Impact of freezing on the physicochemical and functional properties of low-moisture part-skim Mozzarella

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Abstract

Low-moisture part-skim (LMPS) Mozzarella cheeses were held at 4°C for 0, 2 or 8 d before freezing to -20°C. The cheeses were frozen at a rate of 0.6, 2.0 or 8.0°C h^{-1} and held frozen at -20°C for 1, 6, 12 or 44 weeks. After freezing, cheeses were stored at 4°C for 16-37 d, resulting in a total storage time at 4°C (before and after freezing) of 24-37 d (frozen-thawed Mozzarella). Control Mozzarella was stored at 4°C for 25-37 d. The control and frozen-thawed cheeses were assayed for composition, primary proteolysis, moisture distribution, texture profile analysis and melting characteristics after similar storage times at 4°C. Freezing under the evaluated conditions resulted in reduced firmness of the unheated cheese but did not significantly affect the properties of the heated cheese. The results suggest that freezing may be effectively applied to control or extend the functional shelf-life of LMPS Mozzarella shipped to long-distance markets.

1. Introduction

The production of low-moisture part-skim (LMPS) Mozzarella has grown worldwide because of the increasing popularity of pizza. It is a stretched-curd (pasta-filata) cheese, the manufacture of which typically involves kneading and stretching the fermented curd in hot water or dilute brine until it acquires a uniform molten stretchy consistency. The plasticization process confers the cheese with the ability to stretch and undergo limited oiling-off when subsequently baked on pizza (McMahon & Oberg, 2017). Based on the author’s knowledge of the South-East Asian market, some producers import LMPS Mozzarella from Europe, the United States, Australia and/or New Zealand to compensate issues with local milk quality and supply. Guinee, Mulholland, Mullins, Corcoran & Auty (1999) reported that extended storage of LMPS Mozzarella (e.g. > 60 d at 4ºC) resulted in a deterioration in functionality as manifested by the shredded cheese developing an increased susceptibility to clumping/balling and the baked cheese exuding excess free oil, and having a 'soupy' consistency to a degree dependent on cheese composition and proteolysis. Bertola, Califano, Bevilacqua & Zaritzky (1996a) noted that producers freeze LMPS Mozzarella for long-distance export to minimize changes in proteolysis and functionality. Relatively few studies have investigated the effects of freezing on the physicochemical and functional characteristics of LMPS Mozzarella. Ribero, Rubiolo & Zorilla (2007) reported that the freezing point of LMPS Mozzarella ranged between -1.2°C and -2.6°C owing to the presence of solutes (i.e., salts, minerals, N-soluble compounds, lactose and organic acids) in the serum phase. Some studies investigated the effects of freezing on the mechanical characteristics of LMPS Mozzarella (Cervantes, Lund, & Olson, 1983), but did not evaluate other characteristics such as extensibility, consistency and flow of the cooked cheese, which are critical functionalities in pizza application. Potential issues with the freezing of cheese include ice crystallization in the serum phase, mineral deposition, casein dehydration and thereby impairment of the functionality of the frozen-thawed cheese (Everett & Auty, 2008; Kuo & Gunasekaran, 2003; Oberg, Merrill, Brown, & Richardson, 1992). Kuo, Anderson & Gunasekaran (2003) monitored the formation of ice crystals in small LMPS Mozzarella plugs (504 mm^3), exposed to cold air at -40°C, using magnetic resonance imaging (MRI) and found that freezing proceeded symmetrically with the nucleation of ice crystals starting from the outside and progressing inwards during further cooling. The effects of freezing Mozzarella cheeses (5 x 10 x 7 cm) at -20°C on the para-casein matrix were determined using nuclear magnetic resonance (NMR) (Kuo et al., 2003) and scanning electron microscopy in a subsequent study (Kuo & Gunasekaran, 2009). The authors observed a ruptured para-casein network in frozen-thawed Mozzarella and suggested that formation of large ice crystals or recrystallization of ice crystals during frozen storage could potentially weaken the ability of the para-casein matrix to retain moisture, increase serum leakage after thawing, and reduce the melt and stretch of the baked cheese. Kuo & Gunasekaran (2003) noted that the changes in protein structure, and thereby the changes in functionality, could be limited by ripening LMPS Mozzarella before freezing or partially restored by ripening LMPS Mozzarella after thawing. According to Bertola et al. (1996a), LMPS Mozzarella could be frozen without loss of quality provided that the combined storage time of the cheese before and after freezing ranged from 14 to 21 d. These findings suggested that the duration of storage, and hence the level of proteolysis and water binding by the para-casein network of the cheese, is a critical mediator of functionality and should be tightly controlled when freezing LPMS mozzarella to normalize functional performance. Also, the freezing rate could be controlled to limit the size of the formed ice crystals. Bunker (2016) investigated the effects of the freezing rate, expressed as the time to freeze the center of 4 mm thick cheese slabs to -18°C, on LMPS Mozzarella. The author found that the meltability of the cheese, measured by small-strain oscillation rheology and expressed as the maximum loss tangent upon heating the cheese to 100°C, decreased when the time-to-freeze increased from 0 minutes to 95 minutes. In addition, serum relocation from the center of the cheese to its surface was higher when cheeses were frozen to -18°C in 95 minutes as compared to 0 minutes. Conversely, Bertola et al. (1996a) reported that freezing rate, which was defined as the time for the temperature of cheese blocks placed at -20°C to decrease from -1.1 to -6.7°C (0.22 or 10 h), had no effects on LMPS Mozzarella. The inter-study discrepancy on the impact of freezing may be related to differences in Mozzarella composition or freezing conditions. The current study reports on the effects of freezing and key freezing conditions, including freezing rate (FR), storage time in the freezer (TIF) and storage time at 4°C before freezing (TBF), on the properties of commercial LMPS Mozzarella, including proteolysis, ratios of soluble-to-total calcium and mobile serum-to-total serum, and functionality. The effects of freezing on these parameters in LMPS Mozzarella have not been clearly exemplified in literature despite of the fact that they are strongly related to textural, viscoelastic, stretch or melt properties (Banville, Morin, Pouliot, & Britten, 2013; Feeney, Fox, & Guinee, 2001; Guinee, Feeney, Auty, & Fox, 2002; Imm, Oh, Han, Oh, Park & Kim, 2003; Smith, Hindmarsh, Carr, Golding, & Reid, 2017).

2. Materials and methods

2.1. Cheese treatments

LMPS Mozzarella cheeses (2.5kg; 28 cm x 10 cm x 8 cm) were supplied by Milcobel cvba (Langemark, Belgium). Seven cheese vats (A, B, C, D, E, F and G) were sampled over the span of 1.5 years to take the variability in milk composition and cheese processing into account. For each cheese vat sampled, consecutive cheese blocks were removed from the production line, such that the sampled blocks corresponded to the curd from the middle of the cheese vat. This was chosen to minimize the inter-block variability between cheeses taken from the vat. After sampling, the cheeses were sealed in plastic vacuum bags, placed at 4°C and assigned to various treatments: control cheeses which were stored at 4°C for up to 37 d, and frozen-thawed cheeses, which were held at 4°C for 0, 2 or 8 d and frozen to -20°C at different rates (0.6, 2.0 or 8.0°C h^{-1}). The frozen cheeses were held at -20°C for 1, 6, 12 or 44 weeks, and placed at 4°C for a period of 16-37 d. All cheeses were transported chilled to the laboratory (Teagasc, Food Research Centre, Ireland and Ghent University, Belgium), where the characteristics of control and frozen-thawed cheeses were compared after 3 different storage times at 4°C to determine the effects of freezing, storage and possible interaction-effects.

2.1.1. Frozen-thawed LMPS Mozzarella

The effects of the following freezing conditions were investigated as treatments: freezing rate (FR), time in freezer at -20°C (TIF) and storage time at 4°C before freezing (TBF). The various treatments are described in Tables 1, 2 and 3, respectively, and are discussed in detail below. For each treatment, analyses were performed on 2 cheeses at each storage time at 4°C.

2.1.1.1. Effects of the freezing rate (FR)

Twenty-four cheeses were taken from cheese vat A (Table 1). Six cheeses were stored at 4°C and analyzed at 4, 15 or 37 d (control). Eighteen cheeses were held at 4°C for 0 d before freezing to -20°C. To simulate different cooling rates, 6 cheeses were transferred into a Styrofoam box placed in a chest freezer at -20°C (coded M1), 6 cheeses were placed individually in a chest freezer at -20°C (coded M2), and 6 cheeses were transferred into a freezing room at -40°C for 2 hours after which they were transferred to a chest freezer at -20°C (coded M3). Freeze-resistant thermocouples (176T3, Testo, Ternat, Belgium) were used to monitor the temperature at the core and surface of the cheese blocks, and to ensure that the temperature of cheeses placed at -40°C did not decrease to less than -20°C. M1, M2 and M3 resulted in cooling rates of 0.6°C (FR0.6), 2.0°C (FR2.0) and 8.0°C h^{-1} (FR8.0), respectively, as derived from the slope of the cooling curve between the start of cooling and the onset of freezing (i.e., point where latent heat of crystallization became visible). The cheeses were held frozen for 6 weeks, after which they were placed at 4°C and analyzed after total storage times at 4°C of 4, 12 or 37 d (Supplement A, Cheese vat A). The effects of FR were determined by comparing cheeses with different FR after similar total storage times at 4°C, while the effects of freezing were determined by comparing each FR cheese with the corresponding control cheeses after similar total storage times at 4°C. Total storage time is defined as the cumulative time for which the cheese was held at 4°C before analysis, i.e., the sum of storage times at 4°C before and after freezing.

2.1.1.2. Effects of the time in freezer (TIF)

Following manufacture, 40 cheeses were sampled from cheese vat C (Table 2). Eight cheeses were stored at 4°C and analyzed after 4, 10, 16 or 30 d (control). Thirty-two cheeses were held for 2 d at 4°C and transferred to a chest freezer at -20°C. The TIF was varied by holding the cheeses frozen for 1 (TIF1), 6 (TIF6), 12 (TIF12) or 44 weeks (TIF44). After freezing, cheeses were placed at 4°C and analyzed after total storage times at 4°C of 4, 10, 16 or 30 d (Supplement A, Cheese vat C). The effects of TIF were determined by comparing cheeses with different TIF after similar total storage times at 4°C, while the effects of freezing were determined by comparing each TIF cheese with the corresponding control cheeses after similar total storage times at 4°C.

