Effect of freezing and thawing processes on high-moisture Mozzarella cheese rheological and physical properties

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Abstract: High-moisture Mozzarella cheese is a soft, fresh cheese characterized by a short shelf-life, but a freezing process can be effective for improving its storability. In this study, the effects of two freezing/thawing methods (the presence or absence of a covering liquid during the process), three freezing (ranging from -40 °C, 4.1 ± 0.6 m/s, to -25 °C, 1.3 ± 0.2 m/s) and two thawing conditions (+4 °C, 1.3 ± 0.2 m/s, +4 °C, 4.1 ± 0.6 m/s) were evaluated on Mozzarella cheese characteristics. Cheeses processed with a covering liquid were characterized by water absorption during thawing, lower water holding capacity, softer texture and lower rheological moduli. Frozen/thawed cheeses without the covering liquid and stored overnight with a new covering liquid, despite having a lower juiciness, were characterized by a lower degree of freezing-induced modifications and were more similar to the fresh cheese. Cheese properties were not largely affected by the freezing/thawing conditions considered here. Freezing high-moisture Mozzarella cheese has a small impact on a product's properties if it is performed without a covering liquid and is followed by an overnight rehydration step in a fresh covering liquid. Therefore, this should be the preferred method to obtain the best quality results.

*Cover Letter

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Prof. Harald Rohm
Editor
LWT – Food Science and Technology

January 3, 2020

Dear Prof. Harald Rohm,

I would like to re submit the revised article entitled "Effect of freezing and thawing processes on high-moisture Mozzarella cheese rheological and physical properties" by Marcello Alinovi & Germano Mucchetti, for publication in *LWT – Food Science and Technology*.

We appreciate the interest that the editor and reviewers have taken in our manuscript and the constructive criticism they have given. We hope to have addressed the major concerns of the reviewers and editor. In particular, we have tried to improve clarity in the discussion section, and we wrote some comments about the potential role of calcium and pH in the physical and rheological modifications that can occur as a consequence of freezing and thawing. We have also improved language accuracy by mean of a professional revision service. As proof of that, we are submitting the language editing certificate of the service in the page below. These changes have clearly improved our manuscript. We have also included a point-by-point response to the reviewers in addition to making the changes described above in the manuscript. Changes to the manuscript are marked in red or are formatted as revisions.

Our main findings remain unchanged; we evaluated the feasability of various freezing, thawing conditions and methods as preserving techniques for high-moisture Mozzarella cheese quality properties. We discovered that high-moisture Mozzarella cheese can be frozen and thawed with good results in terms of product's quality if the product is processed without the presence of covering liquid. Considering the increasing global demand for high-moisture traditional cheeses such as high-moisture Italian Mozzarella cheese, we believe that this manuscript can have a strong impact in this field of Science, because it discusses for the first time the application of the freezing and thawing processes to improve the storability and the convenience of this kind of product.

This manuscript has not been published and is not under consideration for publication elsewhere and we have no conflicts of interest to disclose. Please address all correspondence concerning this manuscript to me at marcello.alinovi@studenti.unipr.it.

Thank you for your consideration.

Sincerely yours,

Marcello Alinovi

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To whom it may concern

The paper "Effect of freezing and thawing processes on high-moisture Mozzarella cheese rheological and physical properties" by Germano Mucchetti Marcello Alinovi was edited by Elsevier Language Editing Services.

Kind regards,

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***Detailed Response to Reviewers**

Reviewer #1: The manuscript looks at freeze thaw behaviour of mozzarella cheese. The content is new to me but is not a particularly innovative topic for comparison. Nonetheless, I find a real strength in the manuscript, because of the choice of methods. The methods are purely scientific and performed with substantial expertise, for instance, the G' and G" data is interpreted with a power law like a physicist would treat it. Thus it is very clear what each measurement is tells the reader, but it is also clear how it relates to the application and to other measurements.

I do have a few comments that I think could be address
page 1: What is a "lower" viscoelastic behaviour. I think the authors mean proportionally less viscous (as opposed to elastic) behaviour. Just note that lower could also be miss understood as low moduli, so it's important to be specific

Thank you for your comment. With this sentence, we meant both lower storage and loss moduli; but, as this sentence can be misunderstood and could not be clearly interpreted, as correctly pointed out, we changed it in “lower rheological moduli”, in order to highlight that Mozzarella cheeses frozen in the presence of covering liquid were softer, and were characterized by a more viscous, less elastic matrix.

page 6, equation 3. I think the authors mean n'

Thank you for your observation. Yes, we meant n’; we corrected the equation accordingly.

page 9: I think mechanical stress should be replaced with mechanical strain. Surely the stress is the same since the centrifuging conditions are the same. Or have I missed something?

The discussion here (lines 190-191) is to compare the results obtained using two different techniques applied to measure the amount of expressible serum of Mozzarella cheeses; in this term, the amount of expressible serum measured by double compression (TPA) was strongly lower (7.92 ± 2.88 %) compared to the amount separated by centrifugation (57.64 ± 5.95 %), because of the different intensity and nature of force.
As the comparison cannot be made in the previous version of the text, because results concerning expressible serum measured with TPA were not shown, we added figure of ES_{TPA} that can be viewed in supplementary materials.

page 10: line 207-211. mozzarella is known to have channels of fat and water. I'm not sure if the water take up is in these channels, in the protein or both. Change in whiteness would suggest a structure change. Conductivity (measured in Siemens) is 1/R, where R = rho * l / A, where rho is resistivity, l is length A is area. If channels are the major source of conductivity (being salty water I would imagine this was the case) change in volume then this would contribute to the overall conductivity. Overall I'm just looking for a little clarity about why these measurements were made and if the differences are purely due to protein damage as I'm not sure why one sample is more damaged than the other.

Thank you for your comment. We noticed that our discussion in this point was wrong: M_w cheeses did not show difference in electrical conductivity if compared to the control, as you can see in Figure 1D. The only difference for this parameter was between M_d cheese with the control and M_w cheeses as discussed in lines 202-207. So, we decided to delete lines 207-211 according to our mistake.
Anyway, to reply to your answer, a hypothetical increase of electrical conductivity could be related to changes in volume of the serum phase (and changes in volume of serum channels) and a redistribution of water subsequently freezing and thawing (please, have a look, for instance: Gianferri, R., D’Aiuto, V., Curini, R., Delfini, M., & Brosio, E. (2007). Proton NMR transverse relaxation measurements to study water dynamic states and age-related changes in Mozzarella di Bufala Campana cheese. Food Chemistry, 105(2), 720–726.) that could possibly not re equilibrate during the overnight period after thawing.
Moreover, we decided to perform conductivity analyses as we thought that freezing and thawing processes can be related to the depletion of charged molecules from the protein matrix, as it is well known that changes in the equilibrium of calcium can be present as a consequence of temperature variations and/or exchanges with Na+ of the serum fraction; the freezing rate, as well as the presence or absence of covering liquid during both processes could contribute to these changes. In these terms, changes in conductivity, can be indirectly related to a change of the protein matrix structure (colloidal calcium phosphate solubilization, dehydration).

Reviewer #4: This study reported on the use of different freezing/thawing conditions on the textural, rheological and sensory properties of high moisture Mozzarella cheese. The authors also studied the effects of freezing with and without the brine solution (or covering liquid as it was called by the authors). The study is interesting. However, at the present form this manuscript is not acceptable for publication in LWT. The manuscript requires major revisions. The clarity of the manuscript has to be improved as the results and discussion section is quite difficult to follow at times. These are some suggestions/queries that the authors should consider to revise the manuscript accordingly. The manuscript should be reviewed after it has been revised.

Thank you, we tried to improve clarity of the entire manuscript by following your suggestions.

Abstract - suggest stating what the freezing and thawing conditions were. Also include what the "rehydration step" was.

Thank you, we specified the process conditions in the abstract section and that the rehydration step was performed in fresh, new covering liquid. We also tried to re summarize the abstract section, in order to comply with the limit of 200 words.

A brief description of how the cheeses were manufactured should be included. I know the authors gave a reference but it would be easier if a brief description was given in this paper. What type of milk was used? Buffalo milk? Buffalo milk has more fat and protein contents compared to cow's milk. Fat can also be affected by freezing process. What type of acid was used to make the cheeses? Was rennet used during the manufacture?

Thank you. We briefly integrated the description of the manufacturing procedure of Mozzarella cheeses (see lines 68-75 of the revised manuscript), preserving some confidential info, as for example the type of coagulant enzymes, indicating only the general category. Milk used for cheese manufacture was cow’s milk.

Experimental design - more information has to be given. Were the 4 batches obtained on the same day or different days? Only 100g of cheeses obtained? Was this amount sufficient for all the analyses? What shape were the cheeses obtained in? Was 100 g of covering liquid added to 100g of cheese? How much of cheese was packaged in the polyethylene bags? Why were they kept at refrigerated temperature for 5 d prior to freezing? It was also hard to follow the number of cheeses frozen at each condition - maybe suggest doing a flow diagram. That may be easier.

We tried to give more clear information in the revised manuscript. The different batches were manufactured in four different manufacturing days in a period of two months. The batch was composed by 91 cheeses and each cheese was a 100g shape that was individually packaged directly by the manufacturer into a polyethylene bag containing 100g of covering liquid. We specified the shape, that was spheroidal, in line 73 of the revised manuscript.

