH5.2}$ and C) were diluted in a 1:25 proportion in a buffer solution (pH adjusted to 7.0) of 20 mM imidazole (Vetec Química Fina Ltda, Duque de Caxias, RJ, Brazil), 50 mM NaCl and 5 mM $CaCl_2$ (both from Synth, Diadema, SP, Brazil). The buffer solution was previously filtered with 0.2 μm cellulose filter paper. The samples with the buffer were agitated and then taken to the Zetasizer at 25 °C.

2.6. Types of bonds

Specific tests were performed of the different types of bonds responsible for the structure of the curd before the stretching stage and of the cheese after the stretching stage. The method consisted of extractions from the samples of curd at pH 5.2 and cheese with different buffer solutions, to obtain a semi-quantitative estimate of the covalent and non-covalent bonds that stabilise the protein structure.

The analysis of the bond types was performed according to the procedure described by Keim, Kulozik, and Hinrichs (2006) with some changes. The samples (2 g) of curd before the stretching stage and of cheese were transferred to a 50-mL plastic tube with 20 mL of the different buffer solutions (Supplementary material Table S1) with the following reagents: trisodium citrate (Vetec Química Fina Ltda, Duque de Caxias, RJ, Brazil); DTT and SDS (both from Sigma Aldrich, St. Louis, MO, USA); EDTA (Vetec Química Fina Ltda, Duque de Caxias, RJ, Brazil); and monosodium phosphate, NaCl and Tris (all from Synth, Diadema, SP, Brazil). Hydrochloric acid (Synth) was added to adjust the pH of buffer solutions A and B. The buffer solutions were added and homogenised at room temperature for 5 min. Then, the samples were agitated in an orbital agitation table (model MA 140CFT; Marconi, Piracicaba, Brazil) for 30 min and centrifuged (Allegra™ 64R Centrifuge; Beckman Coulter, São Paulo, Brazil) at 15,000 × *g* for 20 min at 20 °C. The supernatant was then filtered with 0.2 μm paper, and the total nitrogen content was determined using the Kjeldahl method (AOAC, 2006g). The nitrogen contents of the buffer solutions (Nbuffer, S) and of the samples (Ncurd and Ncheese) were also determined. The nitrogen content of each buffer solution was calculated using Eq. (2).

$$Nbond,S = \frac{mS+ms}{mS}.Nsup,S - \frac{mS}{ms}.Nbuffer,S \quad (2)$$

where Nsup is the nitrogen content of the supernatant in the curd or in the cheese, mS is the mass of each buffer solution, and ms is the mass of the samples (curd or cheese).

The interpretation of the data on the bond types was based on the solubility of the samples of the different buffer solutions, with the set of equations (Eqs. (3)–(10)) below:

$$\frac{Nbond,A}{Ns} = P(EB) + P(Hy) + P(HB) + P(ub) \quad (3)$$

$$\frac{Nbond,B}{Ns} = P(EB) + P(Hy) + P(HB) + P(SS) + P(ub) \quad (4)$$

$$\frac{Nbond,C}{Ns} = P(EB) + P(HB) + P(ub) \quad (5)$$

$$\frac{Nbond,D}{Ns} = P(EB) + P(HB) + P(CaB) + P(ub) \quad (6)$$

$$P(SS) = \frac{Nbond,B}{Ns} - \frac{Nbond,A}{Ns} \quad (7)$$

$$P(Hy) = \frac{Nbond,A}{Ns} - \frac{Nbond,C}{Ns} \quad (8)$$

$$P(EB) + P(HB) + P(ub) = \frac{Nbond,C}{Ns} \quad (9)$$

$$P(CaB) = \frac{Nbond,D}{Ns} - \frac{Nbond,C}{Ns} \quad (10)$$

where Nbond,A, Nbond,B, Nbond,C, Nbond,D are the nitrogen contents of the supernatant obtained from the treatment of the samples with buffer solutions A, B, C and D (g g^{-1}); Ns is the nitrogen content in the curd (g g^{-1}) or in the cheese (g g^{-1}); and P (j) is the amount of protein (%) stabilised by j interactions, where j corresponds to electrostatic interactions (EB), hydrophobic interactions (Hy), hydrogen bridges (HB), disulphide bridges (SS), calcium bridges (CaB) and free proteins (ub).