2.1.1.3. Effects of the storage time before freezing (TBF)

The TBF was varied by holding LMPS Mozzarella cheeses at 4°C for 0, 2 or 8 d before transferring to a chest freezer at -20°C (Table 3). The effects of TBF were evaluated following a between-subjects design (i.e., cheese of different vats was subjected to one TBF condition) (Supplement A). This approach ensured a similar sample size for each TBF condition, i.e., 24 cheeses with a TBF of 0 d from vats A and B, 32 cheeses with a TBF of 2 d from vat C and 24 cheeses with a TBF of 8 d from vats D, E, F and G. Samples from vats B, D, E, F and G were held frozen for a period of 1 week - 6 weeks. After freezing, all cheeses were placed at 4°C and analyzed after different storage times. The effects of freezing at different TBF were determined by comparing the corresponding control cheeses with each of the TBF treatments after similar storage times at 4°C. Two cheese blocks from each treatment (control and TBF) were compared after each storage time at 4°C.

2.2. Experimental analysis

2.2.1. Cheese sampling

Cheese blocks were divided into four symmetrical quarters by cutting halfway along the length and width. One quarter was shredded (Robot Coupe CL50, shredding disc, aperture 5mm, Voor 't Labo CVBA, Eeklo, Belgium) and grated to a particle size of < 1 mm (Food Processor Russell Hobbs, Spectrum Brands Europe GmbH, Sulzbach, Germany). Grated cheese was used for the analysis of composition, soluble calcium and pH 4.6 soluble N. A second quarter was used to prepare six cube samples (25 mm ± 1 mm) (Cheese Blocker, Bos Kaasgereedschap, Boven graven, the Netherlands) for texture profile analysis. The cubes were wrapped tightly in aluminum foil and stored at 4°C for 4 h prior to analysis. A third quarter was shredded, stored at 4°C for ~1 d and used for measurement of cheese extensibility. The fourth cheese quarter was used to prepare samples for small strain oscillation rheology (2 discs: 50 mm diameter, 2 mm thick) and flow of the heated cheese by the Schreiber–based test (4 discs: 45 mm in diameter, 4 mm thick).

2.2.2. Cheese composition

Grated LMPS Mozzarella was analyzed for moisture, total nitrogen (N), salt and total calcium content in duplicate using International Dairy Federation standard methods as described by Guinee, Auty & Fenelon (2000). The pH was measured on a cheese slurry prepared from 20 g of cheese and 12 g H_2O after 2 d of storage at 4°C (Guinee et al., 2000). Fat was determined by nuclear magnetic resonance (NMR) (Smart Turbo, CEM Corporation, Matthews, North Carolina, USA).

2.2.3. Soluble calcium and pH 4.6 Soluble N (pH4.6SN)

A water-soluble extract (WSE) of the cheese was prepared by blending distilled water (50°C) and grated cheese at a weight ratio of 2:1 (Stomacher, Lab-Blender 400; Seward Medical, London, UK) for 5 min, holding at 50°C for 1 h, centrifuging at 3000 *g* for 20 min at 4°C (Sorvall LYNX 6000 Superspeed centrifuge, Thermo Scientific, Dublin, Ireland), and filtering through glass wool (Acros organics, Geel, Belgium). A portion (4 mL) of filtrate (WSE) was ashed at 550°C and the ash was analyzed for calcium by flame atomic absorption spectroscopy (ISO 8070, IDF 119:2007). Serum-soluble calcium was expressed as a percentage of the total cheese calcium content. A further portion (60 ml) of the WSE was adjusted to pH 4.6 using 10% w/w HCl (Honeywell Fluka™ Chemicals, Offenbach, Germany), centrifuged at 3000 *g* for 20 minutes at 4°C and filtered through glass wool. The resultant pH 4.6 soluble filtrate was analyzed for N using the macro-Kjeldahl method (ISO 8968-1:2014, IDF 20-1:2014) and expressed as a percentage of total cheese nitrogen. Measurements were performed in duplicate per cheese.

2.2.4. Time domain ^{1}H NMR relaxometry

The T_2 relaxation time distribution of LMPS Mozzarella was evaluated by low-field NMR on a benchtop Maran Ultra spectrometer (Oxford instruments, Abingdon, UK), operating at 0.55T (23.4 MHz for ^{1}H). The method was described by Vermeir, Declerck, To, Kerkaert & Van der Meeren (2019) who distinguished three serum fractions comprising liquid oil protons and water protons in LMPS Mozzarella with different T_2 relaxation times (i.e., the time at which the magnetization signal decays to 37% of its original value). The serum fraction characterized with the longest relaxation time was ascribed to weakly interacting serum protons and could be interpreted as 'more-mobile-serum'. In this study, the relative signal intensity of the more-mobile-serum fraction (A_{60ms}), measured as the ratio of the integrated signal area of the 'more-mobile-serum' fraction to the total integrated signal area of all serum fractions, was reported. The latter ratio is indicative of serum that is not immobilized by the calcium-phosphate para-casein network of the cheese, and is therefore available for freezing; hence, cheese with a lower A_{60m} is less likely to be impaired by freezing (Kuo et al., 2003). Relaxometry measurements were performed in one TBF0 and one TBF8 experiment, owing to the constraints of analytical time and equipment availability. Triplicate measurements were performed at two separate locations in one Mozzarella block after 0, 1, 2, 4, 8 or 16 d storage at 4°C. To report the overall effects of freezing on serum behavior, we included the data as an observation only as the measurements were not included in each freezing experiment.

2.2.5. Texture profile analysis (TPA)

Cheese cubes were taken individually from the refrigerator and loaded on a TAHdi texture analyzer fitted with a 100 kg load cell (Stable Micro Systems, Goldalming, UK). Each cube was compressed in two consecutive bites at a speed of 1 mm s^{-1} to 60% of its original height. The method was based on the method applied by Guinee, Pudja, Miocinovic, Wiley & Mullins (2015). The following parameters were derived from the resultant time-force curve: maximum compression force recorded during bite 1 (firmness), the ratio of height to which the cube was compressed at the start of bite 2 relative to the sample's original height (springiness), the ratio of work required to compress the cube in bite 2 relative to that of bite 1 (cohesiveness) and the product of firmness x springiness x cohesiveness (chewiness). Measurements were performed in sextuplicate per cheese.

2.2.6. Extension work (EW)

EW was evaluated by a modification of the method described by Guinee et al. (2015). Shredded cheese (60 g) was weighed in a heat resistant vessel (Stable Micro Systems, Goldalming, U.K.) and heated in a microwave oven (Whirlpool MW201, Fonthill Industrial Estate, Dublin, Ireland) set at 750 W for 60s until the cheese temperature was 85 to 95°C. The vessel containing the heated cheese was then loaded on a TAHDi texture analyzer (Stable Micro Systems, Goldalming, UK) and uniaxially extended at a rate of 10 mm s^{-1} to a height of 380 mm. EW was defined as the cumulative work required to extend the hot molten cheese, directly after heating (EW_0) and after allowing the cheese to cool down for 5 minutes at room temperature (EW_5); EW_5 was used to simulate the impact of cooling-induced stiffening of molten cheese on a pizza during consumption. EW_0 and EW_5 were measured in triplicate and in duplicate, respectively.

2.2.7. Small strain oscillation rheology

Heat-induced changes in viscoelastic characteristics, including storage modulus, G', loss modulus, G", and loss tangent, G"/G', on heating from 25°C to 90°C were measured using low amplitude strain oscillation rheology on a strain-controlled rheometer (MCR501, Anton Paar GmbH, Graz, Austria) (Guinee et al., 2015). Cheese discs (50 mm diameter; 2 mm thickness) were prepared and placed between parallel cross-hatched plates (PP50/P2-SN27902; [diameter = 50 mm]; INSET I-PP50/SS/P2). The exposed surface of the cheese disc was brushed with a thin layer of silicone oil (silicone oil, Sigma-Aldrich, Arklow, Ireland) to prevent surface dehydration during measurement. Samples were equilibrated at 25°C for 15 minutes and subjected to a low amplitude shear strain ($\gamma = 0.0063$) at an angular frequency of 1 Hz, and the temperature was increased from 25°C to 90°C at a rate of 3.25°C min^{-1}. The cross-over temperature (COT), corresponding to the temperature at which G' = G" (i.e., the point at which the solid index of the sample was equal to its liquid index or the point at which the cheese transitioned from the solid phase into the liquid phase) and the maximum value of loss tangent (LT_{max}) (i.e., an index for the fluidity of the cheese during heating) were reported. Measurements were performed in duplicate.

2.2.8. Schreiber flow

Cheese discs (45 mm diameter; 4 mm thickness) were placed on circular glass dishes, heated at 280°C for 4 minutes in a convection oven (Binder FD 35, Binder GmbH, Tuttlingen, Germany), removed, allowed to cool at room temperature for 30 min and measured for length along 4 equidistant diagonals. Flow was defined as the percentage increase in mean diameter during heating. Measurements were performed in quadruplicate.

2.2.9. Baking test

Frozen pizza bases (25 cm diameter) with tomato paste (Bladerdeeg Van Marcke, Belgium) were thawed for 3 hours at room temperature. Control (75g) and frozen-thawed Mozzarella (75g) shreds were each spread uniformly on opposite halves of the base and baked at 245°C for 5.25 min in a conveyor oven (Lincoln Impinger, Fort Wayne, IN, USA). Following baking, the attributes ‘blister color’, ‘blister coverage’, ‘meltability’, ‘oiling off’, ‘stretch’, ‘first chew’ and ‘chewiness’ were scored sequentially by trained laboratory personnel at Milcobel. A score of 2 was awarded if the characteristic was ‘just right’, a score of < 2 was given when the attribute was subpar, and a score > 2 was given if the attribute was more strongly present. Scores of 0 or 4 implied that the measured characteristic was unacceptable because the level of the attribute was either too little or too high, respectively. ‘Blister color’ was indicative of color intensity of the blisters, which ranged from light brown to black, and ‘blister coverage/density’ of the proportion of pizza surface covered by blisters. ‘Meltability’ was a measure of how well the cheese shreds were fused together after baking. ‘Meltability’ scores of < 2 were awarded where individual shreds were visible after baking, while scores > 2 were given where cheese was runny. ‘Oiling off’ was a measure of the amount of oil released as a film on top of the pizza after baking. ‘Stretch’ was manually evaluated by lifting cheese from the baked pizza surface using a fork and extending to a maximum height of 30 cm. ‘First chew’ and ‘chewiness’ were evaluated by tasting a forkful of the molten Mozzarella. ‘First chew’ was a measure of the resistance perceived during the first bite, while ‘chewiness’ coincided with toughness perceived during overall mastication, as moisture and oil were continuously released from the protein matrix.