Cheeses were left 5 d in refrigerated conditions before performing the experimental design with the aim to guarantee the same delay from the day of manufacturing, because for organizational reasons, it was not always possible to transport cheeses from manufacturing site to lab within the same time. So, we preferred to introduce a storage time at 4°C that allowed us to start the freezing process with cheeses equally aged.

As you suggested, we added a flow diagram of sample numerosity and subdivision for each process variable (please, see **figure 1**).

Typically the covering liquid (or brine solution) contains NaCl, calcium chloride and acidulant. Why was only NaCl added in the covering liquid in this study? Wouldn't the calcium leach out and affect the texture of the cheeses? Also not having the acidulant in the covering liquid will affect the pH of the cheeses?

We agree that calcium content and distribution play an important role in defining Mozzarella cheese physical, textural and rheological structure. However, there are manufacturing companies that produce HM Mozzarella cheese without the addition of calcium chloride into covering liquid. We can say the same about the acidulant (citric acid, in this case). The dairy giving us the samples used only a solution of 0.4% of NaCl

for its Mozzarella cheese. Moreover, the purpose of this paper was not to investigate the effect of pH and calcium content on Mozzarella cheese properties, as all the samples were manufactured and stored in covering liquid with the same composition.
The presence of organic acids in the covering liquid can also affect the depletion of calcium from the surface, favoring the peeling of the surface. The concept, for many dairies, is to use a covering liquid which composition is determined managing the potential interactions with the cheese surface (risk of peeling) and the equilibrium with the serum phase of the cheese, which composition depends on the cheesemaking technology.
However, to clarify that calcium and pH play an important role for some properties, we added some considerations in the manuscript, as asked later by the reviewer.

The composition of the cheeses should have been included. It would help in understanding the reasons behind for some of the data/observations.

We included the gross composition of Mozzarella cheese in the material and method section (please, have a look at lines 74-75) that was 17 g of protein, 17 g of fat, 1 g of lactose and 0.4 g of NaCl. However, we were not able to measure the total calcium content and the relative content of the different forms of calcium.

For e.g. composition, pH and calcium contents have important roles to play on the texture, rheological and sensory properties of the cheeses. Both the total calcium and the amount of insoluble calcium associated with the paracasein can alter cheese texture. Both these parameters in addition to proteolysis would have an impact on the textural, rheological and functional properties of the cheeses. Suggest rewriting some part of the manuscript taking this discussion into account. Not having this information has limited the discussion of the results. Most of the discussion has focused on loss of moisture and "protein rehydration".

Ok, thanks, we agree that the role of calcium is an important factor in defining some physical attributes of the cheese; we think that it could be interesting to observe the modifications of calcium equilibria as a consequence of freezing and thawing. However, as we were not able to perform these analyses in the present work, we can only draw hypotheses, but we will take it in consideration for the next studies.
We added some lines to discuss some hypotheses about the role and effects of changes in calcium equilibria during processes, in particular to give a more detailed discussion about water up-take in the case of M_w cheeses.

For clarity, in the present experiment, we can suppose that calcium equilibria were not the main driver in freeze, thaw-induced modifications of M_w-cheeses, as it is well known that a decrease of colloidal calcium phosphate (that is what we can expect in our case, as no $CaCl_2$ was added to the covering liquid) would cause a decrease of protein-protein interactions, a decrease of cheese firmness and an increase of protein hydration (please, have a look, for instance to:

- Guinee, T. P., Feeney, E. P., Auty, M. A. E., & Fox, P. F. (2002). Effect of pH and calcium concentration on some textural and functional properties of Mozzarella cheese. Journal of dairy science, 85(7), 1655-1669.
- Faccia, M., Angiolillo, L., Mastromatteo, M., Conte, A., & Del Nobile, M. A. (2013). The effect of incorporating calcium lactate in the saline solution on improving the shelf life of Fiordilatte cheese. International Journal of Dairy Technology, 66, 373-381.

This hypothesis however does not fit with most of our results, indicating that the driver of the observed modifications was different and was related to dehydration phenomena and structural modifications, that are well documented in cheese freezing studies.

Suggest giving references for some of the methods used: moisture and ES measurements.

Ok, thank you. We have reported a reference for each of the method used (Moisture content, expressible serum measured with compression and expressible serum measured with centrifugation).

How were the cheeses sampled for analyses - being a brined cheeses there must be a gradient created (Lines 216-217 does suggest there was a gradient in moisture between the outer and inner cheese sample). Sampling becomes critical when sampling brined cheeses.

Mozzarella cheese were not salted by brining, as cheeses have been salted directly during stretching by means of salted water. Salt in covering liquid (0.4%) is in equilibrium with the salt content of the cheese (0.4%) to prevent gradients. Moreover, one of the purposes of covering liquid is to limit the moisture gradient in the different zones of the cheese, that is quite lower if compared to ripened cheeses.
However, we agree that gradients of calcium, organic acids, moisture, etc. can be present as a consequence of moisture gradient and are critical in the sampling protocol of this kind of cheese. For this reason, we sampled the cheeses in the same location for a given analysis, in order to take into account concentration/moisture diversities. For moisture content analyses, a whole cheese was sampled, mixed and analyzed in triplicate; for rheological analyses we sampled cylindrical-shape discs from central sections of one cheese, as reported in lines 104-105 of the old manuscript; for expressible serum measured with centrifuge method, we cut an entire Mozzarella cheese into 1-cm cubes and we randomly sampled 90 g of cheese (90 % of the entire shape) to perform a triplicate of analysis; for TPA and expressible serum measured with compression, the inner part of one whole cheese sample was cut into 5 cubes to perform a quintuplicate of analysis; for sensory evaluation, we used 2 Mozzarella cheese shapes: one and a half shape were cut in cubes (by priorly removing the outer part of the cheese) and used for tasting, while the remaining half shape was used for visual evaluation.
For these analyses, we added a brief description of the specific sampling protocol in the revised manuscript.
We also measured the difference in terms of moisture content from the inner to the outer part, as reported in lines 216-217 of the old manuscript. To test moisture gradients, we separately analyzed the skin (defined as the outer part of the cheese with a depth of 4 mm) and the inner part (the remaining part of the cheese).
As the measured gradient, that was around 3%, was not modified by the applied freezing/thawing processes, we did not extensively describe this part in the manuscript to comply with LWT words limitation.

Lines 83-84, how long were the samples equilibrated in the chamber?

They were equilibrated for 1h. We added it to the manuscript.

Line 90, please give a reference of the method used.

We added a reference in the text.

Lines 134-136, what type of sensory methodology used? QDA or spectrum method? How many times were the sensory evaluations repeated for the same sample?

Applied sensory methodology was QDA, we added it to the text. Sensory evaluations were repeated in duplicate, as reported in lines 141-142 of the old manuscript. We tried to improve the clarity regarding the number of repetitions. In dairy sciences, experimental designs considering a blocking variable are usually employed to consider the variability given by the cheese making day or the batch of cheese.
In these cases, the batch of cheese is considered as a sample repetition.
Please, have a look for instance at the following reference:
Bello, N. M., Kramer, M., Tempelman, R. J., Stroup, W. W., St-Pierre, N. R., Craig, B. A., … Gbur, E. E. (2016). Short communication: On recognizing the proper experimental unit in animal studies in the dairy sciences. Journal of Dairy Science, 99(11), 8871–8879. https://doi.org/10.3168/jds.2016-11516

Lines 175-176, did the authors observe or determine whether there was "structural damage" in the samples?

We did not perform microstructural observations in this work. However, we were able to detect microstructural modifications given by freezing and frozen storage on high moisture Mozzarella cheese manufactured following the same protocol in another work in press.

Line 180, please clarify "density variations".

OK, thank you. We specified in the new manuscript that during freezing, there is an increase of volume, associated to the decrease of density of water that changes from liquid to solid (ice).

Line 184, "….concentration's gradient of water molecules during…" is not clear. Please clarify.

Thank you. We agree that this sentence was not clear. We tried to improve clarity of this sentence by modifying it to: "These channels can be responsible for the latter absorption of free water from the covering liquid mediated by capillary forces and by concentration's gradient of water-soluble molecules (e.g. organic acids, salts, etc.) during the thawing process."

Line 187, please specify the "longer thawing time".

We have already reported thawing times obtained with each thawing condition a few lines above. We specified that the process longer time is associated to M_w cheeses. The reason for the longer time is mainly due to the different mass of the product to be thawed. The M_w cheese is immersed into an equal mass of covering liquid.

Lines 193-196, wouldn't the interactions that are formed between caseins and water be dependent on the pH and thus the charges on the proteins? Also the insoluble calcium associated with the proteins will also have a part to play?

Yes, we agree that a part of the water take-up is mediated by the charge of proteins. A change on the charges of proteins can be modulated by a series of factors such as pH, calcium and freeze induced structural modifications. We briefly added the point to the discussion.

Lines 207-211, what are "electrically charged peptides…."? How were they formed? Produced as a result of proteolysis? what is "protein damage phenomena" and how can these compounds contribute to the "protein damage phenomena"? What are the organic salts the authors referring to?