2.7. Assessment of the microstructure of the curds (CC; CR; $C_{pH5.8}$ and $C_{pH5.2}$) and the cheese (C)

The curds and cheese samples were prepared according to the methodology described by Lopez et al. (2007). Initially, the samples were cut in cubes with 5 mm edges with a steel blade and placed in the centre of a glass slide. The samples were stained with the addition of 0.5 mL of acridine orange fluorescent dye and Nile red dye (both from Sigma Aldrich), previously dispersed in anhydrous ethylic alcohol 99.8% (Synth). The solvent was evaporated in the dark. Next, a slide with the sample was covered with a coverslip and stored in the dark for 30 min at 4 °C to evaporate the solvent and then viewed under a microscope (AGZeiss LSM780-NLO; Carl Zeiss, Berlin, Germany). The wavelengths used to excite the Nile red and the acridine orange fluorescent dyes were 568 and 488 nm, respectively. Images of slices of 100–350 μm with a 1024 × 1024 pixel resolution were obtained.

2.8. Experimental design

All experiments were performed in duplicate. The results of composition and apparent zeta (ζ) potential of samples CC, CR, $C_{pH5.8}$, $C_{pH5.6}$, $C_{pH5.4}$, $C_{pH5.2}$ and $C_{pH5.1}$ and of particle size of samples CC, CR, $C_{pH5.8}$, $C_{pH5.2}$ and cheese taken during the manufacturing

stages of mozzarella cheese were analysed using analysis of variance (ANOVA). The Tukey test was applied to verify differences among the means. Differences were considered significant at a 95% probability level ($P \leq 0.05$). The standard deviations are shown as error bars in the figures. The data were analysed using STATISTICA 7.0 software (StatSoft Inc., Tulsa, OK, USA).

3. Results and discussion

3.1. Composition of the standardised and pasteurised milk, whey after partial removal, whey before stretching stage (pH 5.2) and stretching water

The compositions of the standardized and pasteurised milk (PM), whey after partial removal (WR), whey from the stretching stage (WpH5.2) and stretching water (SW) are shown in Table 1. The acidity and total solids contents of the milk meet the technical regulations for pasteurised milk (Brasil, 2011). The pH of samples WR, $W_{pH5.2}$ and SW decreased during the production stages of the mozzarella cheese. In addition, the pH reduction resulted in calcium solubilisation, indicated by the decrease in insoluble calcium from 0.040% in sample CR to 0.020% in sample $W_{pH5.2}$ and by the 7.3% reduction of the apparent zeta potential. The neutralisation of the charges occurs as the pH decreases, which causes a decrease in the electrostatic repulsion, and consequently, decreasing the zeta potential.

The total calcium concentration in the milk was 0.154%, with 58.4% in the colloidal form; this was slightly lower than the value reported by Walstra (2006) of 66% in the colloidal form (bonded to casein) and 34% in the soluble form as Ca^{2+} bonded to citrates and phosphates.

The milk studied in this work had a fat content of 2.3%. A milk fat content of 2.5% is considered optimum for making mozzarella cheese that is to be consumed in slices (Valle, Campos, Yotsunagi, & Souza, 2004), i.e., very close to the value of this study. According to Huppertz and Kelly (2006), fat is initially dispersed in milk as lipid droplets with a size distribution between 0.2 and 15 μm. The size of the fat globules of the standardised and pasteurised milk was determined as $d_{43} = 4.66 \pm 0.65$ μm in this study. Lopez et al. (2007) reported that, in the case of Emmental cheese, the size of the milk fat globules varied between 1.0 and 10 μm, with $d_{43} = 4.04 \pm 0.03$ μm, slightly lower than that of this study.

The total solids and fat in the samples WR, $W_{pH5.2}$ and SW show losses during the production stages of mozzarella cheese. There was a 12.6% loss of fat in whey at pH 5.2, corresponding to whey after its total removal. There was practically no loss of casein. However, the loss of components has a direct influence on the yield and may affect functional, rheological and sensory characteristics of mozzarella cheese.