2.3. Statistical analysis

A factorial design incorporating two factors, A (cheese treatment) and B (total storage time at 4°C), was used for the analysis of response variables. The main effects of A and B and their interaction effect, A x B, on each response variable was determined separately using two-way analysis of variance. Main effects were compared pair-wise using the Least Significant Difference (LSD) test. In presence of significant interaction effects, a simple main effects analysis, which determines the effects of cheese treatments at each level of the storage time at 4°C, was used. To determine treatment impact on sensory properties, a Kruskal-Wallis test was performed. The level of significance was determined at $\alpha = 0.05$ throughout. This approach was used to determine the overall effects of freezing and storage time at 4°C on response variables. The effects of specific freezing conditions (e.g., FR, TIF and TBF) were determined likewise.

3. Results and discussion

3.1. Cheese composition

The mean compositions of the cheeses used for comparing the different treatments are given in Table 4. Slight but significant inter-vat differences were found in dry matter, fat, salt, calcium content and pH. This indicated that determining the effects of TBF, which involved cheeses from different vats, may have been somewhat confounded by such compositional variation. The effects of FR and TIF were not affected by inter-vat compositional variation in cheese as cheeses for each of these treatments were taken from the same vat.

3.2. Overall changes during storage at 4°C of LMPS Mozzarella

The overall comparisons between control and frozen-thawed cheeses, frozen under different conditions, are presented in Fig. 1 and Fig. 2. Each response variable is categorized by two factors: ‘cheese treatment’ (control or frozen-thawed cheese) and ‘storage time at 4°C’. The values presented for frozen-thawed cheeses at the different storage times are means of cheeses frozen under different FR, TIF or TBF conditions. First, the interaction-effects between ‘cheese treatment’ and ‘total storage time at 4°C’ were determined (Table 5). For each response variable, where no significant interaction-effect could be demonstrated, the effect of cheese treatment was determined by comparing the mean values of control cheeses with those of frozen-thawed cheeses, while keeping the factor ‘storage time at 4°C’ fixed. Likewise, the effects of storage time at 4°C were determined by comparing the mean values between the different storage times, while keeping the factor ‘cheese treatment’ fixed. If a significant interaction effect was found, the effect of cheese treatment was determined at each storage time separately.

3.2.1. **Physicochemical changes during storage at 4°C**

Both the control and frozen-thawed cheeses exhibited a reduction in more-mobile-serum fraction (Fig. 1A) and an increase in less-mobile-serum fraction during storage at 4°C (Fig. 1B). This indicated that the more-mobile-serum was gradually ‘immobilized’ during storage at 4°C owing to its uptake into the para-casein network of the cheese matrix. This trend is consistent with the reduction in expressible serum during the storage of LMPS Mozzarella (McMahon and Oberg, 2017). Similarly, proteolysis increased progressively in all cheeses on storage at 4°C, as evidenced by the linear increase in pH4.6SN (Fig. 1C). The proximity of dashed trend lines for pH4.6SN of the control and frozen thawed cheeses showed that freezing had no effect on primary proteolysis. A different trend was reported by Bertola et al. (1996b) for concentration of 12% trichloroacetic acid soluble N (TCAN) in low-moisture Mozzarella, whereby cheeses stored for 6 d at 4°C before freezing at -20°C had higher values than the refrigerated control cheeses at similar storage times. However, in the same study, storage of the cheese for 14 d at 4°C before freezing resulted in similar TCAN values as the control cheeses. The relatively low values of pH4.6SN for all cheeses, for example compared to Cheddar cheese, were consistent with those reported previously for LMPS Mozzarella and reflected the high degree of chymosin inactivation during plasticization (Feeney et al., 2001). The ratio of soluble-to-total Ca varied from 30% to 45% (Fig. 1D) and was not affected by storage time at 4°C or freezing ($P > 0.05$) (Table 5).

Some studies postulated that freezing could affect the behavior of LMPS Mozzarella owing to protein dehydration concurrent with the formation of ice crystals at the exterior of the Mozzarella cheese, which would promote serum relocation from the core to the exterior of the cheese block (Bunker, 2016; Kuo & Gunasekaran, 2003). Moreover, it would be feasible to assume that precipitation of calcium phosphate by migration of soluble Ca and P to the unfrozen serum may further contribute to para-casein aggregation and thereby reduce the susceptibility to proteolysis (Fox, 1970). However, the current results showed that for the current LMPS Mozzarella cheeses, freezing halted storage-related changes in serum distribution (not statistically verified) and pH4.6SN, and did not influence their levels in the frozen-thawed LMPS Mozzarella ($P > 0.05$). It is likely that variation in the composition (e.g., moisture content, calcium and pH) and proteolysis of different commercial Mozzarella cheese variant may alter the susceptibility to freezing.

3.2.2. **Functional characteristics during storage at 4°C**

Increasing storage time of control and frozen-thawed cheeses resulted in lower values of cheese firmness (Fig. 2A), COT (Fig. 2B) and EW (Fig. 2D and Fig. 2E), and higher values of LT_{max} (Fig. 2C) and flow (Fig. 2F). These changes are consistent with the increase in pH4.6SN and the reduction in more-mobile serum (A_{60ms}) during storage at 4°C (Guinee et al., 2002).

Overall, no significant interaction effects could be demonstrated between ‘cheese treatment’ and ‘storage time at 4°C’ for most of the response variables, including firmness of the unheated cheese ($P > 0.05$), and extensibility (EW_0, EW_5) ($P > 0.05$) and viscoelastic properties (COT, LT_{max}) ($P > 0.05$) of the heated cheese (Table 5), which indicated that the rate of storage-related changes of these characteristics at 4°C was similar for the control and frozen-thawed cheeses, as illustrated in Fig. 2. After freezing and thawing, the firmness and chewiness of the unheated cheeses were significantly reduced by 10% and 8%, respectively ($P < 0.001$) (Table 5). However, some studies (Alvarenga, Canada, & Sousa, 2011; Bertola, Califano, Bevilacqua & Zaritzky 1996b) reported that frozen-thawed LMPS Mozzarella had a higher firmness than the corresponding cold-stored cheeses, whereas Cervantes et al. (1983) found that the firmness was unaffected by freezing. No effect of freezing was found for either the cohesiveness ($P > 0.05$) and springiness ($P > 0.05$) of the unheated cheeses, or the extensibility (EW_0, EW_5) ($P > 0.05$) or viscoelastic properties (COT, LT_{max},) ($P > 0.05$) of the heated cheese. A significant interaction ($P = 0.019$) was found for Schreiber flow, as illustrated by Fig. 2f where it can be seen that the effects of freezing, relative to the control, depended on the storage time at 4°C. Hence, the effect of freezing on the flow of the heated cheeses was determined at each level of the storage time but no differences could be demonstrated between control and frozen-thawed cheeses ($P > 0.05$).

3.2.3. **Baking characteristics during storage at 4°C**

No clear differences were detected between the control and frozen-thawed cheeses for ‘blister color’, ‘blister coverage’, ‘meltability’, ‘oiling off’, ‘stretch’ and ‘chewiness’ ($P > 0.05$) after baking on a pizza (Fig. 3). However, the ‘first chew’ of frozen-thawed cheeses received a score of 0.3 units less than that of the corresponding control cheeses after a total storage time at 4°C at 16 d ($P < 0.05$), which suggested that freezing resulted in a slightly softer ‘first chew’. This trend was consistent with the reduction in firmness and chewiness of the unheated cheese after freezing and thawing, as measured by TPA. However, no effects of freezing on the attribute ‘first chew’ could be demonstrated at other storage times.

3.3. Effects of specific freezing conditions

It is possible that the overall effects of freezing, as discussed in Section 3.2, may have been obscured by the effects of specific freezing conditions with opposite effects. Hence, the effects of each of the freezing conditions, i.e., FR, TIF and TBF, were investigated separately and are discussed in detail below.

3.3.1. Effects of freezing rate (FR)

LMPS Mozzarella is commercially frozen in palletized format by placing them in large freezing rooms operating at -20°C. The low heat conductivity of Mozzarella (Dumas & Mittal, 2002), however, results in non-uniform cooling of the pallet with temperatures dropping quickly at the exterior of the pallet and slowly at the core. Mozzarella cheeses were frozen at a rate of 2°C h^{-1} (i.e., individual cheeses placed in a chest freezer at -20°C) or 0.6°C h^{-1} (i.e., individual cheeses placed in a Styrofoam box in a chest freezer at -20°C) to simulate the freezing of LMPS Mozzarella blocks in the exterior and interior portions of palletized cheese, respectively, when placed at -20°C. Cheeses were also frozen at a rate of 8°C h^{-1} to investigate the effects of a faster freezing method (e.g., tunnel freezing).

The statistical significance (*P*) for the effects of freezing at different freezing rates, storage time at 4°C, and their interaction on the properties of Mozzarella is shown in Table 6. No interaction effect between the cheese treatments and storage time at 4°C could be demonstrated for most response variables. The more-mobile-serum fraction (A_{60ms}) of frozen-thawed samples decreased from 4% to 0% during storage at 4°C, and was not affected by the freezing rate ($P < 0.05$). As such, the storage-related changes in more-mobile-serum fraction, i.e. its uptake in the calcium-phosphate para-casein network during storage at 4°C, were similar for all freezing rates. Likewise, the proportion of soluble-to-total calcium, which varied from 31% to 36% during storage, and pH4.6SN were unaffected by the freezing rate ($P > 0.05$). The obtained results further showed no effects of freezing rate on the firmness of the unheated cheese ($P > 0.05$), or the extensibility (EW_0, EW_5) ($P > 0.05$), viscoelastic properties (COT, LT_{max}) ($P > 0.05$) or sensory attributes of the heated cheese ($P > 0.05$). However, a significant interaction was found for the flow of the cheeses, as measured by the Schreiber test. After 4 d storage at 4°C (storage time 1, Table 6), frozen-thawed cheeses subjected to freezing rates 2.0°C h^{-1} or 8.0°C h^{-1} had a mean flow of 36-38% upon heating for 4 min at 280°C whereas cheese frozen at a rate of 0.6°C h^{-1} had a flow of 47%. At storage times of 12 d (storage time 2, Table 6), flow plateaued at ~45-48% for all freezing rates. Overall, the results indicated that the FR did not significantly influence storage-related changes in moisture redistribution, primary proteolysis or functional characteristics. Similar conclusions were found for the measured variables of control and frozen-thawed cheeses frozen at different freezing rates. Potentially, the freezable serum of the current LMPS Mozzarella cheese was too limited to induce an effect of freezing, even when cheeses were frozen directly after production and packaging.