Thank you. We agree that this sentence is quite ambiguous. The discussion about the "charged peptides" may be true in general, but we agree that their presence in HM Mozzarella should be demonstrated and without this demonstration the sentence is too speculative. Moreover, we decided to remove the whole sentence, as also thanks to reviewer #1 we discovered a mistake in the discussion: there is no difference between the control and M_w cheeses as electrical conductivity values are similar (please, have a look to Figure 2).

For clarity: the main organic salt present in the cheese is citric acid, as there is no lactic acid produced by lactic acid bacteria.

Lines 235-236, why?

We suppose that this is due to the differences in freezing/thawing temperatures and freezing times considered in this study, that did not cause a significant modification of Mozzarella cheese rheological properties. We added this hypothesis to the manuscript.

Lines 241-242, it is not clear. Please clarify.

Thank you. We agree that this sentence can be confusing. We would state that in our case we did not observe a decrease of n', n'', n* indexes, that can be associated to the formation of a weaker gel structure. So, we did not observe a weakening of the gel structure. However, as it does not add important information to the discussion, we decided to remove it from the new manuscript in order to comply with the number of words' limitation.

Lines 249-253, is this rearrangement of protein occurring during fast freezing or slow freezing? Did the authors carry out microscopy work to determine whether there were larger fat clusters formed as a result of freezing? Please comment.

We can propose only hypotheses, as we were not able to perform microstructural observations in this study. As reported before, we carried out microscopy observations in another experimental work made on the same matrix and we observed the formation of fat clusters subsequently freezing and frozen storage. We thought that this change was not dependent to the freezing and thawing rate, but was observable, in general, for all the frozen/thawed cheeses, compared to the fresh cheeses, as reported in line 285.

Lines 259-262, it is not clear. Suggest rewriting. Rheological analyses were small deformation tests. TPA is a large deformation test which is dependent on fracture and shear. So sometimes it is not possible to compare these two tests. TPA will be more similar to sensory testing.

Thank you. We agree that this sentence is ambiguous, and we agree on the fact that rheological analyses measure different structural features of a food matrix, in comparison to textural analyses. We decided to remove the reference to tanδ from this part.

Line 265, please elaborate on "the formation of a more plasticized structure".

Thank you, we corrected "more" with "less" plasticized, as it is referred to frozen/thawed cheeses that are characterized by a more rigid, springy and less plastic structure. With "plasticity" we intend the inability of a body to recover its original configuration. As there was no sign of visible rupture or breaks in the matrix, the different springiness was caused by the quickness of recovery when the compressive force is removed.

Lines 268-270, Sensory hardness was not perceived to be different between the samples while TPA hardness values were. So how can they have the similar trend? The r-values seem to be on the low side to be considered as being correlated. Please comment.

With similar trend we would say that both textural and sensory hardness ordered the group of samples in the same order of firmness (M_d had the higher textural and sensory hardness, while control and M_w cheeses had the lowest) as reported in table 5. Of course, these two analyses were not the "same", as textural hardness did not show significance of any considered factor. This resulted in a weak correlation, that yes, is low and weak (we already mentioned it in lines 269-270 of the old manuscript), but that is significant.

Line 295-296, HM Mozzarella has a short shelf-life. So how can it be in the markets because of "longer shelf-life". Please clarify. Are the HM Mozzarella currently being commercially frozen?

HM Mozzarella cheese is typically transported and sold in refrigerated storage conditions; however, the market of frozen HM-Mozzarella cheese has been fastly growing in the last years because of the longer storability. So, it is possible to find both types of product in the same market (e.g. Japan, and also on various WEB sites of Italian dairies, both for B2B and consumers' markets. See for instance: https://www.granarologroup.com/products/frozen-iqf-cheese).

Table 5, how can treatments be different for cohesiveness and springiness when in Table 3 it shows that there were no differences in the treatments? Please clarify.

Thank you. This is because it was not possible to insert the control cheese as a level in the split-split plot ANOVA models due to obvious lack of Fc and Tc conditions. For this reason, we had to evaluate the significant difference between control cheeses and frozen/thawed cheeses by performing post-hoc tests, as reported in line 152 of the old manuscript.
Thus, P values in table 3 refers to the significance value of the freezing/thawing method applied among frozen cheeses, without considering the control. On the contrary, in table 5, letters report differences observed using post hoc tests.

EDITORIAL COMMENTS:

The opinion of reviewer #4 concerning the language is strongly underlined. The revised manuscript must linguistically be checked by a native speaker or a professional service. It is therefore **mandatory to upload a language editing certificate** when submitting the revised version.

- L.29, L.64, elsewhere: do not use % as a concentration unit - replace here by e.g. g/100 g

Thank you. We replaced the notation according to editor's advice throughout the manuscript.

- L.61, elsewhere: check that there is always a space between number and unit

We changed the notation according to editor's advice throughout the manuscript.

- L.65, eslewhere: use wk for weeks, d for days, h for hours, min for minutes, s for seconds throughout the manuscript

We applied the abbreviation throughout the manuscript according to editor's advice.

- L.93, elsewhere: use "L" to abbreviate liter

We changed the notation according to editor's advice throughout the manuscript.

- L.106: change to 0.475/cm.

We changed the notation according to editor's advice.

- L.147, elsewhere: please carefully check citation style

In text references were modified and formatted in the new draft according LWT guidelines using Mendeley software.

- L.165, elsewhere: Fig. not Figure

We changed the notation according to editor's advice throughout the manuscript.

- L.306-309: remove from MS

We removed the "notes" section according to editor's advice.

References: give journal issue numbers (in brackets) for all references, or remove from all

Issue number was missing for some papers, as was not provided by some specific journals (e.g. in the case of "Alinovi, M., Rinaldi, M., & Mucchetti, G. (2018). Spatiotemporal characterization of texture of Crescenza cheese, a soft fresh Italian cheese. Journal of Food Quality, 2018, 1–8"). According to Editor's indication, we removed the issue number from all the references and we corrected mistakes made in reference style.

- Fig. 2, 3: Legends to symbols shpuld be in the caption and not as figure insert

Thank you, we modified figure captions and figures according to Editor's advice. We also edited X-axis (M_w and M_d, instead W and D), in graphs reported in Figure 1, in order to have a clearer correspondence with figure caption.

***Highlights (for review)**

- Quality of Mozzarella cheese frozen with or without covering liquid is different
- The presence of covering liquid causes cheese water absorption during thawing
- Frozen Mozzarella cheese without covering liquid shows good quality results
- Applied freezing/thawing rates have no effects on measured cheese properties

Effect of freezing and thawing processes on high-moisture Mozzarella cheese rheological and physical properties

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Abstract

High-moisture Mozzarella cheese is a soft, fresh cheese characterized by a short shelf-life, but a freezing process can be effective~~;~~ ~~to improve~~for improving its storability~~ and convenience, the freezing process can be effective~~. In this study, the effects of two freezing/thawing methods (the presence~~,~~ or absence of a covering liquid during the process~~es~~), three freezing (ranging from -40 °C, 4.1 ± 0.6 m/s, to -25 °C, 1.3 ± 0.2 m/s) and two thawing conditions (+4 °C, 1.3 ± 0.2 m/s, +4 °C, 4.1 ± 0.6 m/s) were evaluated on Mozzarella cheese characteristics~~. The presence of the~~ the ~~covering liquid during the processes influenced many Mozzarella~~the ~~cheese physicochemical, rheological and sensory characteristics of the Mozzarella~~. Cheeses ~~processed~~ with a covering liquid were characterized by water absorption~~ phenomena~~ during thawing, ~~a~~ lower water holding capacity, ~~a~~ softer texture and ~~a lower viscoelastic behavior~~ lower rheological moduli. ~~On the contrary, f~~Frozen/thawed cheeses without ~~the~~ the covering liquid and stored overnight with a new covering liquid, despite having a lower ~~sensory~~ juiciness, were characterized by a lower degree of freezing-induced modifications and were more similar to the fresh cheese. Cheese properties were not largely affected by the freezing/~~and~~thawing conditions considered here. ~~Results of this study highlighted that f~~Freezing~~ of~~ high-moisture

Mozzarella cheese has a small impact ~~over~~ on a product's properties if it is performed without a covering liquid and is followed by an overnight rehydration step in a fresh covering liquid.~~;~~ ~~thus~~Therefore, this should be the preferred method to obtain the best quality results.

Keywords: Mozzarella cheese; Cheese texture; Sensory analysis; Expressible serum; Air-blast freezing

1. Introduction

Mozzarella cheese, one of the most consumed cheese worldwide, can be divided into two categories related to its final utilization: for consumption as a fresh cheese or as an ingredient for pizza or other prepared foods (Francolino, Locci, Ghiglietti, Iezzi, & Mucchetti, 2010). ~~As f~~Functional characteristics, such as the shreddability and meltability, largely depend on the cheese moisture content (Bertola, Califano, Bevilacqua, & Zaritzky, 1996), and they are poor in the case of high-moisture (HM) Mozzarella cheese, ~~having~~ which has a moisture content between 52 and 60 g/100 g ~~% (w/w)~~ (U.S. FDA, 2018) or higher in the case of the Italian-type. Thus, HM Mozzarella cheese is preferably consumed as a fresh cheese and is packed and stored with ~~the~~ a covering liquid that is a brine containing mono, divalent salts ~~(NaCl, $CaCl_2$)~~ and/or organic acids (NaCl, $CaCl_2$, Calcium lactate) (Faccia, Angiolillo, Mastromatteo, Conte, & Del Nobile, 2013).~~;~~ ~~t~~This liquid is useful to maintain the high moisture of the cheese, to avoid the formation of a rind and eventually to complete cheese salting ~~brining~~. Because of its high moisture content and the fresh taste expected by the consumer, HM Mozzarella cheese is characterized by a short shelf-life that can vary from one to thirty days~~ays~~ (Mucchetti, Pugliese, & Paciulli, 2016).

