Impact of stretching mode on chemical, rheological and microstructural properties of low-moisture Mozzarella cheese analogue

Running title: Low-moisture Mozzarella cheese analogue

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ABSTRACT

In this work, the curd plasticization of low-moisture Mozzarella cheese analogue (LMMCA) was carried out through a continuous dipping-arms cooker-stretcher or a batch twin-screw extruder. The chemical composition of the LMMCA samples obtained with the two machines was similar. During refrigerated storage (at 8°C) up to 50 days, samples processed with cooker-stretcher were slightly less proteolysed and harder. Confocal laser scanning microscopy pointed out similar microstructures. The study demonstrated that after fine-tuning of cheese making parameters both stretching machines allowed an extension of LMMCA shelf life up to 50 d.

Keywords: Low-moisture Mozzarella cheese analogue; Cooker-stretcher; Twin-screw extruder; Steam stretching; Refrigerated storage.

INTRODUCTION

Cheese is being used increasingly in a wide variety of food formulations. When adopted as an ingredient, cheese is required to exhibit specific performance attributes, both in the raw and cooked forms (Lucey 2008). In particular, the demand for Mozzarella cheese with customized functional characteristics is steadily growing in several food trade sectors (i.e., burger, pizza, sandwich and snack) (Fox *et al.* 2017). In the United States, low-moisture Mozzarella cheese (LMMC), also known as Mozzarella cheese or pizza cheese, is used as a pizza topping. LMMC is a rennet-coagulated semi-hard variety and, similarly to Mozzarella, it belongs to pasta filata cheeses. Fibrous texture formed during the cooking-stretching step characterizes this variety (Feng *et al.* 2021). Due to the lower moisture content, LMMC is more suitable (after ageing for 1 to 3 weeks) than traditional Mozzarella when used on pizza (Bertola *et al.* 1996). Generally, the desired firmness of LMMC to allow it machinability is observed between 2 and 6 weeks of its refrigerated storage (Moynihan *et al.* 2014).

Cheese analogue (CA) is a cheese substitute with similar overall properties and intended use. Low-moisture Mozzarella cheese analogue (LMMCA) is a valuable alternative to LMMC, finding application as an ingredient too (e.g., slices in stuffed burgers). Generally, in CA vegetable counterparts partly or fully replace milk components, most commonly fat (Guinee 2016). These cheese-like products have been introduced to meet the consumers' demand for innovative foods with modified dietary properties. Advantages of CA over the counterpart consist in compositional and nutritional flexibility, health and dietary benefits, tailored manufacture, convenient packing, ease-of-use, as well as functional and storage properties as per the market and consumer needs (Masotti *et al.* 2018). Over the years, the commercial trend of these products has been directed towards an extension of shelf life up to two months under refrigerated conditions. During this lapse of time, it is a priority that the cheese keeps the required functionalities for its intended use. Generally, functional properties are classified in two groups referring to unmelt or melt cheeses. Machinability is a generic term to indicate the ability of the unmelted Mozzarella cheese or its analogue to be cut or size-reduced by specific equipment (i.e., cubing, dicing, grating or slicing) (Lucey 2008). In this case, the requested functionalities include shreddability, sliceability and spreadability, which are related to texture-based attributes (e.g., hardness, elasticity, adhesiveness, brittleness) (Guinee 2016). In particular, sliceability plays an important role when LMMC or LMMCA are provided for retail and food service. The cheese must be cut cleanly into thin slices without fracturing, crumbling or sticking to cutting tool. Slices must resist fracture at outer zones on contacting packing equipment. Furthermore, slices have to exhibit a high degree of peelability (e.g., from stacks of slice-on-slice in food service applications) (Fox *et al.* 2017).