3.3.2. Effects of time in freezer (TIF)

After manufacture and freezing of LMPS Mozzarella, the duration of frozen storage depends on various commercial factors including the dispatch time (i.e., released from the producer to the distributor or harbor), the loading time (i.e., loading of Mozzarella on the ship), the transportation time on the boat, the docking time (i.e., release of Mozzarella at the harbor of the country of destination), the transportation time to the customer and the storage time at -20°C at the customer. To simulate these conditions, LMPS Mozzarella was held at 4°C for 2 d before freezing to -20°C and stored frozen for 6 to 12 (TIF6 and TIF12) weeks to mimic the duration of frozen export, and for 44 weeks (TIF44) to simulate the duration of long-term frozen storage as applied by some customers who on receipt of frozen Mozzarella maintain it frozen for a relatively long time prior to thawing and using. Cheeses were also kept frozen for 1 week (TIF1) to evaluate short periods of frozen storage.

Overall, the duration of TIF (1, 6, 12 or 44 weeks) had no effect on most of the evaluated parameters (Table 7), including pH4.6SN ($P > 0.05$), ratio of soluble-to-total Ca ($P > 0.05$), LT_{max} ($P > 0.05$), extensibility (EW_0, EW_5) ($P > 0.05$), Schreiber flow ($P > 0.05$) and sensory attributes ($P > 0.05$).

However, extending the storage from 12 weeks to 44 weeks reduced the firmness ($P < 0.001$) and chewiness ($P < 0.001$) of the unheated cheese by 23% on average over the 30 d of total storage time at 4°C, and reduced the COT ($P < 0.01$) of the heated cheese by 2% on average, i.e., the onset-temperature for melting Mozzarella was reduced by 1.3°C. The reduction in melting point was not reflected in the baking test, where panel members gave all TIF treatments similar scores for each sensory attribute ($P > 0.05$) (Table 5). Moreover, the COT of TIF12 samples did not significantly differ from those of TIF1, TIF6 or TIF44 samples ($P > 0.05$), which suggested that the effect of 44 weeks of frozen storage on the COT of frozen-thawed Mozzarella cheeses was limited.

Relative to the control, holding the cheeses at 4°C for 2 d before freezing to -20°C and keeping them frozen for a period between 1 and 12 weeks did not influence the response variables ($P > 0.05$) (Table 7). However, when the cheeses were stored frozen for 44 weeks, firmness and chewiness of cheeses were reduced by 29% ($P < 0.001$) and 26% ($P < 0.001$), respectively, whereas the COT of the heated cheese was reduced by 1.7% ($P < 0.01$). Overall, freezing under these conditions did not affect LT_{max} ($P > 0.05$), extensibility (EW_0, EW_5) ($P > 0.05$), flow ($P > 0.05$) or sensory attributes ($P > 0.05$) of the heated cheese.

3.3.3. Effects of time before freezing (TBF)

The TBF was varied in a systematic way to evaluate whether the potential detrimental effects of direct freezing could be mitigated by prolonging the storage at 4°C before freezing and thereby allowing the uptake of more-mobile-serum into the calcium-phosphate para-casein network of the cheese (Kuo & Gunasekaran, 2003). Freezing as soon as possible after manufacturing could minimize storage costs. Cheeses were held at 4°C for 0 (TBF0), 2 (TBF2) or 8 d (TBF8) before freezing to -20°C; these cheeses were sampled from vats A and B (TBF0), vat C (TBF2) or vats D, E, F and G (TBF8) (Table 3). Control cheeses, sampled from the different vats (A-G), differed in terms of pH4.6SN ($P < 0.001$), cohesiveness ($P < 0.05$), springiness ($P < 0.01$), LT_{max} ($P < 0.001$)

and EW_0 ($P < 0.05$) after 16 d storage at 4°C, and differed in pH4.6SN ($P < 0.001$), firmness ($P < 0.001$), cohesiveness ($P < 0.05$), springiness ($P < 0.05$), LT_{max} ($P < 0.05$) and Schreiber flow ($P < 0.01$) after 30-37 d storage at 4°C, which implied that the effects of TBF were somewhat confounded. Nevertheless, it was possible to compare each TBF treatment with the corresponding control cheese from the same cheese vat (Table 3).

No significant differences were found between control cheeses, obtained from vats A or vat B, and the corresponding frozen-thawed cheeses which were held at 4°C for 0 d before freezing to -20°C (TBF0) (Table 5) (discussed in Section 3.3.1). A similar trend was found when comparing the control and frozen-thawed cheeses from vat C (TBF2) (Table 5) (discussed in Section 3.3.2). Likewise, TBF8 cheeses, obtained from 4 different vats, did not significantly differ from the corresponding control cheeses ($P > 0.05$) (Table 5) with the exception of a significant interaction effect between freezing and storage time at 4°C for firmness of the unheated cheese ($P < 0.01$). Compared to the corresponding controls, TBF8 cheeses exhibited lower firmness after 10 d storage at 4°C ($P < 0.01$), but not after other storage times ($P > 0.05$).

Overall, as evident from Fig. 3 and Fig. 4, the current results indicated that there was little effect of holding the cheeses at 4°C for 0, 2 or 8 d before freezing to -20°C on the physicochemical and functional properties of the current variant of LMPS Mozzarella.

4. Conclusions

A total of 132 blocks of LMPS Mozzarella cheese were sampled from a commercial manufacturer over a 1.5 year period. The cheeses were assigned to 2 groups, namely control cheeses which were stored at 4°C for up to 37 d, and frozen-thawed cheeses which were held at 4°C for different times (TBF: 0, 2 or 8 d) before freezing to -20°C at different rates (FR: 0.6, 2.0 or 8.0°C h^{-1}). The frozen cheeses were held at -20°C for different times (TIF: 1, 6, 12 or 44 weeks), and then placed at 4°C for up to 37 d to achieve total storage times at 4°C similar to the control. The effects of freezing were determined by comparing the control and frozen-thawed cheeses taken from the same vat, and the effects of different freezing conditions (FR and TIF) by comparing the frozen-thawed cheeses subjected to the different levels of condition. The control and frozen-thawed cheeses were evaluated after similar total storage times at 4°C for composition, primary proteolysis, moisture distribution, texture profile (firmness, springiness, cohesiveness), functional properties (extensibility, viscoelastic behavior and flow of the heated cheese) and baking performance on pizza. Overall, freezing *per se* did not significantly affect the properties of the cheese. Likewise, there was little difference between frozen-thawed cheeses frozen under the following conditions: FR (0.6, 2.0 or 8.0°C h^{-1}) or TIF (1, 6 or 12 weeks). Extending the TIF from 1, 6 or 12 weeks to 44 weeks reduced the firmness and chewiness of the unheated frozen-thawed cheese (by 23% on average), and reduced the melting temperature by 2% during a total storage time at 4°C of 30 d. However, there was no detectable difference in baking performance when the TIF was varied from 1 to 44 weeks.

Considering the overall effects observed in this study, we conclude that freezing of commercial LMPS Mozzarella cheese (with respective dry matter, fat and protein levels of ~52, 22 and 25 g 100 g^{-1}, and a calcium level of ~740 mg 100 g^{-1}) under the applied conditions, halted the physico-chemical changes that occur on storage at 4°C without having significant effects on functionality and baking performance. However, the applicability of the findings to commercial Mozzarella in general may vary depending on the manufacturing and compositional characteristics of the cheese, which are likely to impact the degree of aggregation of the calcium-phosphate para-casein matrix and its ability to bind serum. Critical factors affecting aggregation are likely to include cheese moisture, pH, calcium content, ratio of soluble-to-total calcium, and degree of proteolysis. In practice, changes in make procedure which affect cheese composition may therefore necessitate tailoring of freezing conditions to ensure comparable functionality of control and frozen-thawed Mozzarella.

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Declaration of interests

There are no conflicts of interest.

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Figure captions

Fig. 1: Overall changes during storage at 4°C in relative signal intensity of (A) more-mobile-serum (A_{60ms}) and (B) less-mobile-serum (A_{3ms}) of frozen-thawed LMPS Mozzarella (●) or control LMPS Mozzarella (●).

Overall changes during storage at 4°C in (C) pH 4.6 Soluble N and (D) ratio of soluble-to-total Ca of frozen-thawed LMPS Mozzarella cheeses, which were held at 4°C for 0 (O), 2 (Δ) or 8 d (☐) before freezing, and of corresponding control cheeses (●, ▲ and ■). Trendlines represent the overall dynamic behavior of frozen-thawed (---) and control (---) cheeses during storage at 4°C. The cheeses were obtained from 7 vats and were frozen under different conditions.

Fig. 2: Overall changes during storage at 4°C in firmness of the unheated cheese, cross-over temperature (COT), maximum value of the loss tangent (LT_{max}), extension work at 0 (EW_0) or 5 (EW_5) min after melting and Schreiber flow of frozen-thawed LMPS Mozzarella cheeses, which were held at 4°C for 0 (O), 2 (Δ) or 8 d (☐) before freezing, and of corresponding control cheeses (●, ▲ and ■). Trendlines represent the overall dynamic behavior of frozen-thawed (---) and control (---) cheeses during storage at 4°C. The cheeses were obtained from 7 vats and frozen under different conditions.

Fig. 3: Overall appearance of Mozzarella shreds after baking on a pizza after 2, 16 or 35 d of storage at 4°C. Top row pictures present control Mozzarella and bottom row pictures present frozen-thawed Mozzarella, held at 4°C for 0 days before freezing to -20°C. The cheese was held frozen for 6 weeks. After freezing, cheeses were placed at 4°C for up to 35 d.