To improve the storability, ~~the application of~~ freezing has been assessed in the last few years for several cheeses (Alberini, Miccolo, & Rubiolo, 2015; Alvarenga, Canada, & Sousa, 2011; Conte et al., 2017; Reid & Yan, 2004; Kuo, Anderson, & Gunasekaran, 2003). Considering the globalization of food markets and the increasing demand for highly perishable cheeses, freezing can be a good strategy to decrease waste by improving, for example, a product's convenience and supply chain efficiency (Pollack, 2001).

Patents concerning HM Mozzarella cheese or curd freezing have been published (Coker, Gillies, Havea, & Taylor, 2017; Zambrini & Bernardi, 2017),~~;~~ ~~nowadays,~~and the process is currently performed

~~in ~~on an industrial scale, despite HM Mozzarella cheese ~~is not~~not being considered ~~to be ~~suitable for freezing in the US (USDEC, 2016). However, there is a lack of scientific data about the effects of freezing and thawing on HM Mozzarella cheese characteristics. HM Mozzarella cheese can be frozen by an ~~I~~individual ~~Q~~quick ~~F~~freezing (IQF) method as a packaged product immersed in its covering liquid or as a non-packaged product.

Conte et al., (2017)~~,~~ compared the effects of freezing rate and 2-months of frozen storage ~~of ~~on "fiordilatte" Mozzarella cheeses. Higher freezing rates preserved better cheese quality. However, the authors measured a decreased~~e of~~ pores volume and overall sensory quality probably caused by the increase in firmness that can be related to ice formation and subsequent~~ly~~ protein dehydration, as reported by other authors for different cheeses (Alvarenga et al., ~~Canada, & Sousa, ~~2011; Diefes, Rizvi, & Bartsch, 1993; Reid & Yan, 2004). Considering its high moisture content, HM Mozzarella cheese can be sensitive to freezing,~~;~~ thus, the process must be closely controlled and tailored~~,~~ in terms of the freezing rate and methods (Alvarenga, Ferro, Almodôvar, Canada, & Sousa, 2013).

In this context, the objective of this work was to evaluate the effects of different freezing/thawing methods and processing conditions over HM Mozzarella cheese characteristics~~,~~ to find the best process parameters that can lead to optimal quality results.

2. Material and methods

2.1 Experimental design

Four 100 g batches of fresh~~,~~ HM Mozzarella cheese~~ of 100 g~~ were kindly provided by Alival S.p.a. (Nuova Castelli S.p.a. RE, Italy). The ~~C~~cheeses used for the study were produced ~~in ~~on different days in a two-months period ~~according to the usual manufacturing method (Francolino et al., Locci, Ghiglietti, Iezzi, & Mucchetti, 2010)~~. Cheeses were manufactured using standard~~ized~~ cow~~'s~~ milk (3.30 g/100 g protein, 3.50 g/100 g fat)~~; milk was ~~pasteurized at 74 °C for 25 s; 1.2 g/100 g of citric acid and

Microbial rennet were added to start milk coagulation. After cheese curd stretching with salted boiling water, cheeses were moulded ~~as~~ into 100- g individual spheroidal shapes and cooled by immersion in~~to~~ tap water. Each ~~P~~product's final gross composition was 17.0 g of protein, 17.0 g of fat, 1.0 g of lactose and 0.4 g of NaCl. Cheeses were individually packaged into polyethylene bags containing 100 g of covering liquid (0.4 g/100 g~~% w/w~~ NaCl) and then were kept at 4 ± 1 °C for 5 d~~ays~~ before being frozen. For each manufacturing batch, 91 cheeses were considered (**Fig. 1**).

Samples were frozen by applying two freezing/thawing methods (***M***).~~:~~ ~~c~~Cheeses were frozen with (***M**_w_*) or without (***M**_d_*) covering liquid using an air blast freezer (MF 25.1, Irinox, TV, Italy) until a temperature of -20 °C in the core of the product was reached. M_d-treatments were separated from the covering liquid before freezing, while M_w-treatments were unpackaged and poured into truncated cone-shaped~~,~~ polypropylene containers (r_1 = 10 cm r_2 = 8 cm, height = 9 cm) with their original covering liquid.

Three freezing conditions (***Fc***), governed by the air temperature and velocity in~~to~~ the freezing chamber, were applied, as reported in **Fig. 1**~~: **C1)** -40 °C, 4.1 ± 0.6 m/s; **C2)** -30 °C, 2.5 ± 0.4 m/s; **C3)** -25 °C, 1.3 ± 0.2 m/s~~. Cheeses were stored at -18 °C in~~to~~ a freezer for a maximum of 10 d~~ays~~, and then were thawed by applying two thawing conditions (**Fig. 1**) ~~(***Tc***): **S1)** +4 °C, 1.3 ± 0.2 m/s; **S2)** +4 °C, 4.1 ± 0.6 m/s~~. The temperature of a cheese sample per freezing/thawing cycle was monitored in the centre and outer part of the cheese using ~~thermocouples~~ K-type thermocouples (Ni/Al–Ni/Cr) connected to a multimeter (mod. MV100, Yokogawa Electric Corporation, Tokyo, Japan). As HM Mozzarella cheese is characterized by its~~a~~ high moisture content, rapid drying occurs if the cheese remain separated from its covering liquid; for this reason, a~~A~~fter thawing, M_d-treated~~ments~~ cheeses were immersed into a freshly prepared covering liquid.~~;~~ A~~a~~ll samples were stored at 4 °C for 1 d~~ay~~ before analysis.

Trials were performed according to a completely randomized block design. For each batch, fourteen M_d and M_w-cheeses were frozen for each *Fc* (**Fig. 1**) in two separate freezing runs.; fFor each *Fc*, the group of frozen cheeses was divided, and 7 cheeses were thawed for each *Tc*. For every batch, measurements were also performed on the fresh, non-frozen cheese that was considered as the control, the same dayay of the treatments' freezing. Before analyses, samples were equilibrated in a climate chamber (mod. ICH 256L, Memmert, Schwabach, Germany) at 25 °C for 1 h.

2.2 Physicochemical analyses

Changes in weight caused by the processes were assessed by a laboratory scale (mod. BCE 5200, Orma, Milan, Italy) with an accuracy of ± 0.01 g. Cheeses were weighted before, and after freezing and subsequently again after the overnight period in a covering liquid after thawing. Changes of to the weight were expressed as percentage changes of from the original weight.

The Mmoisture Content (**MC**) was measured according to the IDF standard method (1982) by sampling and mixing a whole cheese and performing the analysis in triplicate by the oven drying method at 102 °C until constant weight was reached.

Expressible serum (**ES**) of Mozzarella cheese was measured in triplicate by centrifuging 30 g of each sample that were was previously cut from a whole cheese in 1-cm cubes, at 12,500 g per 75 min in 50 mlL tubes using a benchtop centrifuge (mod. 5810R, Eppendorf, Hamburg, Germany) (**ES_{CT}%**), according to Guo & Kindstedt (1995). After centrifugation, ES_{CT}% was measured subsequently after fat layer removal. ES was also measured in quintuplicate by weighting the amount of serum separated after a tTexture Pprofile Aanalysis (TPA) double compression test (**ES_{TPA}%**). ES_{TPA}% was determined as the weight lost after the compressioncompressing of the sample, similarly to Riebroy, Benjakul, & Visessanguan (2008). The inner part of one whole cheese Sample was cut into five cubes a cubic shape (with 15 mm sides) using a knife.,; cCubes were placed between double layers of filter papers (type

11106, Sartorius, Goettingen, Germany) and subjected to ~~a~~ double compression, as reported in section 2.4. ES_{TPA}% and ES_{CT}% were calculated as the ratio of ~~the~~ apparent expressible serum (ES_{app}) weigh~~t~~ed using an analytical scale (mod. AR 2140, Ohaus Corporation, New Jersey, USA) to the MC, according to the following equations (1) and (2):

$$ES_{TPA}\% = \frac{ES_{TPA\,app}}{MC} \times 100 \tag{1}$$

$$ES_{CT}\% = \frac{ES_{CT\,app}}{MC} \times 100 \tag{2}$$

The e~~E~~lectrical conductivity of ES_{CT}% was measured with a Portamess conductometer (mod. 913, Knick Elektronische, Berlin, Germany) and a TetraCon 325 probe (WTW Xylem Analytics, Weilheim, Germany) having a cell constant (K) of 0.475/cm~~$^{-1}$~~.