It is recognized that Mozzarella performance capabilities are affected by several factors including milk composition, manufacturing parameters as well as post-manufacturing conditions (Ma *et al.* 2013). In cheese, minerals play a major role, and their significant levels are associated with the protein network resulting in an important structural unit (Lucey and Fox 1993; Lamichhane *et al.* 2018). Colloidal calcium affects the texture and cooking properties by promoting casein-casein interactions, increasing the structural rigidity of the cheese matrix and conferring an elasticity, which is important for effective sliceability (Lucey and Fox 1993; Guinee 2016). In addition, processing parameters (such as the stretching step) play an important role in the fibrous structure formation of pasta filata cheese (Feng et al. 2021). Today, mechanical mixers of various configurations are adopted including batch or continuous processes, based on single or twin screw systems designed with different materials, barrel geometries and heating methods (Feng *et al.* 2021). Usually, the curd is stretched with mechanical mixers with one or several screws and in hot water at temperatures ranging from 82 to 85°C. Twin-screw cooker-stretchers with recirculating hot water prevail. Nevertheless, this treatment causes a heterogeneous distribution of moisture in the Mozzarella cheese, and increased stirring speeds result in higher fat loss and lower moisture content. For these reasons, nowadays stretchers using direct steam injection are preferred (Gonçalves *et al.* 2021).

Only few studies have investigated the effect of thermo-mechanical stretching parameters such as water temperature and screw speed on the cheese texture and subsequent functionalities (Renda *et al.* 1997; Yu and Gunesakaren 2005; Feng *et al.* 2022). For instance, Renda et al. (1997) reported that screw speed influenced moisture, fat-in-dry-matter (FDM) and free-oil formation of Mozzarella cheese. These authors observed that an increase in the screw speed resulted in a cheese with a lower moisture and FDM contents and a higher fat loss. In this regard, confocal laser scanning microscopy (CLSM) has been often adopted to gain a detailed comprehension of the evolution of cheese microstructure.

In the present study, we focused on the feasibility of two mechanical stretchers using direct steam injection for the manufacture of LMMCA obtained from skim milk supplemented with palm oil as milk fat substitute. We used a continuous dipping-arms cooker-stretcher (trial A) or a batch twin-screw extruder (trial B) in the manufacture of LMMCA. The experimentation was justified by the current interest of food manufacturers in using the twin-screw extruder as an alternative to the conventional cooker-stretcher. The aims were to evaluate the effect of both machines on targeted chemical (moisture, fat and protein content, concentration of mineral elements, pH, heat load and proteolysis) and physical (microscopic structure and hardness) properties of LMMCA, and to understand whether these characteristics changed over 50 d refrigerated storage.

MATERIALS AND METHODS

LMMCA production and sampling procedure

Samples of LMMCA were manufactured by a local factory adopting two different stretching devices, as detailed in the flow chart of Figure 1.

Preliminary experiments were performed to set the stretching conditions, which allowed delivering enough heat to plasticize the curd. Each cheese making trial was repeated on two different days. Briefly, 81550 kg raw milk were centrifuged, and the skimmed milk (0.05 % w/w in fat) supplemented with 2038 kg of palm oil (2.5 %, w/w) to a protein-to-fat ratio of 1.35. After pasteurization at 72°C for 15 s, the mixture was cooled to 36°C and poured in a 10200 L capacity horizontal vat. Subsequently, freeze-dried lactic acid bacterial starter *Streptococcus thermophilus* STI (Chr. Hansen, Hoersholm, Denmark) was added directly into the vat. To allow coagulation, 390 mL of pure (100%) chymosin (Maxiren 600, DSM, Heerlen, The Netherlands) were added. After 28 min, the coagulated gel was cut into cubes (approximately 0.9 cm^3 in size) and left under agitation in the cheese vat for 5 min. The resulting curd grains were heated at 41°C for 22 min and kept under agitation for additional 30 min. Overall, the cheesemaking process in the vat lasted 100 min.