3.2. Influence of the production stages on the curds and mozzarella cheese composition

The compositions of the curds at different production stages and of the mozzarella cheese are shown in Table 2. The acidification of the curd resulted in more whey release and in the consequent reduction of 31.87% of the curd moisture after the cutting point (CC) when compared with the cheese moisture (C). The mozzarella cheese had 17.07% ± 1.07 fat, 52.63% ± 0.39 moisture and 1.63% ± 0.04 salt. The low fat % in the cheese is a result of the casein/fat ratio of the milk and the high moisture content of the cheese. After the stretching stage ($C_{pH5.2}$), only 66.87% of the insoluble calcium in the curd after the cutting point (CC) was still bonded to the casein (insoluble calcium/casein ratio expressed in dry basis). This value decreased to 58.20% in the cheese after the stretching stage and was 59.75% in the curd at pH 5.1, below the ideal for stretching (Table 2). These results confirm that calcium bonded to casein must be partially solubilised to promote the stretching of the curd.

The insoluble calcium/casein*DB ratio of the cheese (C) was 1.88, a 41.79% ($P = 0.00$) reduction of the insoluble calcium of the curd after cutting (CC) (Table 2). According to Guinee, Feeney, Auty, and Fox (2002), calcium and pH are interdependent and are the primary factors that influence the microstructure of mozzarella cheese. The pH defines the amount of soluble calcium lost in the curd after whey removal and the soluble calcium/insoluble calcium ratio in the final cheese. The proportion of soluble calcium increases with decreasing pH, which helps neutralise the protein charges. This allows the association of proteins through hydrophobic interactions; thus, at a low pH, the degree of solvation of the protein is thought to decrease. In turn, at a high pH, the protein matrix will swell and absorb more water and the size of the whey channels will decrease. Fig. 2 shows the evolution of pH and soluble calcium during the processing stages of mozzarella cheese. The pH from CC until $C_{pH5.2}$ decreased ($P < 0.0001$) and the calcium bonded to casein solubilised more with the pH reduction.

3.3. Microstructure of the curd, apparent (ζ) potential, particle size and predominant bond types before the stretching stage and of the cheese after the stretching stage

The confocal microscopy technique enabled the analysis of the fat globules without modifying the internal structures of the curds during production as well as that of the cheese, so it was possible to visualise what occurs during the different processing stages of mozzarella cheese. Fig. 3 shows the microstructure of the curds during the production stages (cutting, whey removal, acidification and stretching) and of the mozzarella cheese after the stretching. The images of the curd after cutting (Fig. 3A) and after partial whey

Table 1
Composition of the standardised and pasteurised milk, whey after partial removal and whey at stretching pH (pH 5.2)[a].

Chemical composition	Samples			
	PM	WR	$W_{pH5.2}$	SW
Acidity (%)	0.14 (0.00)	0.11 (0.00)	0.20 (0.00)	0.025 (0.00)
pH	6.50 (0.02)	6.32 (0.03)	5.22 (0.04)	5.26 (0.01)
Total solids (%)	10.9 (0.11)	5.99 (0.22)	6.38 (0.19)	0.75 (0.10)
Casein (%)	2.17 (0.16)	0.01 (0.00)	0.01 (0.00)	0.01 (0.00)
Fat (%)	2.31 (0.17)	0.36 (0.03)	0.28 (0.07)	0.27 (0.10)
Total calcium (%)	0.150 (0.005)	0.071 (0.009)	0.072 (0.011)	0.024 (0.007)
Insoluble calcium (%)	0.090 (0.019)	0.040 (0.014)	0.020 (0.003)	0.020 (0.007)
Apparent zeta-potential, mV	−8.48 (0.65)	−8.78 (0.36)	−8.14 (0.18)	−6.45 (0.20)

[a] Abbreviations are: PM, standardised and pasteurised milk; WR, whey after partial removal; $W_{pH5.2}$, whey at stretching pH; SW, stretching water. The batch was repeated twice. Values are means with standard deviations in parentheses.

Table 2
Composition of the curds and cheese during the production stages of mozzarella cheese[a].