The ~~Color~~colour of the inner and outer parts of the cheese ~~was~~ were measured using a CR-2600d spectrophotometer (Minolta Co., Osaka, Japan) equipped with a D65 ~~illuminant~~light. ~~Considering t~~The CIE L*a*b* ~~color~~colour space~~-~~, the lightness of the ~~color~~colour (L*, from 100 ~~of~~ for white to 0 ~~of~~ for black), redness (a*, from +120 ~~of~~ for red to -120 ~~of~~ for green), yellowness (b*, from +120 ~~of~~ for yellow to -120 ~~of~~ for blue) were measured in quintuplicate.

2.3 Rheological analysis

Frequency sweep tests were performed using an ARES rheometer (TA instruments, New Castle, USA) equipped with a 25 mm parallel plate geometry according to Alinovi, Cordioli, et al., (2018~~a~~) with slight modifications. Disk-shaped samples (thickness 4-5 mm, diameter 30 mm) were portioned from the centr~~e~~re of Mozzarella cheese using a slicer and a borer. Sandpaper was applied to the plates to eliminate sample slippage. A solvent trap was used to minimize sample drying during analysis. The temperature during the analysis was set at 25 °C.

Measurements were performed at a constant strain of 0.05~~ ~~% that was into the linear viscoelastic region of the fresh and frozen/thawed cheeses. The ~~F~~frequency~~-~~ dependence of the storage modulus (G'), loss modulus (G'') and complex viscosity (η^*) were evaluated using power-laws equations (3), (4) and (5) (Yilmaz et al., 2016):

$$G' = k'(f)^{n'} \quad (3)$$

$$G'' = k''(f)^{n''} \quad (4)$$

$$\eta^* = k^*(f)^{n^*-1} \quad (5)$$

Measurements were performed in quadruplicate.

2.4 Textural analysis

Cheese textural properties were measured at room temperature using a TA.XT2plus texture ~~analyzer~~analyser (Stable Micro Systems, Godalming, UK). Sample preparation was the same as used to measure the ES_{TPA}% (section 2.2). A TPA double compression test was performed using a stainless-steel cylindrical probe with a diameter of 30 mm.~~;~~ ~~a~~A crosshead speed of 1.5 mm/s was applied to compress the cube samples (15 mm side) to 60~~ ~~% strain. The textural parameters considered were hardness (N), cohesiveness, springiness and gumminess (N).

2.5 Sensory analysis

Sensory descriptive analysis was performed by eight ~~panelists~~panellists (5 males, 3 females). A reduced list of cheese descriptors (**Table 1**) from the list ~~of~~ from (Pagliarini, Monteleone, & Wakeling, (1997) was considered. ~~Panelists~~Panellists were trained by performing 8 training sessions of 1 h~~our~~ to evaluate each descriptor according to three or more reference samples. Scores of the reference samples were adjusted and fixed according to ~~panelists'~~panellists' comments and opinions. The intensity of every Mozzarella sensory descriptor was evaluated between 1 (absence of the attribute) and 9 (extreme intensity of the attribute). After removing the skin, one and a half cCheesess werewere was portioned in 10 mm cubes (with 10 mm sides) for taste and aroma evaluation, while the remaining a half portion of the cheeses was used for visual evaluation. Analyses were performed in duplicate by each panel member, by evaluating tTwo of the four batches of cheese were assessed by the panel group.

2.6 Statistical analysis

To evaluate the main effects of the freezing/thawing method (M_i, i=1, 2), freezing conditions (Fc_k, k=1, 2, 3) and thawing conditions (Tc_l, l=1, 2), and of their interactions for every measured parameters, split-split plot ANOVA models were created using PRC GLM of SAS (SAS Inst. Inc., NC, USA) according to (Alinovi, Rinaldi, & Mucchetti, (2018b). Batches of cheese (B_j, j=1, 2, 3, 4) was were used as the blocking factor of the models (equation 6):

$$Y_{ijkl} = \mu + M_i + B_j + \delta_{ij} + Fc_k + (Fc \times M)_{ik} + \gamma_{ijk} + Tc_l + (M \times Tc)_{il} + (Fc \times Tc)_{kl} + (Fc \times Tc \times M)_{ikl} + \varepsilon_{ijkl} \qquad (6)$$

wWhere δ_{ij}, γ_{ijk} and ε_{ijkl} are the main plot and the two subplot error terms, respectively; and Y_{ijkl} is the selected response variable. Multiple comparisons (LSD adjustment) were performed among means when significant effects were found and to compare frozen/thawed and control cheeses.

To perform a classification of cheeses based on their characteristics, Pprincipal component analysis (PCA) was carried out using normalized variables on two of the four cheese batches. Only important cheese variables were included into the final model, based on their contribution in to the definition of PCs calculated from their loadings. Hierarchical cluster analysis (**HCA**) was carried out on PCs to highlight possible groups of samples based on their PCs scores;. Cclustering was performed considering Euclidean distances and Ward's method. Pearson's correlation coefficients (r) were also calculated to find relations among evaluated variables. Multivariate analyses were performed using SPSS v.25 (IBM, Armonk, USA).

3. Results and discussion

3.1 Physicochemical properties

The freezing/thawing methods (*M*) caused ~~a~~ significant (P < 0.05) weight changes ~~of~~ in the cheese (**Table 2**). M_w-Mozzarella cheeses showed a strong increase ~~of~~ in weight (+6.~~9 %~~9%), while M_d-Mozzarella cheeses did not show a strong variation of their average weight (100.~~2 %~~2%) ~~because of the processes~~ (**Fig.~~ure~~ ~~2~~1A**). Drip losses, moisture evaporation and sublimation during freezing (Delgado & Sun, 200~~0~~1) caused a weight decrease for M_d-treatments (-2.~~0 %~~0%), ~~that~~ which was balanced by subsequent~~ly~~ rehydration when the cheese was immersed and stored overnight in the covering liquid after thawing. ~~On the contrary,~~ In contrast, M_w-treatments were not affected by weight losses during freezing, thanks to the covering liquid that acts as a glaze (Jaczynski, Tahergorabi, Hunt, & Park, 2016).~~;~~ ~~d~~During thawing, M_w-cheeses absorbed water from the covering liquid and increased their weight.

Accordingly, a significant MC variation (P < 0.05) caused by freezing/thawing was associated ~~to~~ with these weight changes. M_w-Mozzarella cheeses showed higher MC than the control and M_d-treatments~~, respectively~~ (**Fig.~~ure~~ ~~2~~1B**). ~~On the contrary,~~ In contrast, ~~both~~ neither *Fc* ~~and~~ nor *Tc* ~~did not highlight~~ had statistical significance ~~neither~~ in terms of weight variation ~~nor of~~or MC (P > 0.05). The w~~W~~ater absorption of M_w-cheeses can be possibly due to ~~the higher structural damage caused by~~ the longer freezing (C1: 44.7 ± 3.1 min, C3: 67.3 ± 3.4 min for M_d-cheeses, C1: 116.2 ± 9.1 min, C3: 157.2 ± 7.6 min for M_w-cheeses) and thawing times (S1: 180.1 ± 11.9 min, S2: 309.0 ± 17.8 min for M_d-cheeses, S1: 376.0 ± 84.5 min, S2: 593.0 ± 69.0 min for M_w-cheeses) than for M_d-cheeses. It is well known that lower freezing rates can promote the formation of ice crystals ~~of~~ with bigger dimensions that damage the cheese structure. The increased~~ of~~ volume of water fraction ~~Density,~~ ~~variations~~ associated ~~to~~ with the formation of ice crystals~~,~~ forces the casein fibr~~er~~es to be in close contact and promotes the formation of bigger serum channels (Bertola et al.~~, Califano, Bevilacqua, & Zaritzky~~, 1996; Reid & Yan, 2004). These channels can be responsible for the latter absorption of free water from the covering liquid mediated by capillary forces and by the concentration's gradient of water-soluble molecules (~~e.g.~~ e.g., organic acids, salts, etc.) during the thawing process. During thawing, ice first~~ly~~ melts in the covering liquid, generating a zone with a higher temperature and a lower concentration of solutes, promoting the diffusion of water from this zone to the higher solute concentration zone represented by the frozen cheese. In this context, ~~a~~ the longer thawing time associated ~~to~~ with M_w-cheeses~~, that is~~ caused by the asymmetry of thermal food properties~~,~~ can improve water absorption (Gonzalez-Sanguinetti, Anon, & Calvelo, 1985). Moreover, changes ~~of~~ to the colloidal calcium phosphate content induced by the increasing ionic strength of the medium during freezing (Kljajevic, et al., 2016) can reduce protein-protein interactions and ~~consequently~~ increase protein hydration (Faccia et al., 2013; Guinee, Feeney, Auty, & Fox, 2002).