Curd grains rested for acidification for 105 min to reach a pH value of curds of 5.05 and 5.10 in trial A and B, respectively). The stretching step was achieved alternatively using two stretchers through which curds were heated by steam at 62°C in the core for 10 or 15 min in trial A and B, respectively. The steam stretching step was carried out with a dipping arms continuous cooker-stretcher (trial A) or a batch twin-screw extruder (trial B) (Figure 1). The plasticized stretched curds were shaped in parallelepiped blocks (2.5 kg), brine-salted and packed in polyethylene bags. Processing output capacity of both machines was 1000 kg per hour.

Cheese samples were stored at 8 ± 1°C and sampled for analyses after 22, 35 and 50 d. Each cheese block was firstly sampled for compression test (Figure 2). Subsequently, a 3 cm-thickness slice was cut perpendicularly to the principal axis of the cheese and sampled for CLSM. The LMMCA slice was cut, finely ground and used for chemical composition and furosine analyses.

Sample analysis

Chemical composition and furosine analysis

Fat and protein (total nitrogen x 6.38) of bovine milk samples were determined according the gravimetric (ISO 1211:2010) and the Kjeldahl methods (ISO 8968-1:2014), respectively.

The pH of cheese samples was evaluated using the potentiometric method on the cheese water-soluble extract. to the Ten g of grated LMMCA were added 10 mL of Milli Q-treated water and homogenized by Ultra-Turrax . The measure of pH was performed on the supernatant after centrifugation (5000 g at 10°C for 10 min) by using a pH meter (Crison Instruments, Barcelona, Spain). The following chemical analyses were carried out on LMMCA: moisture by gravimetric method (ISO 5534:2004), fat by gravimetric method (ISO 3433:2008), total nitrogen (TN) and pH 4.4 soluble nitrogen (pH 4.4 SN) by Kjeldahl method (ISO 27871:2011). Nitrogenous fractions were determined with an automatic digestor K-439 and a distillation and titration unit K-375 (Büchi Labortechnick, Flawil, Switzerland). Proteolysis was expressed through the maturation index calculated as a percentage of pH 4.4 SN in TN according the following equation:

$$\text{Maturation index} = \text{pH 4.4 SN} * 100/\text{TN} \qquad (1)$$

Furosine analysis was carried out according to the ISO Standard (ISO 18329:2004).

Concentrations of mineral elements were measured (in triplicate) by inductively coupled plasma mass spectrometry (ICP–MS; Bruker AURORA M90 ICP–MS, Bruker Daltonik, Leipzig, Germany).

LMMCA structure

Confocal laser scanning microscopy (CLSM) was used to examine the changes in microstructure of the LMMCA samples stored at 8°C for 35 d and 50 d. Two cheese cross-sections perpendicular to the flow direction (2 x 2 x 1 mm) were taken from a cheese slice at 3-cm depth using a razor blade (Figure 2). Samples were prepared for CLSM by staining with either Nile Red (Merck, Darmstadt, Germany) to stain fat and Fast Green FCF (Merck) to visualize the protein matrix. The staining was performed as follows: stock solution of

Nile red (1 mg mL^{-1} in dimethylsulfoxide, Merck) and stock solution of Fast Green FCF (1 mg mL^{-1} in MilliQ-treated water) were tenfold diluted just prior to 5-min staining (D'Incecco *et al*. 2020). Confocal laser scanning microscopy observation was carried out using an inverted CLSM A11+ from Nikon (Minato, Japan). The excitation/emission wavelengths were set at 488 nm/520–590 nm for Nile Red and at 638 nm/660–740 nm for Fast Green FCF. Images were presented as maximum projection of 23 optical sections stacked together with separation between layers set at 0.30 µm.

Compression test

Block-shaped LMMCA samples were evaluated by a compression test in the outer (1-cm in depth) and central areas of the cheese block (Figure 2). In each zone, measures were taken in three points in-depth. Briefly, two consecutive cycles of compression were performed with a dynamometer (Z005, ZwickRoell, Ulm, Germany) (Rossi *et al.* 2016). Each compression cycle accounted for a maximum deformation of the sample of 5 mm, using a cylinder probe with a 2 mm diameter, at a crosshead speed of 2 mm per s. Maximum compression force (i.e., F_{max}, expressed in N, as the peak force of the first compression cycle) was calculated from the force-time plots using the software TestXpert V10.11 Master (ZwickRoell). All tests were carried out at 23.0 ± 0.5°C and 40.0 ± 2.5% relative humidity, immediately after the cheese sample extraction from the refrigerator.