Composition	Samples							
	CC	CR	$C_{pH5.8}$	$C_{pH5.6}$	$C_{pH5.4}$	$C_{pH5.2}$	$C_{pH5.1}$	Cheese
Moisture (%)	86.83 (0.68)^{a}	76.50 (0.65)^{b}	66.00 (1.44)^{c}	63.13 (0.80)^{c}	56.88 (0.87)^{d}	54.50 (0.07)^{de}	54.95 (0.56)^{de}	52.63 (0.39)^{f}
Total calcium (%)	0.105 (0.008)^{c}	0.425 (0.035)^{b}	0.475 (0.003)^{ab}	0.467 (0.008)^{ab}	0.483 (0.003)^{ab}	0.499 (0.009)^{a}	0.502 (0.008)^{a}	0.498 (0.00)^{a}
Insoluble calcium (%)	0.089 (0.008)^{c}	0.393 (0.035)^{b}	0.436 (0.004)^{ab}	0.422 (0.011)^{ab}	0.429 (0.006)^{ab}	0.449 (0.016)^{ab}	0.433 (0.013)^{ab}	0.456 (0.011)^{a}
Casein (%)	2.77 (0.15)^{g}	9.30 (0.52)^{f}	15.48 (0.39)^{e}	17.43 (0.41)^{ed}	18.56 (0.38)^{cd}	20.78 (0.19)^{bc}	22.39 (0.01)^{ab}	24.25 (1.54)^{a}
Total calcium/casein	0.038 (0.005)^{ab}	0.045 (0.001)^{a}	0.030 (0.00)^{bc}	0.026 (0.00)^{cd}	0.026 (0.00)^{cd}	0.024 (0.00)^{cd}	0.022 (0.00)^{d}	0.020 (0.009)^{d}
Insoluble calcium/casein	0.032 (0.004)^{b}	0.042 (0.001)^{a}	0.028 (0.00)^{bc}	0.024 (0.00)^{cd}	0.023 (0.00)^{cd}	0.021 (0.00)^{cd}	0.019 (0.00)^{d}	0.018 (0.009)^{d}
Insoluble calcium/casein, dry basis	3.23 (0.47)^{b}	4.22 (0.14)^{a}	2.83 (0.04)^{bc}	2.40 (0.00)^{cd}	2.31 (0.01)^{cd}	2.16 (0.04)^{cd}	1.93 (0.05)^{d}	1.88 (0.18)^{d}
d_{43} (μm)	7.58 (0.82)^{a}	4.32 (0.27)^{bc}	5.73 (0.35)^{ab}	-	-	3.74 (0.01)^{bc}	-	2.79 (0.83)^{c}

[a] Abbreviations are: CC, curd after cutting; CR, curd after partial whey removal; $C_{pH5.8}$, curd at pH 5.8; $C_{pH5.6}$, curd at pH 5.6; $C_{pH5.4}$, curd at pH 5.4; $C_{pH5.2}$, curd at pH 5.2; $C_{pH5.1}$, curd at pH 5.1; d_{43} (μm), average volume-weighted diameter. Values are means with standard deviation in parentheses; means with different superscript letters in the same row are significantly different by the Tukey's test ($P \leq 0.05$). The batch was repeated twice.