The effect of the freezing/thawing processes also led to different results ~~also~~ in terms of ES. $ES_{CT}\%$ showed higher values than $ES_{TPA}\%$, as the amount of mechanical stress ~~impressed to~~on the samples ~~by~~ during centrifugation was strongly higher. $ES_{CT}\%$ (**Fig.~~ure~~ 2~~1~~C**) and $ES_{TPA}\%$ were higher for M_w than for the control and M_d-treatments~~, respectively~~ ($P < 0.05$). Frozen M_w-cheeses showed a lower ~~W~~water ~~H~~holding ~~C~~capacity (WHC), probably ~~caused by~~because of the minor interactions between caseins and the additional water absorbed from the covering liquid.~~,~~ Related~~consequently~~ to the damage caused by the formation and growth of ice crystals and the subsequently rearrangement of water into the product,~~;~~ interactions between water and caseins can be modulated by a series of factors, such as the pH and colloidal calcium content, that regulate the hydrophilic and hydrophobic interactions (Faccia, Gambacorta, Natrella, & Caponio, 2019; Faccia et al., 2013) and consequently can have an effect on the tertiary and quaternary casein structure. ~~In facts,~~ F~~f~~reezing can promote casein dehydration phenomena (Reid & Yan, 2004) and an increase of water mobility (Kuo et al.~~, Anderson, &~~

Gunasekaran, 2003), contributing to the increase of serum separated by the application of physical forces. Also,Additionally, *Fc* showed awas significantly main effectdependant on on ES_{CT}% (P < 0.05), as the amount of ES separated by the centrifuge method was directly related to the freezing rate, as because longer freezing times can promote higher degrees of freezing damage.

Serum collected from M_w-cheeses showed a higher conductivity than from control and M_d, respectively (**Fig.ure 21D**). The lowest conductivity of came from M_d serum, which can be explained fromas M_d-samples were being immersed in a freshly prepared covering liquid during the overnight storage after thawing. As the new covering liquid had the same initial composition of the original one, the decrease of conductivity can be caused by salts and organic acids diffusingon from the cheese to covering liquid during the refrigerated storage before the freezing-thawing processes (Ghiglietti et al., 2004). On the contrary, in the case of M_w treatments, as the covering liquid was always the same, an increase in electrical conductivity of ES compared to the control can be related to protein damage phenomena: electrically charged peptides, amino acids or organic salts (Mucchetti, Gatti, & Neviani, 1994) can be deployed by the casein matrix and be found in ES.

ColorColour coordinates (L^*, a^*, b^* values) did not exhibit any modification (P > 0.05) for all the factors evaluated (**Table 2**). In general, Mozzarella cheese exhibited a high lightness and a dominant yellowish colorcolour, that which were respectively higher and lower in the externally than in the inner part of the cheese (L^*ext = 93.5 ± 0.5, L^*int = 91.8 ± 0.5; b^*ext = 12.9 ± 1.56, b^*int = 16.0 ± 1.2). and that isThis was related to the measured higher MC of the outer part (results not shown), as differences in lightness can be caused by different amounts of free water droplets on the analyzedanalysed surface (Sánchez-Macías et al., 2010).

3.2 Rheological properties

Frequency-dependence curves of dynamic moduli (**Fig~~.ure~~ 3~~2~~A, B**) highlighted the predominance of the elastic ~~behavior~~behaviour in all the ~~analyzed~~analysed cheeses, as G' was higher than G'' in the whole frequency range. Both G' and G'' were linearly dependent ~~to~~ on the applied frequency variation in a log-log scale. Reported power law equations (equations 3, 4, 5) fitted ~~well~~ the experimental rheological data well as the coefficients of determination (R^2) were higher than 0.97.

In the case of G', the power law coefficient k' showed significant differences between ~~the~~ cheeses with ~~a~~ different freezing/thawing methods (*M*) ($P < 0.05$), despite the differences among the batches (**Table 3**). M_d-treatments were characterized by a higher k' value than M_w-treatments (**Table 4**), ~~; on the contrary, the~~while control cheeses ~~was~~ were not significantly different from ~~both the~~either of the differently treated cheeses ($P > 0.05$). ~~A~~ The lower k' of M_w-treatments can be related to their higher MC if compared to the other samples, ~~that~~ which can be related to a higher extent of protein hydration caused by calcium phosphate depletion from caseins (Guinee et al., 2002). As water acts as a plasticizer in a viscoelastic system, an increase of its content can promote a decrease ~~of~~ in the elastic forces of the cheese body (Alberini et al.~~, Miccolo, & Rubiolo~~, 2015; Diefes et al.~~, Rizvi, & Bartsch~~, 1993). Moreover, a decrease ~~of the~~to k' can be partially explained by ~~a~~ the higher degree of freezing damage caused by the longer freezing~~,~~ and thawing rates. In accordance with the k' variations, differences in ~~terms of~~the η^* curves were highlighted (**Fig~~.ure~~ 3~~2~~D**), as it is also possible to observe from the k^* values (**Table 4**). Moreover, ~~also~~ k'' also followed the same trend described for k', despite the estimated P-value for the *M* factor ~~was~~ being at the limit of significance ($P = 0.050$). ~~On the contrary,~~ In contrast, no significant main effects ~~of~~ relating *Fc* and *Tc* ~~on~~ to k', k'' ~~and~~ or k^* were highlighted, probably because the freezing and~~/~~ thawing rates considered in this study did not cause a significant modification ~~of~~ to the rheological properties.

The frequency-~~-~~dependence of dynamic rheological parameters, ~~that~~ which can be estimated from n', n'' and n^* values (**Table 4**), was not influenced by the different process factors, ~~as~~ because the slope of the regression curves was not different (P > 0.05). ~~As a higher frequency dependence (higher n', n'' and n^* values) would indicate the presence or the formation of weak gels (Banville, Morin, Pouliot, & Britten, 2014), a lower structured cheese matrix that could originate from the application of the processes was not observed.~~

The tangent of the phase angle (tanδ) (**Fig.~~ure~~ 3~~2~~C**) confirmed the viscoelasticity of the samples, as it was always lower than 1. Tanδ did not showed differences related to the *M* factor. ~~On the contrary,~~ In contrast, control cheeses exhibited a significantly different tanδ curve than the frozen/thawed cheeses, ~~that~~ which can also be observed from the tanδ data reported at ~~the~~ a frequency of 1 Hz (**Table 4**)~~.;~~ ~~l~~The lower values of tanδ ~~of~~ for frozen/thawed cheeses is ~~can be~~ probably related to the caseins dehydration phenomena ~~as~~ already discussed in other studies (Alberini et al.~~, Miccolo, & Rubiolo~~, 2015; Alvarenga et al.~~, Canada, & Sousa~~, 2011)~~.;~~ D~~dD~~uring freezing, a rearrangement of the protein matrix may cause~~s~~ the formation of a more compact texture~~,~~ containing aggregates of casein that interact with each other and are intercalated by serum channels and fat clusters of bigger dimensions. In our case, the increased ~~of~~ rigidity seems to not ~~to~~ be ~~in relation with~~related to any studied factor ~~and~~ or with the different MC of the cheeses but is only ~~with~~ related to the application of any freezing ~~and~~ or thawing process (**Table 4**).

3.3 Textural and sensory properties

Texture analyses partially confirmed the rheological measurements, as M_w-cheeses had a significantly lower hardness and gumminess (P < 0.05) than M_d-treated~~ments~~ cheeses (**Table 3**). Moreover, as observed with the rheological analyses, control cheeses did not show ~~statistical difference~~significant differences ~~with both~~between the freezing/thawing treatments (**Table 5**). ~~Contrarily,~~The cohesiveness and springiness did not show differences related to any factor considered in the models (**Table 3**). However, ~~accordingly to tanδ,~~ the control cheese exhibited a significant difference (P < 0.05) with the frozen/thawed cheeses for both the cohesiveness and springiness values (**Table 3**).~~;~~ ~~i~~In the case of cohesiveness, the difference was independent ~~to~~ of the applied freezing/thawing process~~ applied~~, while in the case of springiness the control was different only with M_w-treatments (**Table 5**). Frozen/thawed ~~control~~ cheeses showed a ~~higher~~ lower cohesiveness and a ~~lower~~ higher springiness than ~~frozen~~ control cheeses~~,~~ ~~that~~. This can be related to changes in the moisture organization subsequent~~ly~~ to freezing~~,~~ because of the caseins dehydration phenomena and the formation of a more rigid, less plasticized structure, as the frozen/thawed cheeses had a slightly higher ability to recover their original configuration.

Concerning sensory evaluation, the panel group was not able to detect significant differences related to the freezing/thawing factors for most of the attributes considered (P > 0.05) (**Table 3**). ~~Sensory Differently from~~Contrary to the textural hardness, sensory hardness was not perceived as being different among the samples.~~;~~ ~~h~~However, ~~despite~~ as it highlighted a similar trend to textural hardness among the samples, (**Table 5**), ~~and~~ the sensory hardness was significantly (P < 0.01) but weakly correlated to the TPA hardness, gumminess and MC (r=0.48, 0.55 and -0.65, respectively).~~;~~ A~~a~~ccordingly, ~~also~~ saltiness was also not perceived as different despite the significantly different electrical conductivity of ES. The only sensory parameter ~~that highlighted a~~with significant ~~main effect~~differences related to the *M* factor was juiciness, ~~that~~ which showed a significantly lower score for M_d-treatments ~~rather~~ than control cheese and M_w-treatments. This observation can be related to the ES of the different cheeses, ~~that~~which was the lowest for M_d-cheeses.