Statistical Analysis

Two replicates were performed with each sample. Statistical differences between mean values were determined by Student's t-test, with a significance level (P) < 0.05, using Statgraphics Plus 4.0 software (Statgraphics, Rockville, USA). Data were analyzed by analysis of variance (ANOVA) with the same software. When differences between treatment effects were significant (P < 0.05), a multiple comparison of means was performed using the least significant differences test.

RESULTS AND DISCUSSION

Manufacturing of LMMCA

Chemical composition of cheese milk and LMMCA samples

The manufacturing protocol of LMMCA samples included the fat standardization with palm oil to a value of 2.5% (w/w), this level being considered optimal for processing Mozzarella cheese to be consumed in slices (Figure 1) (Gonçalves and Cardarelli 2019). Standardized and pasteurized bovine milk samples for continuous cooker-stretcher and batch extruder processing had similar composition; they contained 2.44±0.12% (w/v) of fat and 3.47±0.15% (w/v) of proteins (TN x 6.38). Cheese making included the in-vat addition of a thermophilic starter culture suitable for obtaining functionalities typical for LMMCA (Kindstedt 2007). The milk temperature was set to 36°C and the curd was stirred and heated to 41°C to obtain the low moisture content in the final product. Curds showed a mean pH value of 5.07 which was suitable to allow the subsequent stretching phase. At this pH value, it is recognised that the high dissociation of calcium phosphate and the decrease of the net charge of the proteins increase the degree of hydrophobic interactions among caseins (Gonçalves and Cardarelli 2019). As observed in previous findings (Guinee *et al.* 2000), a large increase in pH occurred between curd milling (mean pH value = 5.15) and the 4 d cheese (mean pH value = 5.41) for both trials. This increase in pH was assigned to the loss of lactic acid and changes in the calcium-phosphate moiety of the curd during plasticization (Feeney *et al.* 2001).

Overall, the chemical composition of LMMCA samples was similar to that reported in the literature for LMMC (Feeney *et al.* 2001; McMahon and Oberg 2011). Only slight compositional differences were recorded between LMMCA obtained by the two protocols (Table 1). These data were expected as the milk-in-vat was standardized to a fixed protein-to-fat ratio (1.35) and subjected to similar cheese making processes. Mean moisture values fitted with the broad compositional range reported in the literature (from 45 to 52%), which allows to meet the functional performances when LMMC is heated during pizza preparation (Kindstedt 2007). Samples from trial A showed slightly lower moisture content. To exclude the variability of fat on cheese moisture, the FDM parameter was calculated. Its levels almost overlapped and were within the typical interval for LMMC (from 30 to 50%). Mineral contents were higher in samples from trial A. Nevertheless, the difference between values of the replicated trial samples was not significant.

We measured the furosine to evaluate the heat load applied during cheese making. This index may be adopted for the quality control of pasta filata fresh cheeses, i.e. Mozzarella. Previous research studies (Pellegrino *et al.* 1995) highlighted that the industrial production of Mozzarella shaped in hot water was characterized by furosine values lower than 8 mg per 100 g protein. Such low levels are explained by the fact that during the extrusion process water temperature is typically in the range from 60 to 85°C, and the cheese temperature as it exits the mixer is from 50 to 65°C (Renda *et al.* 1997). In our study, the final products obtained through steam-based stretching at 62°C (for 10 min in trial A and 15 min in trial B) induced furosine levels slightly higher (10.2 in trial A and 9.2 mg in trial B per 100 g protein).