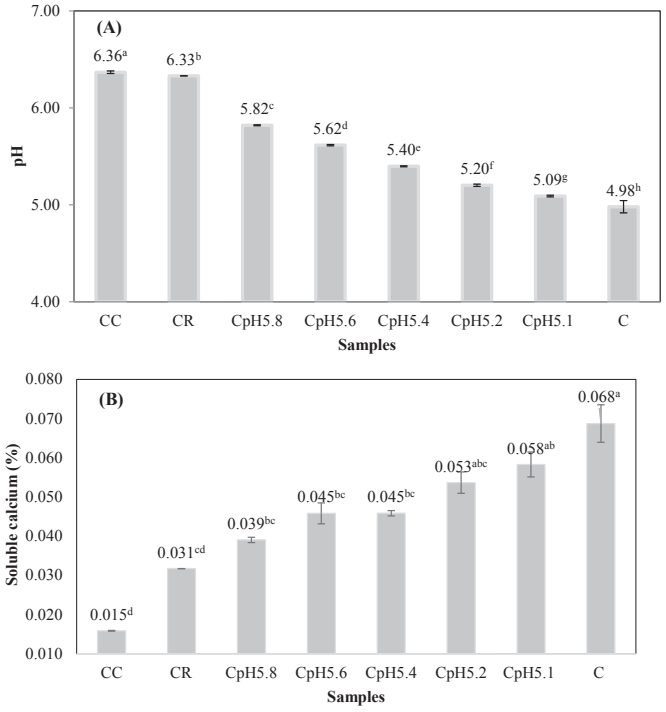

Fig. 2. Evolution of pH (A) and soluble calcium (B) during the processing stages of mozzarella cheese. Curd after cutting (CC), curd after whey removal (CR), samples of the curds during acidification ($C_{pH5.8}$, $C_{pH5.6}$, $C_{pH5.4}$, $C_{pH5.2}$, $C_{pH5.1}$) and cheese (C). Average values with different superscript letters are significantly different by the Tukey's test ($P \leq 0.05$). The cheese processing was repeated twice.

removal (Fig. 3B) show fat globules dispersed in an open protein matrix with an amorphous and unorganised structure. Because the pH was near 6.3 during cutting and whey removal, the membrane of the fat globule was negatively charged, which resulted in electrostatic repulsion between the fat globules and their consequent separation from each other (Fig. 3A,B).

The curd was agitated and heated after cutting. The thermomechanical treatment of the curd caused syneresis, a result of the grain's contraction that enables whey expulsion, which caused changes in the structure with the formation of a more compact protein matrix and, consequently, fat globules closer to each other (Fig. 3B). This approach was favoured by the reduction of the apparent (ζ) potential (Fig. 4).

The apparent (ζ) potential measures potential in volts at the surface of hydrodynamic shear. High values of apparent (ζ) potential mean a high level of charge–charge interactions or repulsion. This means that the high the apparent (ζ) potential, the greater the strength of the electrostatic interactions. In turn, a lower apparent (ζ) potential decreases the repulsion among the particles. Fig. 4 shows the apparent (ζ) potential of the curd after cutting, curd after whey removal, curd at pH 5.8, curd at stretching pH (pH 5.2) and the cheese, the apparent (ζ) potential decreased with increasing acidity. In parallel, the negative charges of the casein reduce with the pH decreases caused by the dissociation of the calcium ions from the micelles.

The sharp 56.04% increase in total solids from the curd after cutting to that after whey removal (Table 2), as a result of heating, caused the syneresis and contraction of the protein matrix. The increase in density of the protein matrix during the heating reduced the size of the whey pores (Fig. 3B); therefore, the fat globules and/or fat globules aggregates became very large and separated the protein matrix. The fat retention inside the cheese matrix is strongly influenced by the initial fat and protein content of milk and by the curd cutting (Johnston, Luckman, Lilley, & Smale, 1998). In addition, native fat globules with hydrophilic surfaces that did not interact with the protein network were expelled with the whey and the stretching water during the cheese processing stages (Table 1). Therefore, the external region of the curd grains had a low concentration of fat globules (Fig. 3B).

Fig. 3C shows the curd at pH 5.8 ($C_{pH5.8}$), with a protein matrix even more compact, with 24% of whey lost compared with the curd after cutting, and with few voids compared to the protein matrix after cutting (Fig. 3A) and after whey removal (Fig. 3B). The average fat globules sizes were 7.58 ± 0.82 μm and 5.73 ± 0.35 in the curd after cutting and in the curd at pH 5.8 ($C_{pH5.8}$), respectively (Fig. 3A,C). After the stretching stage, the protein matrix was more organised and porous, interwoven with fat globules of different sizes (Fig. 3D). At the end of the mozzarella cheese production, the protein matrix became more compact, with more agglomeration of fat particles and with the incorporation of small individual fat globules (of approximately 2.79 ± 0.83 μm, Table 2) (Fig. 3E). The mozzarella cheese had a lower fat content because of the standardisation of the casein and fat ratio (Table 1); therefore, the higher volume fraction of the casein matrix formed thicker para-casein fibres with reduced fat and whey channel inclusions, as observed in the micrographs.

The average particle sizes varied from 7.58 ± 0.82 μm in the curd after cutting to 3.74 ± 0.01 μm in the curd at the stretching pH (Table 2). The membranes of the fat globules are negatively charged at the cutting point (pH 6.37), which increases the electrostatic repulsions among the fat globules. The average size of the fat particles decreased to 2.79 ± 0.83 μm after the stretching stage due to the thermomechanical treatment during this process.