3.4 Samples classification according to PCA and HCA

Multivariate statistics were considered ~~to have~~for an overview and a classification of the cheeses. ~~Of the totality of measured parameters, s~~Sixteen variables were selected from all the measured parameters (**Fig.~~ure~~ ~~4~~3B**). PCA generated five PCs that explained 80.47~~-~~% of the variance of the dataset. The first two PCs used for sample classification and visualization~~,~~ explained the largest amount of variance, ~~that was~~ at 28.8~~-~~% and 21.7~~-~~%~~, respectively~~. The relatively low variance explained by the model can be caused by batch variability issues as already reported for univariate analyses. However, M_d and M_w-cheeses were clearly classified considering the first two PCs (**Fig.~~ure~~ ~~4~~3A**).~~;~~ ~~a~~According to HCA, the control cheese was grouped with M_d-cheeses. ~~On the contrary,~~ In contrast, no classification of the samples based on the different levels of *Fc* and *Tc* was obtained. According to the samples classification, PC1 was mainly represented by negative loadings of textural, rheological and sensory parameters such as TPA and sensory hardness, gumminess, k', and k^* (**Fig.~~ure~~ ~~4~~3B**).~~;~~ ~~coherently,~~ ~~p~~Parameters such as the weight change, MC, tanδ, ES_{CT}%, ES_{TPA}%, and juiciness showed strong positive loadings on PC1. Interestingly, the sensory and their respective physical parameters showed similar loadings as they were significantly correlated ($P < 0.05$) (saltiness and electrical conductivity, $r=0.59$; sensory and textural hardness, $r=0.48$); the only exception was represented by sensory juiciness, ~~that~~ which was not correlated with ES but was significantly correlated with weight change ($r=0.60$) and MC ($r=0.44$). M_w compared to control and M_d-cheeses~~,~~ ~~were~~ was classified according to their lower structural and textural properties and the higher MC and ES.

4. Conclusions

Frozen HM Mozzarella cheese is a product increasingly present ~~both~~ in both retail and ingredient markets~~,~~ because of its longer shelf-life. Its quality at consumption largely depends on the applied freezing method ~~applied~~. HM Mozzarella cheese characteristics were not affected by the freezing and thawing conditions evaluated in this study,~~; on the contrary~~ however, the presence or ~~the~~ absence of ~~the~~

a covering liquid during the processes influenced some of the Mozzarella cheese physicochemical, rheological and sensory characteristics. Cheeses processed with a covering liquid were characterized by longer freezing times and showed water absorption phenomena during thawing.~~;~~ ~~t~~Thus, these cheeses were characterized by freezing-induced modifications. ~~On the contrary,~~ In contrast, cheeses frozen/thawed without a covering liquid were more similar to the control cheeses. As a higher moisture content in ~~a~~ fresh cheese is often associated ~~to a~~with lower storage stability, the results of this study highlighted that freezing and ~~/~~thawing without a covering liquid followed by a rehydration step should be the preferred method to obtain the best results in terms of quality.

~~Notes~~

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Figure Captions

Figure 1. Schematic representation of the ~~applied~~ experimental design to study Mozzarella cheese freezing and thawing processes.

Figure 2. Representation of weight change (A), moisture content (B), expressible serum measured by the centrifuge method (ES_{CT}%) (C), and electrical conductivity (D) of fresh, non-frozen Mozzarella cheese (control) and of frozen Mozzarella cheese without (M_d) and with (M_w) a covering liquid. Columns with different letters are significantly different ($P < 0.05$).

Figure 3. Frequency-dependent~~ce~~ dynamic curves of the storage modulus (G') (A), loss modulus (G'') (B), tangent of the phase angle (tanδ) (C), and complex viscosity (η^*) (D) of Mozzarella cheeses.~~;~~ (□) ~~of~~ ~~f~~Fresh, non-frozen Mozzarella cheese (control),~~;~~ (◆) ~~and~~ ~~of~~ frozen Mozzarella cheese without (M_d),~~;~~ and (●) frozen Mozzarella cheese ~~and~~ with (M_w) a covering liquid.

Figure 4. Principal component analysis (PCA) score and loading plots of the first two principal components of the model (PC1, PC2). Principal components were calculated considering a reduced list of chemical, physical, rheological and sensory parameters,~~;~~ and cheese samples were clustered according to the results obtained from the hierarchical cluster analysis based on Euclidean distances and Ward's method. Samples were indicated and labelled according to the different freezing/thawing methods (Control (□): non-frozen cheese; M_d (◆): cheese frozen without covering liquid; M_w (●): cheese frozen with covering liquid), freezing conditions (C1: -40 °C, 4.1 ± 0.6 m/s; C2: -30 °C, 2.5 ± 0.4 m/s; C3: -25 °C, 1.3 ± 0.2 m/s) and thawing conditions (S1: +4 °C, 1.3 ± 0.2 m/s; S2: +4 °C, 4.1 ± 0.6 m/s).

Table 1

Table 1. Sensory descriptors, meaning and reference samples (with relative scores in brackets) considered during Mozzarella cheese sensory evaluation.

Sensory descriptor	*Meaning*	Reference samples (score)
Texture and taste		
Sensory hardness	*Strength required to compress but not destroy a sample between the molars during the first bite*	Santa Lucia ciliegie-type Mozzarella cheese (1), Conad bocconcini-type Mozzarella cheese (3), Conad 400g-pizza cheese (7), Conad 250g-pizza cheese (9)
Juiciness	*Degree of humidity perceived in the mouth during mastication*	Conad 250g-pizza cheese (1), Conad 400g-pizza cheese (4), Santa Lucia ciliegie-type Mozzarella cheese (6), Vallelata Fiordilatte-type Mozzarella cheese (9)
Acidity	*One of the basic tastes that is perceived on the central part of the tongue*	Conad UHT skimmed milk (0), Conad UHT skimmed milk with 25% of Yomo plain skimmed yogurt (5), onad UHT skimmed milk with 50% of Yomo plain skimmed yogurt (9)
Saltiness	*One of the basic tastes that is perceived on the tip and side parts of the tongue*	Alival citric Mozzarella cheese (1), Alival citric Mozzarella cheese with 0.3% of salt added (5), Alival citric Mozzarella cheese with 0.6% of salt added (9)
Appearance		
Whiteness	*White colour perceived by the human eye*	Conad 400g-pizza cheese (1), Alival citric Mozzarella cheese (3), Alival lactic Mozzarella cheese (9)
Translucency	*Degree of wetness of cheese inner surface*	Conad 400g-pizza cheese (1), Alival citric Mozzarella cheese (5), Alival lactic Mozzarella cheese (9)
Paste smoothness	*Inversely related to the abundance of holes and cracks in the inner part of the cheese*	Alival 5-d old citric Mozzarella cheese (1), Alival 15-d old citric Mozzarella cheese (5), Alival 25-d old citric Mozzarella cheese (7)
Surface smoothness	*Inversely related to the amount of imperfections on the surface of the cheese*	Alival 5-d old citric Mozzarella cheese (1), Alival 15-d old citric Mozzarella cheese (5), Alival 25-d old citric Mozzarella cheese (7)

Table 2

Table 2. P values obtained from split-split plot ANOVA models of the evaluated physical and chemical parameters for each of the factors considered: Freezing/thawing method (*M*), freezing condition (*Fc*), thawing condition (*Tc*). Measured parameters were the relative weight variation of the cheese (WGT), moisture content (MC), expressible serum measured with centrifuge method (ES_{CT}) and TPA (ES_{TPA}), electrical conductivity of expressible serum (COND) and external and internal colorimetric coordinates (L^*ext, a^*ext, b^*ext and L^*int, a^* int, b^* int).

Parameter	WGT	MC	ES_{CT}	ES_{TPA}	COND	L^*ext	a^*ext	b^*ext	L^*int	a^*int	b^*int
Batch (Block)	0.967	0.031	0.057	0.028	0.605	0.202	0.006	0.023	0.034	0.001	0.004
Freezing/Thawing Method (*M*)	0.004	0.042	0.023	0.054	0.009	0.694	0.077	0.930	0.715	0.110	0.306
B x *M*											
Freezing condition (*Fc*)	0.349	0.101	0.044	0.703	0.104	0.501	0.135	0.543	0.615	0.134	0.431
M × *Fc*	0.439	0.606	0.831	0.752	0.003	0.873	0.182	0.586	0.319	0.445	0.613
B x *M* x *Fc*											
Thawing condition (*Tc*)	0.087	0.728	0.328	0.000	0.559	0.597	0.939	0.971	0.084	0.411	0.779
Tc × *M*	0.207	0.544	0.634	0.485	0.988	0.037	0.755	0.080	0.213	0.142	0.814
Tc × *Fc*	0.416	0.128	0.543	0.501	0.074	0.190	0.414	0.326	0.161	0.547	0.082
M × *Fc* × *Tc*	0.103	0.680	0.688	0.888	0.763	0.593	0.219	0.666	0.913	0.080	0.186

Table 3

Table 3. P values obtained from split-split plot ANOVA models of the evaluated rheological, textural and sensory parameters for each of the factors considered: Freezing/thawing method (*M*), freezing condition (*Fc*), thawing condition (*Tc*). Measured parameters were power law regression parameters from storage modulus (k', n'), loss modulus (k'', n'') and complex viscosity (k^*, n^*); hardness (HAR TPA), cohesiveness (COH), gumminess (GUM), springiness (SPR) measured with TPA double compression; perceived whiteness (WHI), translucency (TRA), hardness (HAR sens), juiciness (JUI), paste and surface smoothness (PAS-SMO, SUR-SMO), acidity (ACI) and saltiness (SAL) from sensory evaluation.