Evaluation of LMMCA proteolysis

Several factors influence the rate and extent of casein hydrolysis: cheese composition (e.g., moisture in non-fat substance), type and residual amount of coagulant, cooking/stretching conditions, as well as ripening temperature. The breakdown of the intact caseins by rennet is thought to be one of the main factors affecting the age-related changes which occur in LMMC (Feeney *et al.* 2001). Thermophilic starters are generally inactivated during stretching at approximately the same temperature as the coagulant enzymes (McMahon and Oberg 2011). We monitored the degree of proteolysis of LMMCA samples aged 22, 35 and 50 d through the maturation index (Figure 3). We chose these sampling times, because we were interested in monitoring the evolution of analytical parameters exceeding the 2-4 weeks of storage conventionally used for LMMC.

Over the 4-week storage period at 8°C, the level of pH 4.4 SN almost doubled from 5–7% (at 22 d) up to 10–11% (at 50 d). Small differences in stretching temperature can cause large changes in proteolysis during cheese storage (McMahon and Oberg 2001). It is generally accepted that the type of starter culture and coagulant affect the rate of proteolysis and the subsequent changes in LMMC properties during refrigerated storage. Under the conditions adopted in this work, the overall extent of casein hydrolysis was relatively low due to the partial inactivation of the residual chymosin following curd cooking at 41°C and cooking-stretching at 62°C (Feeney *et al.* 2001). Almost equal slope characterized the pH 4.4 SN-time curves, thus suggesting that the stretching machine did not selectively influence the evolution of proteolysis over the storage period. Cheese analogue samples from trial B distinguished for slightly higher levels of pH 4.6 SN. This was in line with the fact that samples with higher moisture typically undergo faster proteolysis (McMahon and Oberg, 2001).

We recorded an increasing time dependant proteolytic behaviour (Figure 3). Storage temperature is a contributing factor explaining the trend of casein hydrolysis curve through storage time. Our data were consistent with those reported by other authors for LMMC (Folkertsma *et al.* 1996; Feeney *et al.* 2001). Feeney et al. (2001) observed that the interaction between ripening temperature and time had a significant effect on the increase in the maturation index. In detail, the authors recorded an increase in the maturation index from approximately 6% at 20 d storage to 10.5% after 50 d at 10°C. These authors also reported that, in the ripening period from 20 d to 50 d, the levels of maturation index in samples stored at 10 and 15°C were similar and significantly higher ($P < 0.05$) than those kept at 4°C. The reasons of the higher susceptibility of caseins to hydrolysis may be ascribed also to the decrease of insoluble calcium, which promotes a reduction of the electrostatic interactions between caseins, thus leading to a more open protein matrix (Gonçalves *et al.* 2021).

Compression test

Mechanical properties of LMMCA estimated through the compression test allow obtaining information about its structure. In our experiment, the deformation test was applied in the outer and central regions of the samples to quantify potential differences in mechanical properties between the two areas. In both trials at all sampling times slightly higher values of F_{max} were recorded in the core cheese area (Figure 4). This hardness homogeneity in each block cheese is relevant in terms of structural effects. Indeed, hardness influences cheese machinability. Two distinct profiles of F_{max} values were observed, being cheese analogue samples from trial A approximately two times harder than those manufactured through the twin-screw extruder (trial B). This behaviour fitted well with the higher levels of proteolysis measured in trial B samples during all time-points of ripening. In other words, the use of twin screw cooker-stretcher allowed to obtain a LMMCA lighter and with a more breakable structure. Differently, cheese manufacturing with dipping-arms stretcher resulted in samples less prone to be deformed and more capable to absorb an applied stress. Under the adopted processing conditions, during storage we recorded a substantial flat progress of hardness-time curves in both experimental samples. This trend was not in accordance with data reported by Guinee *et al.* (2001). These authors observed a steep decrease of hardness in LMMC stored at 0 to 15°C. They also found a strong correlation between the age-related reduction of intact casein content and the cheese softening. Indeed, storage temperature is just one of several parameters affecting cheese structure throughout refrigerated ripening.