The changes in the zeta potential (Fig. 4) suggest protein adsorption on the surface of the fat globules, which may induce the

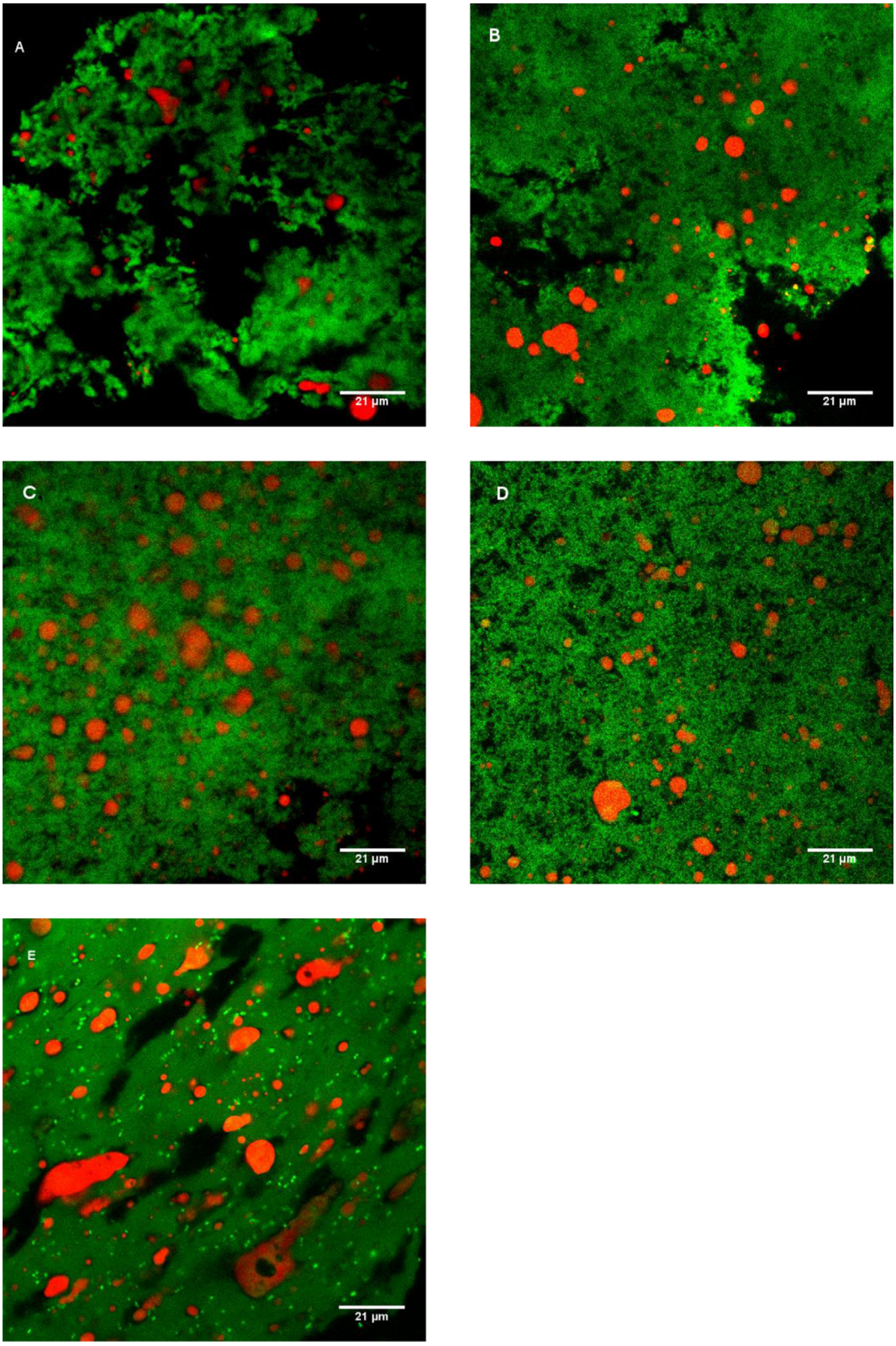

Fig. 3. Micrographs of the curds and cheese: A, curd after cutting (CC); B, curd after partial whey removal (CR); C, curd during acidification ($C_{pH5.8}$); D, curd during acidification ($C_{pH5.2}$); E, cheese after the stretching stage (C).

formation of aggregates of fat globules. However, some globules seem to be trapped in the protein matrix (Fig. 3A).