Fig. 4: Changes during storage at 4°C in pH 4.6 Soluble N, firmness, cross-over temperature (COT), maximum value of the loss tangent (LT_{max}), extension work at 0 min after melting (EW_0), and Schreiber flow of control and frozen-thawed LMPS Mozzarella cheeses, which were held at 4°C for 0 (TBF0) or 8 d (TBF8) before freezing to -20°C.

TBF0 samples, sampled from vat A or vat B, were used to determine the effects of holding the cheese at 4°C for 0 d before freezing to -20°C (○); the cheeses were frozen at 0.6, 2 or 8°C h^{-1} and held in the freezer for 6 weeks. Control samples were taken from the same vat (○).

TBF8 samples, sampled from vat D, E, F or G, were used to determine the effects of holding the cheeses at 4°C for 8 d before freezing to -20°C (□); the cheeses were frozen at 2°C h^{-1} and held in the freezer for 6 weeks. Control samples were taken from the same vats (□).

Supplement A. Overview of the sampling design per cheese vat.

Twenty-four cheeses were sampled from vat A. Six cheeses were control cheeses. Eighteen cheeses were held at 4°C for 0 d before to freezing to -20°C (TBF0) at a rate of 0.6°C (FR0.6), 2.0°C (FR2.0) or 8.0°C h^{-1} (FR8.0) to evaluate the effects of freezing at different freezing rates (FR). All cheeses were held frozen for 6 weeks (TIF6).

Forty cheeses were sampled from vat C. Eight cheeses were control cheeses. Thirty-two cheeses were held at 4°C for 2 d before to freezing to -20°C (TBF2) at a rate of 2.0°C h^{-1}. The frozen cheeses were held for 1 (TIF1), 6 (TIF6), 12 (TIF12) or 44 weeks (TIF44) in the freezer to evaluate the effects of freezing at different holding times in the freezer (TIF).

To evaluate the effects of holding time at 4°C before freezing (TBF), cheeses from each vat were subjected to one TBF condition. This approach ensured a similar sample size for each TBF condition. A total of 24, 32 or 24 cheeses were held at 4°C for 0 (TBF0), 2 (TBF2) or 8 d (TBF8) before freezing to -20°C, respectively. After freezing, cheeses were held at 4°C for up to 16 to 37 d, depending on the vat they were sampled from. Frozen-thawed cheeses (FT cheeses) and control cheeses were evaluated at various storage times at 4°C (ST1, ST2, ST3 or ST4) (total storage time at 4°C) as described in Table 3.

Table 1: Experimental design to determine the effects of freezing at different rates (FR) on LMPS Mozzarella[a,b]

	Control cheeses		Frozen-thawed cheeses						
Cheese vat	Number of cheese blocks	Storage time at 4°C (d)	Number of cheese blocks	Storage time at 4°C before freezing (d) (TBF)	Freezing rate (°C h^{-1}) (FR)	Time in freezer (weeks) (TIF)	Storage time at 4°C after freezing (d)	Total storage time at 4°C (d)	Sample code
Vat A	6	4 - 15 - 37	6	0	0.6	6	4 - 12 - 37	4 - 12 - 37	**FR0.6**|TIF6|TBF0
			6	0	2.0	6	4 - 12 - 37	4 - 12 - 37	**FR2.0**|TIF6|TBF0
			6	0	8.0	6	4 - 12 - 37	4 - 12 - 37	**FR8.0**|TIF6|TBF0

[a] The effects of FR were investigated by freezing 18 cheeses from vat A to -20°C at a rate of 0.6°C (FR0.6), 2.0°C (FR2.0) or 8.0°C h^{-1} (FR8.0). All cheeses were held at 4°C for 0 d before freezing (TBF0) and held in the freezer for 6 weeks (TIF6). After freezing, the frozen cheeses were placed at 4°C and stored at 4°C for up to 37 d.

[b] To evaluate the effects of freezing on the characteristics of the cheese, frozen-thawed cheeses were compared to control cheeses after similar storage times at 4°C (total storage time at 4°C).

Table 2: Experimental design to evaluate the effects of freezing at different storage times in the freezer (TIF) on LMPS Mozzarella[a,b]

	Control cheeses		Frozen-thawed cheeses						
Cheese vat	Number of cheese blocks	Storage time at 4°C (d)	Number of cheese blocks	Storage time at 4°C before freezing (d) (TBF)	Freezing rate (°C h^{-1}) (FR)	Time in freezer (weeks) (TIF)	Storage time at 4°C after freezing (d)	Total storage time at 4°C (d)	Sample code
Vat C	8	4 - 10 - 16 - 30	8	2	2.0	1	2 - 8 - 14 - 28	4 - 10 - 16 - 30	FR2.0|**TIF1**|TBF2
			8	2	2.0	6	2 - 8 - 14 - 28	4 - 10 - 16 - 30	FR2.0|**TIF6**|TBF2
			8	2	2.0	12	2 - 8 - 14 - 28	4 - 10 - 16 - 30	FR2.0|**TIF12**|TBF2
			8	2	2.0	44	2 - 8 - 14 - 28	4 - 10 - 16 - 30	FR2.0|**TIF44**|TBF2

[a] The effects of TIF were investigated by freezing 32 cheeses from vat C to -20°C and holding the cheeses in the freezer for 1 (TIF1), 6 (TIF6), 12 (TIF12) or 44 weeks (TIF44). All cheeses were held for 2 d at 4°C before freezing (TBF2) and were frozen at a rate of 2.0°C h^{-1} (FR2.0). After freezing, the frozen cheeses were held at 4°C and kept at 4°C for up to 28 d.

[b] To evaluate the effects of freezing on the characteristics of the cheese, frozen-thawed cheeses were compared to control cheeses after similar storage times at 4°C (total storage time at 4°C).

Table 3: Experimental design to determine the effects of freezing at different storage times at 4°C before freezing (TBF) on LMPS Mozzarella[a,b]

	Control cheeses		Frozen-thawed cheeses						
Cheese vat	Number of cheese blocks	Storage time at 4°C (d)	Number of cheese blocks	Storage time at 4°C before freezing (d) (TBF)	Freezing rate (°C h^{-1}) (FR)	Time in freezer (weeks) (TIF)	Storage time at 4°C after freezing (d)	Total storage time at 4°C (d)	Sample code
Vat A	6	4 - 15 - 37	6	0	0.6	6	4 - 12 - 37	4 - 12 - 37	FR0.6|TIF6|**TBF0**
			6	0	2.0	6	4 - 12 - 37	4 - 12 - 37	FR2.0|TIF6|**TBF0**
			6	0	8.0	6	4 - 12 - 37	4 - 12 - 37	FR8.0|TIF6|**TBF0**
Vat B	6	2 - 16 - 35	6	0	2.0	6	2 - 16 - 35	2 - 16 - 35	FR2.0|TIF6|**TBF0**
Vat C	8	4 - 10 - 16 - 30	8	2	2.0	1	2 - 8 - 14 - 28	4 - 10 - 16 - 30	FR2.0|TIF1|**TBF2**
			8	2	2.0	6	2 - 8 - 14 - 28	4 - 10 - 16 - 30	FR2.0|TIF6|**TBF2**
			8	2	2.0	12	2 - 8 - 14 - 28	4 - 10 - 16 - 30	FR2.0|TIF12|**TBF2**
			8	2	2.0	44	2 - 8 - 14 - 28	4 - 10 - 16 - 30	FR2.0|TIF44|**TBF2**
Vat D	8	2 - 8 - 17 - 36	6	8	2.0	1	2 - 8 - 28	10 - 16 - 36	FR2.0|TIF1|**TBF8**
Vat E	8	2 - 8 - 17 - 25	6	8	2.0	1	2 - 10 - 17	10 - 18 - 25	FR2.0|TIF1|**TBF8**
Vat F	8	2 - 8 - 16 - 32	6	8	2.0	6	2 - 8 - 16	10 - 16 - 24	FR2.0|TIF6|**TBF8**
Vat G	8	2 - 8 - 16 - 32	6	8	2.0	6	2 - 8 - 16	10 - 16 - 24	FR2.0|TIF6|**TBF8**

[a] The effects of TBF were investigated by holding: (i) 18 cheeses from vat A and 6 cheeses from vat B at 4°C for 0 d before freezing (TBF0), (ii) 32 cheeses from vat C at 4°C for 2 d before freezing (TBF2) and (iii) 6 cheeses from each of vats D, E F and G at 4°C for 8 d before freezing (TBF8). All cheeses were frozen to -20°C at a rate of 0.6 (FR0.6), 2.0 (FR2.0) or 8.0°C h^{-1} (FR8.0) and held in the freezer for 1 (TIF1), 6 (TIF6), 12 (TIF12) or 44 (TIF44) weeks. After freezing, frozen cheeses were held at 4°C for up to 16 to 37 d, depending on the vat they were sampled from. This approach ensured a similar sample size for each TBF condition.

[b] To evaluate the effects of freezing on the characteristics of the cheese, frozen-thawed cheeses were compared to control cheeses after similar storage times at 4°C (total storage time at 4°C).

Table 4: Composition of LMPS Mozzarella used in freezing studies

Cheese vat	Dry matter (g 100g^{-1})	Fat (g 100g^{-1})	Protein (g 100g^{-1})	Salt (g 100g^{-1})	Calcium (mg 100g^{-1})	pH
A	$52.1^{a,b}$	21.7^{a}	24.4^{a}	0.9^{a}	$766^{a,b}$	5.53^{a}
B	52.6^{b}	$21.8^{a,b}$	25.3^{a}	$1.2^{b,c}$	809^{a}	5.51^{a}
C	$52.0^{a,b}$	21.7^{a}	24.8^{a}	1.1^{b}	697^{c}	$5.41^{b,d}$
D	$52.0^{a,b}$	22.1^{c}	24.5^{a}	1.1^{b}	$712^{b,c}$	5.43^{b}
E	$51.9^{a,b}$	21.7^{a}	25.1^{a}	1.2^{b}	696^{c}	5.34^{c}
F	$52.2^{a,b}$	$22.1^{b,c}$	24.8^{a}	1.3^{c}	$735^{a,b,c}$	5.34^{c}
G	51.7^{a}	21.6^{a}	24.7^{a}	1.3^{c}	784^{a}	$5.36^{c,d}$

[a,b,c] Values in columns with different superscripted letters denote a significant difference ($P < 0.05$). Presented data of dry matter, fat, protein, salt and calcium content are mean values measured on at least four different cheeses per vat. The pH of the cheese was measured on two cheeses per vat after 2 d of storage at 4°C.