Cheese structure

Microscopy has been widely applied to monitor structural changes during cheese storage (Ong *et al.* 2022). CLSM has been successfully used to study the microstructural development of Mozzarella cheese during ripening enabling rapid and simultaneous imaging of fat and protein distributions (Auty *et al.* 2001). Unidirectional fibrous structure characterizes pasta filata cheeses. The plasticization step transforms the curd from an open-celled structure, consisting of a network of protein strands containing interspersed serum and fat globules, into parallel protein fibers separated by long channels of accumulated fat and free serum (McMahon 2011). The manufacturing process determines this microstructure, as it affects molecular assembly and the specific arrangement of proteins and fat (Ong *et al.* 2022). It is known that storage or ripening conditions can further alter cheese microstructure and influence cheese properties. During this period, the structure of the protein network of the LMMCA matrix alters due to complex physical and biochemical changes, such as proteolysis, demineralization of casein and hydration of the casein networks, as reviewed by Guinee (2016). Typically, the microscopic analysis of LMMC shows the para-casein in the form of longitudinally aligned protein fibres with entrapped fat columns as coalesced globules. The micrographs of LMMCA samples after 35 d storage at 8°C clearly show that the protein matrix completely encases the fat globules (Figure 5). A limited presence of serum pockets (black) immersed in the cheese matrix is visible in samples from both trials. It is likely that as the hydrophobic regions inside the proteins get closer, some of the water is forced to change into a free state in the interstitial spaces (Gonçalves *et al.* 2021). In detail, a uniform and homogeneous protein matrix (green) with a majority of small spherical fat globules (red) similar in size in both sampling areas was clearly visible, whereas only a low number of coalesced fat domains (up to 30–40 µm in diameter) appeared (Figure 5). This pattern supports the findings of Banville *et al.* (2016) who verified that light stretching conditions result in small fat globules. Under processing conditions of our study, the steam temperature (62°C) was lower than the conventional one adopted with hot water (70–75°C). Only slightly more close-packed fat clusters characterized the 35-d aged samples of trial B. Based on these data, we can infer that the processing conditions adopted with the two stretching systems supplied a similar mechanical energy, which resulted in a comparable impact on cheese structure.

Fifty-day-old samples distinguished for the progress of fat coalescence in the form of misshapen fat or droplet clustering, independently of the implemented plasticizing equipment. The increase in fat size is an important attribute that has been associated with cheese functional properties such as meltability (Noronha *et al*. 2008). Concomitantly, the protein phase of the LMMCA became more continuous following gradual swelling of the para-casein fibres. We hypothesized that the increase in hydration of the para-casein is a result of proteolysis and solubilization of casein-bound calcium. The increased water binding-capacity is important as it affects the ability of the cheese to retain moisture and hence the ability to flow and stretch (Auty *et al.* 2001). Overall, the slight structural changes observed by CLSM through storage were consistent with the limited textural changes detected with the compression test.

CONCLUSIONS

Properties of LMMCA depend on ingredients, processing parameters as well as on post-manufacturing conditions, which are an additional factor affecting the composition and rheological properties of the final product. This work investigated the impact of a continuous dipping-arms cooker-stretcher and a batch twin-screw extruder on targeted chemical and physical LMMCA properties throughout prolonged storage at 8 °C. Following proper set up of equipment, LMMCA samples showed similar compositional and microstructural properties. Under the adopted conditions, samples obtained using the dipping-arms extruder were only slightly harder in comparison to those obtained using the cooker-stretcher, and both were steadily machinable up to 50-d storage. A comparative assessment of equipment was not possible due to the fact that changes in the processing conditions interact with composition changes, resulting in a combined and complex effect on properties of the final product.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

ACKNOWLEDGEMENTS

Confocal laser scanning microscopy was performed by the staff of the Unitech NOLIMITS facility at the University of Milan (Italy). We acknowledge the technical manager dr. Nadia Santo and dr. Miriam Ascagni for their expertise and kind assistance. We thank dr. Paolo D’Incecco for sample staining. Ringraziare il tecnico che ha fatto le analisi ICP-MS.

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