The rupture of the fat globules entrapped with the protein matrix were also observed in the confocal microscopy (Fig. 3E). The heating during the stretching stage induced some changes in the microstructure of the fat globules, such as coalescence and rupture. The stretching process stretched the fat globules that filled the voids between the protein fibres. The density increase in the

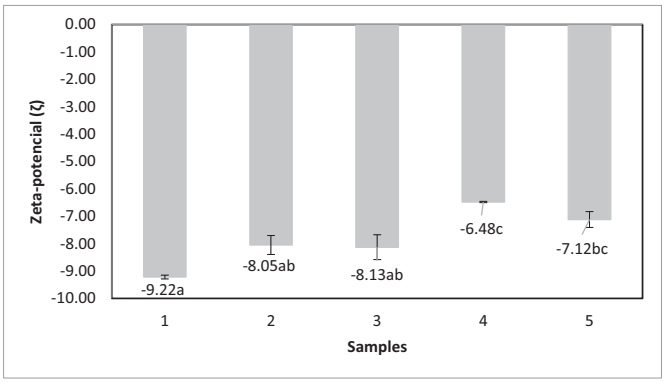

Fig. 4. Apparent zeta-potential of curd after cutting (1; CC), curd after partial whey removal (2; CR), curd during acidification (3; $C_{pH5.8}$), curd during acidification (4; $C_{pH5.2}$) and cheese after the stretching stage (5; C). Average values with different superscript letters are significantly different by the Tukey's test ($P \leq 0.05$).

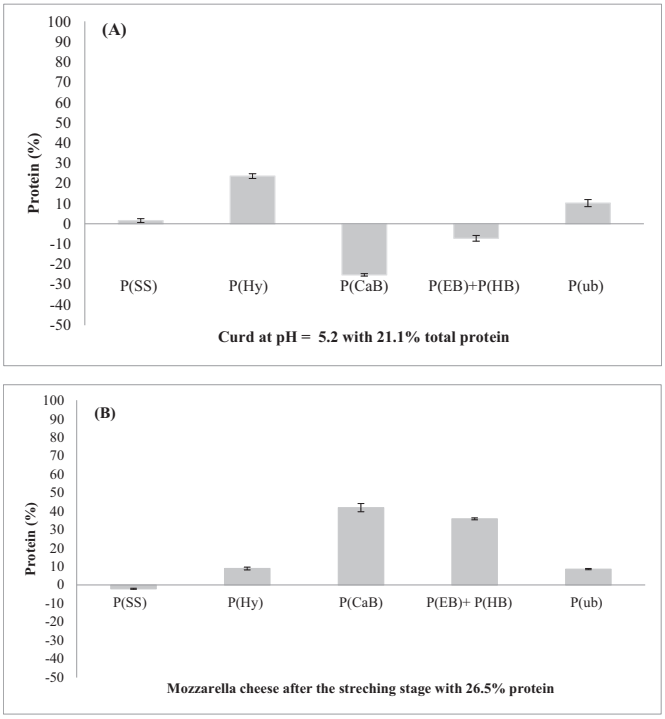

Fig. 5. Predominant bond types in (A) the curd at stretching pH ($C_{pH5.2}$) and (B) the cheese after the stretching stage: P(SS), disulphide bonds; P(Hy), hydrophobic interactions; P(CaB), calcium bridges; P(EB + HB), electrostatic interaction + hydrogen bonds; P(ub), unbound protein. The cheese processing was repeated twice.

protein matrix reduced the space occupied by the whey, and therefore, the fat globules and/or the fat globule aggregates became very large and separated the protein matrix. In addition, fat globules with hydrophilic surfaces that had not interacted with the protein network may have been expelled with the whey during the contraction of the globules. The concentrations of the fat expelled from the heated curd grains were 0.36 ± 0.03% and 0.28 ± 0.07% in the whey partially removed (WR) and in the whey at pH 5.2 ($S_{pH5.2}$; Supplementary material Table S1), respectively.