Table 5: Effects of freezing treatments (FR, freezing rate; TIF, storage time in freezer; TBF, storage time at 4°C before freezing), total storage time at 4°C and their interaction on the characteristics of LMPS Mozzarella[a, b, c, d, e, f, g]

Cheese characteristic	Overall effects of freezing at different conditions			Effects of freezing cheeses held at 4°C for 0 d before freezing			Effects of freezing cheeses held at 4°C for 2 d before freezing			Effects of freezing cheeses held at 4°C for 8 d before freezing		
	Freezing (F)	Storage Time at 4°C (ST)	Interaction (F*ST)	Cheese Treatments (CT)	Storage Time at 4°C (ST)	Interaction (CT*ST)	Cheese Treatments (CT)	Storage Time at 4°C (ST)	Interaction (CT*ST)	Cheese Treatments (CT)	Storage Time at 4°C (ST)	Interaction (CT*ST)
Unheated cheese												
More-mobile-serum (A_{60ms})	n/d	n/d	n/d	-	***	-	n/d	n/d	n/d	-	***	-
Ratio soluble-to-total Ca	-	-	-	-	-	-	-	-	-	-	-	-
pH 4.6 Soluble N	-	***	-	-	***	-	-	***	-	-	***	-
Firmness	***	***	-	-	***	-	***	*	-	-	***	**
Springiness	-	***	-	-	***	-	-	***	-	-	*	-
Cohesiveness	-	***	-	-	***	-	-	***	-	-	***	-
Chewiness	***	***	-	-	***	-	***	***	-	-	***	*
Heated cheese												
COT	-	***	-	-	***	-	**	***	-	-	***	-
LT_{max}	-	***	-	-	***	-	-	***	-	-	**	-
EW_0	-	***	-	-	***	-	-	***	-	-	***	-
EW_5	-	***	-	-	***	-	-	***	-	-	***	-
Shreiber flow	-	***	*	-	***	***	-	**	-	-	***	-
'Blister color'	-	***	n/a	-	***	n/a	-	-	n/a	-	***	n/a
'Blister coverage'	-	***	n/a	-	***	n/a	-	-	n/a	-	***	n/a
'Meltability'	-	***	n/a	-	***	n/a	-	***	n/a	-	***	n/a
'Oiling off'	-	***	n/a	-	***	n/a	-	***	n/a	-	***	n/a
'Stretch'	-	***	n/a	-	***	n/a	-	-	n/a	-	***	n/a
'First chew'	***	***	n/a	-	***	n/a	-	***	n/a	-	***	n/a
'Chewiness'	-	***	n/a	-	***	n/a	-	***	n/a	-	***	n/a

[a] The statistical significance (P) for treatment effects across the evaluated properties of control and frozen-thawed cheeses is given where $P > 0.05$, $P < 0.05$, $P < 0.01$ and $P < 0.001$ is denoted by -, *, ** and ***, respectively.

[b] The effects of freezing (Fr) were determined by comparing the characteristics of the control and frozen-thawed cheeses.

[c] The effects of total storage time at 4°C (ST) were determined for all cheeses. Cheeses were stored at 4°C for up to 37 d.

[d] Cheese treatments where cheeses were held at 4°C for 0 d before freezing to -20°C (TBF0) correspond to cheeses frozen at a rate of 0.6, 2.0 or 8.0°C h^{-1}. The frozen cheeses were held frozen for 6 weeks in the freezer. Control and frozen-thawed cheeses were sampled from vats A or B.

[e] Cheese treatments where cheeses were held at 4°C for 2 d before freezing to -20°C (TBF2) correspond to cheeses frozen at a rate of 2.0°C h^{-1}. The frozen cheeses were held frozen for 1, 6, 12 or 44 weeks in the freezer. Control and frozen-thawed cheeses were sampled from vats C.

[f] Cheese treatments where cheeses were held at 4°C for 8 d before freezing to -20°C (TBF8) correspond to cheeses frozen at a rate of 2.0°C h^{-1}. The frozen cheeses were held frozen for 6 weeks in the freezer. Control and frozen-thawed cheeses were sampled from vats D, E, F or G.

[g] n/d = not determined; n/a = not applicable

Table 6: Effects of freezing at different rates (FR), total storage time at 4°C and their interaction on the characteristics of LMPS Mozzarella[a, b, c, d]

Cheese characteristic	Control	FR0.6	FR2.0	FR8.0			*P*
Unheated cheese							
More-mobile-serum (%)							
Storage time 1	3.8 ± 0.2	3.6 ± 0.5	3.3 ± 0.6	3.4 ± 0.9	Cheese treatment	(CT)	-
Storage time 2	1.9 ± 0.7	1.8 ± 0.5	1.4 ± 0.3	2.1 ± 0.5	Storage time	(ST)	***
Storage time 3	0.1 ± 0.4	0.3 ± 0.4	0.3 ± 0.2	0.3 ± 0.2	Interaction	(CT x ST)	-
pH 4.6 Soluble N (% TN)							
Storage time 1	2.6 ± 0.5	2.8 ± 0.1	2.5 ± 0.3	2.3 ± 0.4	Cheese treatment	(CT)	-
Storage time 2	3.8 ± 5.4	3.8 ± 0.1	3.6 ± 0.2	3.5 ± 0.1	Storage time	(ST)	***
Storage time 3	5.4 ± 0.1	6.2 ± 0.6	6.6 ± 0.1	6.0 ± 0.3	Interaction	(CT x ST)	-
Soluble Ca (% total Ca)							
Storage time 1	33 ± 1	35 ± 2	34 ± 2	35 ± 4	Cheese treatment	(CT)	-
Storage time 2	35 ± 1	33 ± 2	34 ± 4	33 ± 2	Storage time	(ST)	-
Storage time 3	33 ± 3	33 ± 2	33 ± 2	33 ± 1	Interaction	(CT x ST)	-
Firmness (N)							
Storage time 1	115 ± 13	106 ± 12	108 ± 20	125 ± 14	Cheese treatment	(CT)	-
Storage time 2	111 ± 10	113 ± 16	102 ± 12	84 ± 10	Storage time	(ST)	**
Storage time 3	88 ± 14	88 ± 11	84 ± 10	76 ± 7	Interaction	(CT x ST)	-
Heated cheese							
COT (°C)							
Storage time 1	58 ± 1	57 ± 1	59 ± 2	59 ± 3	Cheese treatment	(CT)	-
Storage time 2	56 ± 0	56 ± 1	56 ± 0	56 ± 1	Storage time	(ST)	***
Storage time 3	54 ± 0	54 ± 0	54 ± 1	55 ± 1	Interaction	(CT x ST)	-
Lt_{max}							
Storage time 1	1.8 ± 0.2	2.0 ± 0.2	1.9 ± 0.3	1.7 ± 0.4	Cheese treatment	(CT)	-
Storage time 2	2.6 ± 0.0	2.6 ± 0.1	2.6 ± 0.1	2.6 ± 0.0	Storage time	(ST)	***

Storage time 3	2.8 ± 0.2	2.6 ± 0.3	2.8 ± 0.1	2.7 ± 0.2	Interaction	(CT x ST)	-
EW_0 (mJ)							
Storage time 1	221 ± 44	207 ± 36	222 ± 22	222 ± 21	Cheese treatment	(CT)	-
Storage time 2	164 ± 17	130 ± 20	119 ± 18	135 ± 18	Storage time	(ST)	***
Storage time 3	81 ± 14	96 ± 7	109 ± 12	105 ± 20	Interaction	(CT x ST)	-
EW_5 (mJ)							
Storage time 1	708 ± 183	769 ± 63	830 ± 173	764 ± 54	Cheese treatment	(CT)	-
Storage time 2	510 ± 73	506 ± 67	462 ± 126	591 ± 64	Storage time	(ST)	***
Storage time 3	272 ± 70	336 ± 19	341 ± 16	383 ± 57	Interaction	(CT x ST)	-
Schreiber flow (%)							
Storage time 1	39 ± 4	47 ± 5	36 ± 6	38 ± 6	Cheese treatment	(CT)	-
Storage time 2	47 ± 6	45 ± 4	46 ± 5	48 ± 4	Storage time	(ST)	**
Storage time 3	43 ± 5	38 ± 4	46 ± 6	41 ± 4	Interaction	(CT x ST)	**

[a] The statistical significance (*P*) for treatment effects across the evaluated properties of LMPS Mozzarella is given where $P > 0.05$, $P < 0.01$ and $P < 0.001$ are denoted by -, ** and *** respectively. Control and frozen-thawed cheeses were sampled from vat A.

[b] The different cheese treatments correspond to cheeses frozen to –20°C at 0.6 (FR0.6), 2.0, (FR2.0) or 8.0°C h^{-1} (FR8.0). The frozen cheeses were held at 4°C for 0 d before freezing and held in the freezer for 6 weeks.

[c] The different storage times at 4°C (total time at 4°C) correspond to: 4 d (storage time 1), 12-15 d (storage time 2) or 37 d (storage time 3) with the exception for the characteristic more-mobile-serum, which was analyzed at 2, 4 or 9 d storage at 4°C

[d] Data presented are means ± standard deviation of two Mozzarella blocks per ripening point.