The high temperature applied to the curd during the stretching stage favoured the hydrophobic interactions (protein–protein), which caused the aggregation and contraction of the protein matrix. The contraction initiates the partial separation between the protein phase and the water inside the curd structure. The application of shear forces on the heated curd during the stretching stage aligned the aggregated protein matrix in dense elastic fibres separated by free fat and whey channels (Fig. 3E).

The change induced by the temperature resulted in the increase in the insoluble calcium content, which presumably has the effect of reinforcing the protein interactions and strengthening the dense para-casein fibres through bonds with calcium [calcium bridges (CaB), Fig. 5B]. According to Joshi, Muthukumarappan, and Dave (2003), non-covalent bonds, such as calcium bridges, are responsible for stabilising the gel structure obtained by enzymatic coagulation. During enzymatic coagulation, the soluble calcium, in its ionic form, helps form the curd and favours cross bonds among the casein micelles. In cheese, the decrease in the insoluble calcium content reduces the electrostatic interactions among the casein molecules and improves the softness, while making the casein molecules more susceptible to proteolysis (Fathollahi, Hesari, Azadmardb, & Oustan, 2010). Some authors suggest that the calcium bridges and the hydrophobic interactions are the bonds responsible for stabilising the structure of the cheese's protein network, whereas other authors indicate an additional effect of the electrostatic interactions and the hydrogen bridges (Keim et al., 2006).

The predominant bonds among the proteins in the curd at the stretching pH (pH 5.2) were the hydrophobic interactions (Fig. 5A). The number of CaB was minimal, as was the number of disulphide bridges, which allowed the curd to undergo the stretching process, considering that, at this pH, the number and/or the sum of the forces of all types of bonds was weaker. When the curd reached a pH of approximately 5.25, the number of calcium phosphate bonds in the micelles (insoluble calcium) was minimal, which indicated that the percentage of stabilised protein was minimal (Fig. 5B). The bonds among the micelles became weaker with the pH decrease associated with the increase in the soluble calcium content, as a combined effect, which caused the rigidity curd diminish, favouring fluidity when the tension of the stretching process was applied in the curd.

According to Yun, Kiely, Kindstedt, and Barbano (1993), the stretching stage of mozzarella cheese is easier to achieve in the pH range between 5.0 and 5.3. In this range, calcium phosphate becomes more dissociated and a decrease in the net charge on the proteins helps increase the degree of hydrophobic interaction among the casein molecules, as also shown in this study (Fig. 5). The stretching stage is probably responsible for the calcium bridges and the electrostatic interactions and the hydrogen bridges (Hinrichs & Keim, 2007) that are also predominant in mozzarella cheese (Fig. 5B). The additional effect of the electrostatic interactions and/or the hydrogen bridges may have occurred because of the partial calcium removal during the stretching stage of the curd.

There was an increase in electrostatic interactions, hydrogen bonds and calcium bonds between the curd at the stretching pH ($C_{pH5.2}$) and the cheese after the stretching stage (Fig. 5A,B). Therefore, the predominant bonds in the cheese were the calcium bonds, the electrostatic interactions and the hydrogen bridges. These results agree with the casein micelle model described by Horne (1998) and developed by Lucey et al. (2003) to explain the cheeses' texture. The same authors affirm that those bonds are the primary interactions that control the cheeses' melting and behaviour at high temperatures. The force or the contribution of each type of interaction is regulated by the residual charge in the casein molecule, the casein fractions and the storage temperature of the cheese. This residual charge is directly influenced by the pH, the ionic force and the calcium bond (Lucey et al., 2003).

4. Conclusions

There were changes in the structure and composition of the curd grains with acidification. The protein matrix became more compact, and the size of the fat globules decreased. The predominant bonds in the curd at the stretching pH were hydrophobic interactions, whereas the number of calcium bridges was minimal, which allowed the stretching stage of the curd. The predominant bonds after the stretching stage were calcium bridges, electrostatic interactions and hydrogen bridges. The additional effect of the electrostatic interactions and hydrogen bridges were a consequence of the partial calcium removal during the stretching stage of the curd. These results clarify important aspects of the bonds involved in the production of this type of cheese.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.idairyj.2018.07.005.

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