Table 7: Effects of freezing at different storage times in the freezer (TIF), total storage time at 4°C and their interaction on the characteristics of LMPS Mozzarella[a, b, c, d]

Cheese characteristic	Control	TIF1	TIF6	TIF12	TIF44			*P*
Unheated cheese								
pH 4.6 Soluble N (% TN)								
Storage time 1	2.4 ± 0.1	2.4 ± 0.0	2.5 ± 0.1	2.5 ± 0.0	2.5 ± 0.1	Cheese treatment	(CT)	-
Storage time 2	3.7 ± 0.1	4.0 ± 0.2	3.8 ± 0.1	3.8 ± 0.1	3.9 ± 0.7	Storage time	(ST)	***
Storage time 3	5.2 ± 0.1	4.7 ± 0.3	4.2 ± 0.2	4.4 ± 0.5	5.6 ± 0.1	Interaction	(CT x ST)	-
Soluble Ca (% total Ca)								
Storage time 1	37 ± 1	39 ± 1	38 ± 2	38 ± 1	39 ± 1	Cheese treatment	(CT)	-
Storage time 2	39 ± 2	40 ± 2	39 ± 1	39 ± 1	38 ± 2	Storage time	(ST)	-
Storage time 3	41 ± 1	40 ± 5	38 ± 2	38 ± 2	40 ± 2	Interaction	(CT x ST)	-
Firmness (N)								
Storage time 1	116 ± 18	96 ± 14	101 ± 22	110 ± 13	84 ± 14	Cheese treatment	(CT)	***
Storage time 2	134 ± 5	90 ± 17	114 ± 16	108 ± 15	83 ± 7	Storage time	(ST)	*
Storage time 3	93 ± 12	100 ± 9	78 ± 13	97 ± 8	76 ± 7	Interaction	(CT x ST)	-
Heated cheese								
COT (°C)								
Storage time 1	59 ± 2	59 ± 1	58 ± 1	59 ± 0	58 ± 1	Cheese treatment	(CT)	**
Storage time 2	57 ± 1	57 ± 1	57 ± 1	56 ± 1	55 ± 1	Storage time	(ST)	***
Storage time 3	54 ± 1	55 ± 0	56 ± 1	55 ± 1	55 ± 1	Interaction	(CT x ST)	-
Lt_{max}								
Storage time 1	2.0 ± 0.2	2.0 ± 0.3	2.0 ± 0.1	1.9 ± 0.0	1.9 ± 0.1	Cheese treatment	(CT)	-
Storage time 2	2.7 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.7 ± 0.1	2.6 ± 0.1	Storage time	(ST)	***
Storage time 3	3.0 ± 0.1	2.8 ± 0.1	2.8 ± 0.1	2.8 ± 0.1	2.7 ± 0.1	Interaction	(CT x ST)	-
EW_0 (mJ)								
Storage time 1	197 ± 26	204 ± 19	195 ± 32	212 ± 16	200 ± 20	Cheese treatment	(CT)	-
Storage time 2	113 ± 8	106 ± 9	106 ± 16	107 ± 12	101 ± 6	Storage time	(ST)	***

Storage time 3	75 ± 7	83 ± 10	83 ± 12	90 ± 12	83 ± 13	Interaction	(CT x ST)	-
EW_5 (mJ)								
Storage time 1	544 ± 81	591 ± 60	625 ± 93	605 ± 87	683 ± 67	Cheese treatment	(CT)	-
Storage time 2	308 ± 10	351 ± 38	366 ± 66	340 ± 27	363 ± 49	Storage time	(ST)	***
Storage time 3	274 ± 13	311 ± 28	264 ± 24	296 ± 46	286 ± 30	Interaction	(CT x ST)	-
Schreiber flow (%)								
Storage time 1	39 ± 6	42 ± 5	43 ± 7	38 ± 6	41 ± 5	Cheese treatment	(CT)	-
Storage time 2	52 ± 5	53 ± 7	49 ± 5	52 ± 4	47 ± 4	Storage time	(ST)	**
Storage time 3	53 ± 10	47 ± 8	48 ± 5	49 ± 6	46 ± 5	Interaction	(CT x ST)	-

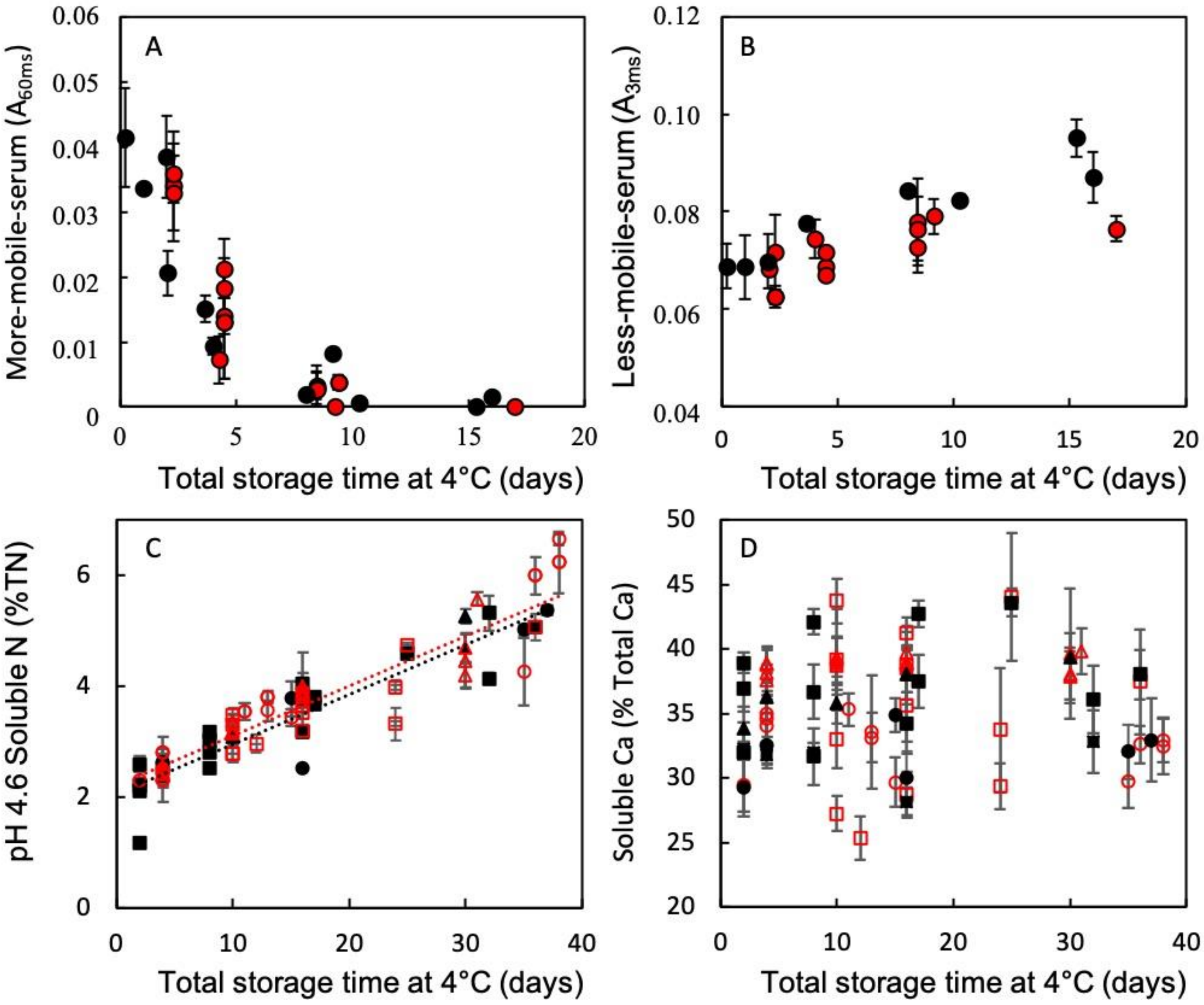
[a] The statistical significance (*P*) for treatment effects across the evaluated properties of LMPS Mozzarella is given where $P > 0.05$, $P < 0.05$, $P < 0.01$ and $P < 0.001$ are denoted by -, *, ** and ***, respectively. All cheeses were sampled from vat C.

[b] The different cheese treatments correspond to cheeses stored frozen for 1 (TIF1), 6 (TIF6), 12 (TIF12) or 44 (TIF44) weeks. The cheeses were held at 4°C for 2 d before freezing to -20°C at a rate of 2°C h^{-1}.

[c] The different storage times at 4°C (total time at 4°C) correspond to: 4 d (storage time 1), 10 d (not included in table), 16 d (storage time 2) or 30 d (storage time 3).

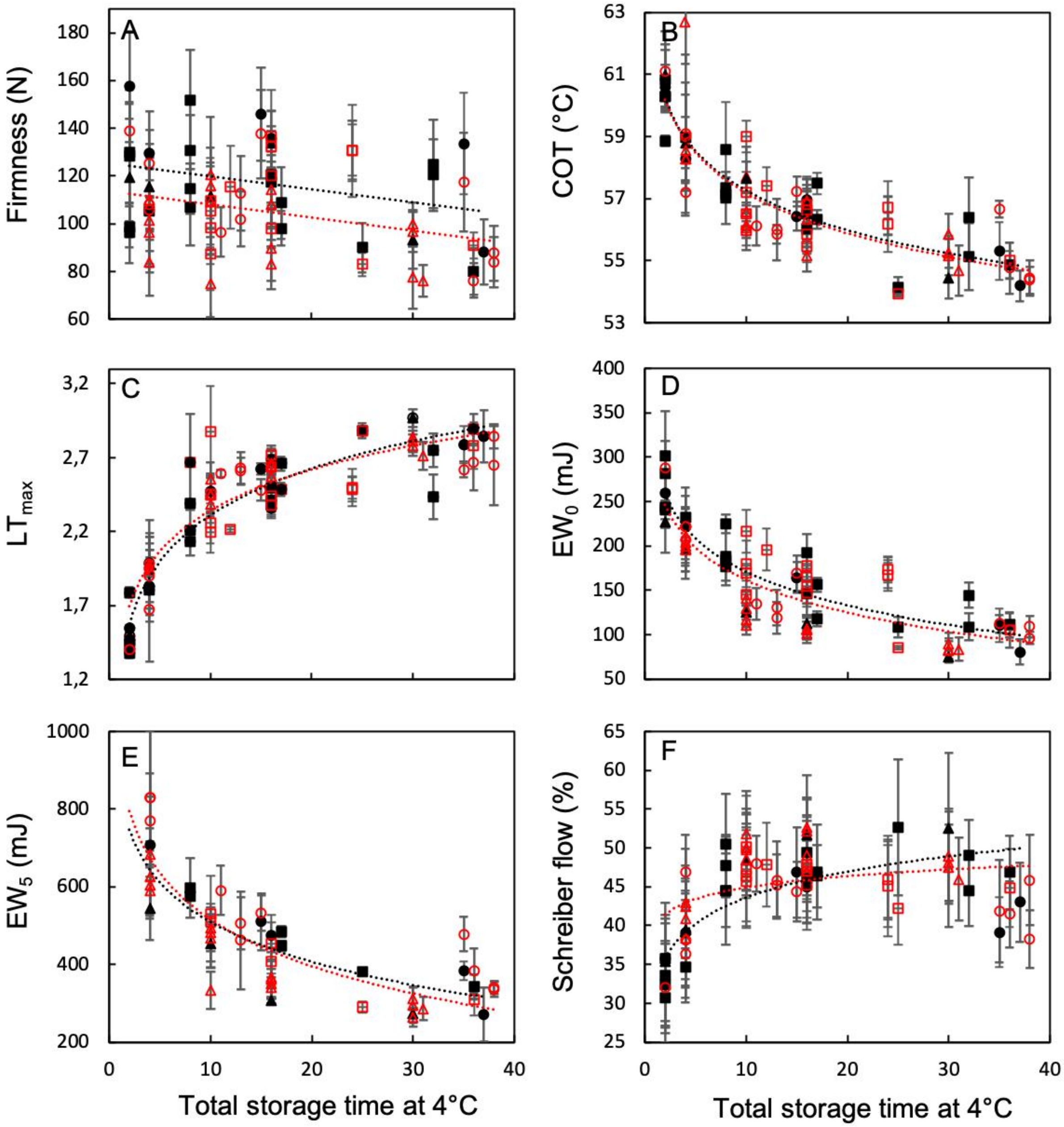
[d] Data presented are means ± standard deviation of two Mozzarella blocks per ripening point.

Fig. 1



A
More-mobile-serum (A_{60ms})
0.06
0.05
0.04
0.03
0.02
0.01
0
0
5
10
15
20
Total storage time at 4°C (days)
B
Less-mobile-serum (A_{3ms})
0.12
0.10
0.08
0.06
0.04
0
5
10
15
20
Total storage time at 4°C (days)
C
pH 4.6 Soluble N (%TN)
6
4
2
0
0
10
20
30
40
Total storage time at 4°C (days)
D
Soluble Ca (% Total Ca)
50
45
40
35
30
25
20
0
10
20
30
40
Total storage time at 4°C (days)

676 Fig. 2



A
Firmness (N)
180
160
140
120
100
80
60
B
COT (°C)
63
61
59
57
55
53
C
LT$_{max}$
3,2
2,7
2,2
1,7
1,2
D
EW$_0$ (mJ)
400
350
300
250
200
150
100
50
E
EW$_5$ (mJ)
1000
800
600
400
200
0
10
20
30
40
Total storage time at 4°C
F
Schreiber flow (%)
65
60
55
50
45
40
35
30
25
0
10
20
30
40
Total storage time at 4°C

677

Fig. 3

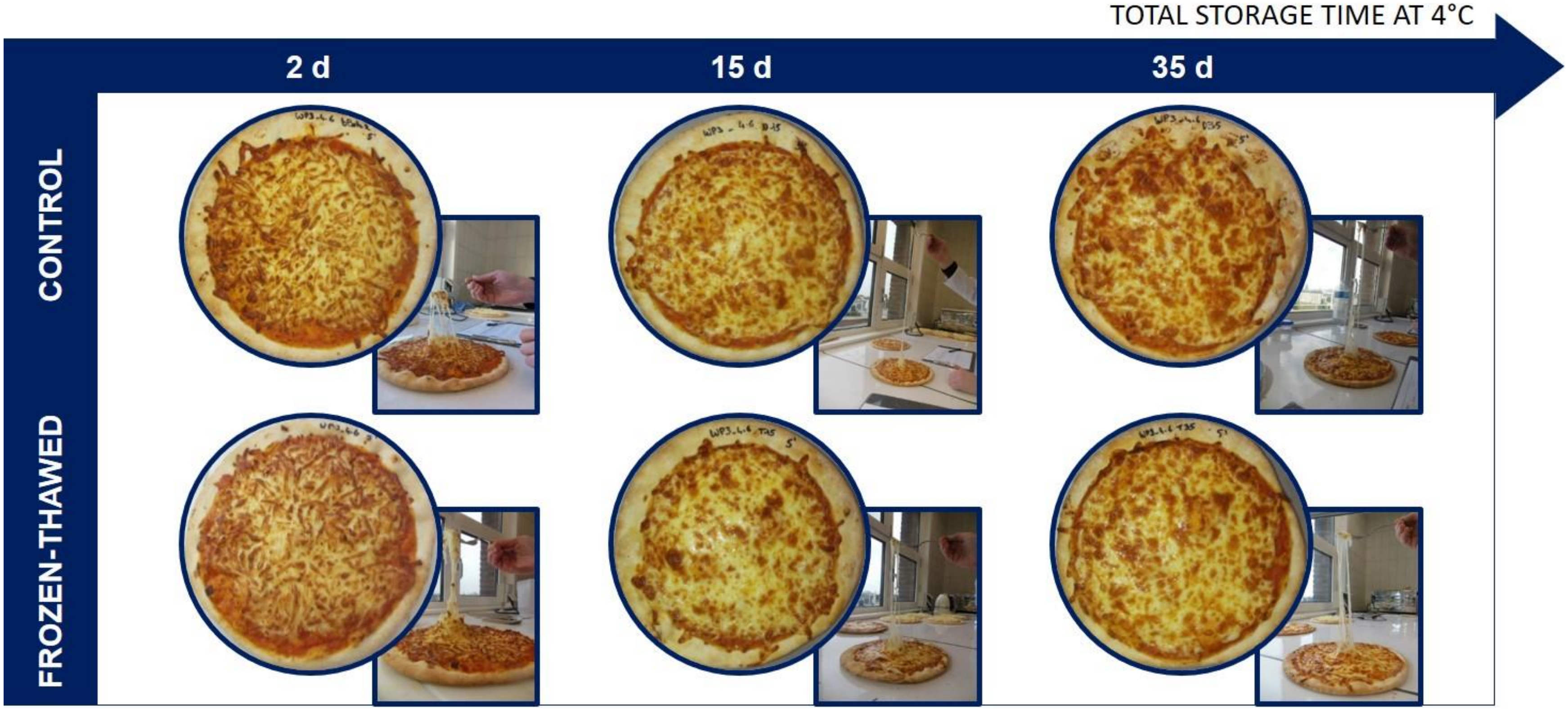
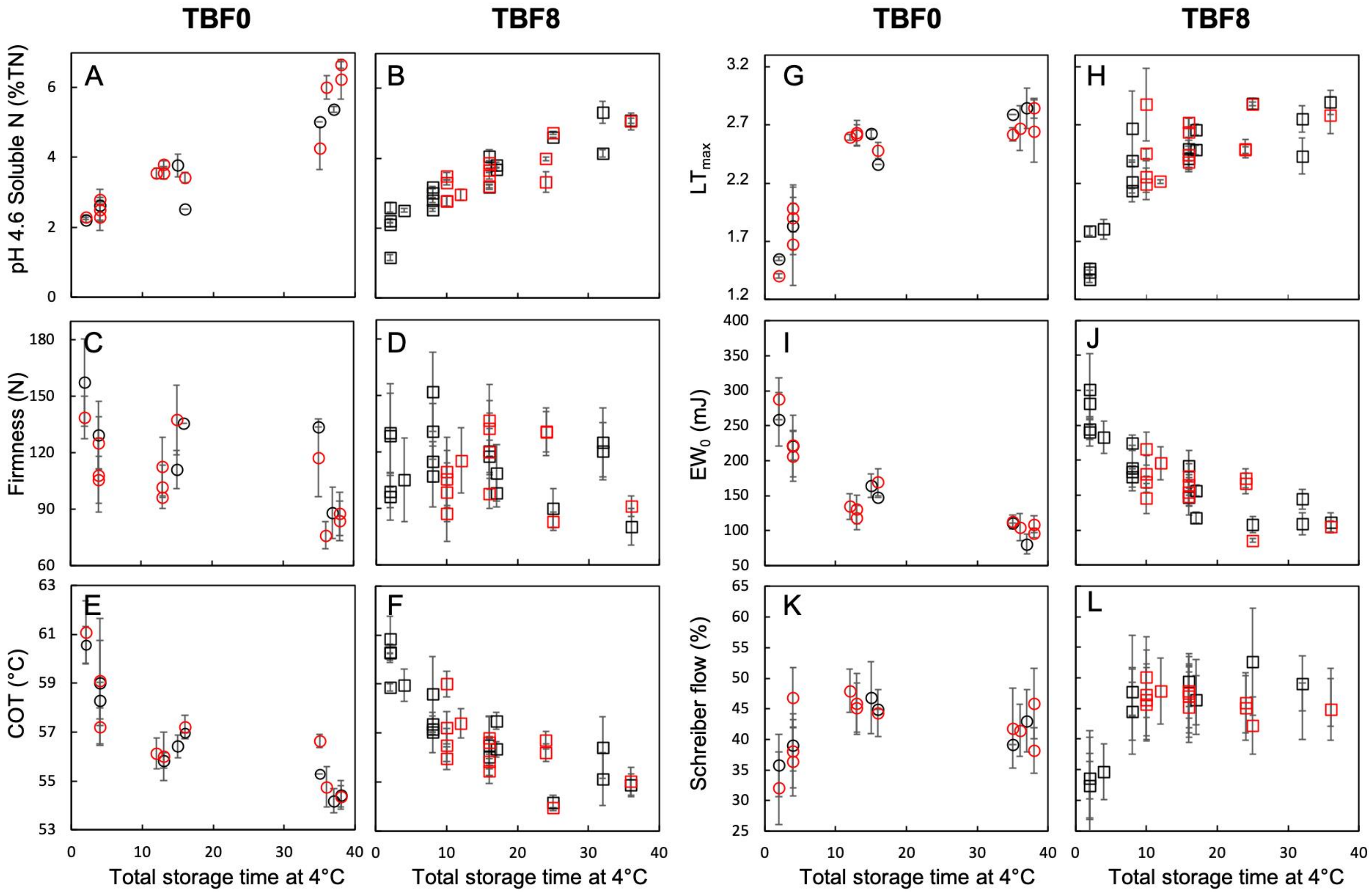


Fig. 4


TBF0
TBF8
TBF0
TBF8
A
B
C
D
E
F
G
H
I
J
K
L
pH 4.6 Soluble N (%TN)
0
2
4
6
Firmness (N)
60
90
120
150
180
COT (°C)
53
55
57
59
61
63
LT$_{max}$
1.2
1.7
2.2
2.7
3.2
EW$_0$ (mJ)
50
100
150
200
250
300
350
400
Schreiber flow (%)
25
30
35
40
45
50
55
60
65
0
10
20
30
40
Total storage time at 4°C
Total storage time at 4°C
Total storage time at 4°C
Total storage time at 4°C

Supplement A