Parameter	k'	k"	k^*	n'	n"	n^*	HAR TPA	COH	GUM	SPR	WHI	TRA	HAR sens	JUI	PAS-SMO	SUR-SMO	ACI	SAL
Batch (Block)	0.002	0.061	0.012	0.400	0.912	0.105	0.003	0.138	0.002	0.037	0.443	0.858	0.091	0.108	0.459	0.565	0.564	0.593
Freezing/Thawing Method (*M*)	0.013	0.050	0.024	0.899	0.420	0.349	0.048	0.175	0.028	0.176	0.564	0.352	0.188	0.024	0.176	0.204	0.472	0.236
B x *M*																		
Freezing condition (*Fc*)	0.338	0.250	0.772	0.967	0.900	0.159	0.989	0.289	0.925	0.131	0.519	0.073	0.284	0.278	0.486	0.241	0.077	0.264
M × *Fc*	0.036	0.796	0.406	0.894	0.125	0.300	0.934	0.265	0.951	0.124	0.556	0.024	0.137	0.968	0.274	0.846	0.215	0.069
B x *M* x *Fc*																		
Thawing condition (*Tc*)	0.438	0.075	0.822	0.048	0.733	0.949	0.476	0.797	0.546	0.927	0.537	0.340	0.463	0.017	0.230	0.059	0.143	0.688
Tc × *M*	0.996	0.485	0.271	0.625	0.708	0.469	0.500	0.844	0.506	0.458	0.919	0.411	0.606	0.826	0.852	0.578	0.512	0.691
Tc × *Fc*	0.318	0.491	0.835	0.580	0.905	0.520	0.861	0.064	0.690	0.391	0.431	0.658	0.894	0.299	0.546	0.284	0.941	0.262
M × *Fc* × *Tc*	0.504	0.504	0.137	0.435	0.104	0.906	0.999	0.484	0.954	0.977	0.986	0.529	0.589	0.035	0.947	0.153	0.744	0.748

Table 4

Table 4. Power law coefficients (mean ± standard deviation) derived from dynamic rheological curves of G', G'' and η^* of control Mozzarella cheese and Mozzarella cheese samples frozen and thawed with (M_w) and without (M_d) covering liquid.

Treatment	k' (Pa s)	k'' (Pa s)	k^* (Pa s)	n' (-)	n'' (-)	n^* (-)	$\tan\delta_{(1\ Hz)}$ (-)
Control	$9310^{ab} \pm 3952$	$3213^{ab} \pm 1289$	$1566^{ab} \pm 627$	0.1913 ± 0.0204	0.1787 ± 0.0051	0.1932 ± 0.0385	$0.3343^{a} \pm 0.0157$
M_d cheese	$9839^{a} \pm 3819$	$3860^{a} \pm 2076$	$1785^{a} \pm 532$	0.1815 ± 0.0106	0.1750 ± 0.0160	0.1918 ± 0.0143	$0.3106^{b} \pm 0.0193$
M_w cheese	$7897^{b} \pm 3715$	$2836^{b} \pm 1122$	$1344^{b} \pm 631$	0.1822 ± 0.0136	0.1791 ± 0.0116	0.1960 ± 0.0118	$0.3128^{b} \pm 0.0144$

[a-b] Mean values within a column with different superscript letters are significantly different ($P < 0.05$).

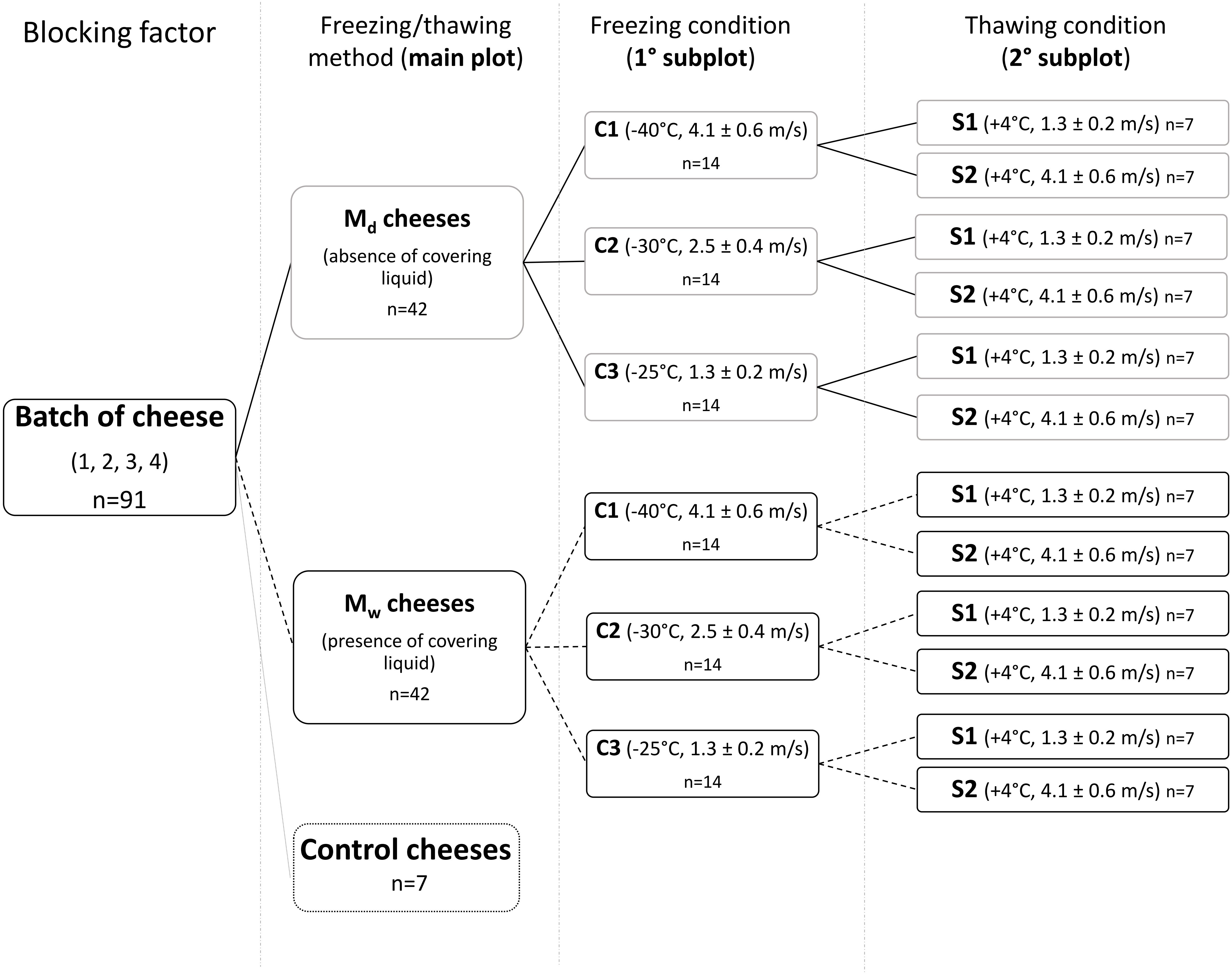
Table 5

Table 5. Textural and sensory parameters of control Mozzarella cheese and Mozzarella cheese samples frozen and thawed with (M_w) and without (M_d) covering liquid. Evaluated parameters were hardness (HAR tpa), cohesiveness (COH), gumminess (GUM), springiness (SPR) measured with TPA double compression; perceived whiteness (WHI), translucency (TRA), hardness (HAR sens), juiciness (JUI), paste and surface smoothness (PAS-SMO, SUR-SMO), acidity (ACI) and saltiness (SAL) from sensory evaluation.

Treatment	HAR TPA (N)	COH (-)	GUM (N)	SPR (-)	HAR SENS (-)	JUI (-)	ACI (-)	SAL (-)	WHI (-)	TRA (-)	PAS-SMO (-)	SUR-SMO (-)
Control	$9.12^{ab} \pm 3.93$	$0.65^{a} \pm 0.01$	$5.97^{ab} \pm 2.58$	$0.69^{b} \pm 0.03$	3.6 ± 0.6	$5.1^{a} \pm 1.2$	2.1 ± 0.1	2.8 ± 0.5	2.9 ± 0.5	5.2 ± 0.4	3.1 ± 0.9	4.2 ± 2.0
M_d	$10.07^{a} \pm 2.65$	$0.61^{b} \pm 0.02$	$6.19^{a} \pm 1.69$	$0.71^{ab} \pm 0.05$	4.2 ± 1.1	$3.9^{b} \pm 0.9$	1.8 ± 0.4	2.1 ± 0.4	2.7 ± 0.8	4.9 ± 1.1	3.8 ± 1.4	3.9 ± 0.6
M_w	$9.07^{b} \pm 2.76$	$0.60^{b} \pm 0.02$	$5.51^{b} \pm 1.78$	$0.73^{a} \pm 0.04$	3.5 ± 1.0	$5.4^{a} \pm 1.0$	2.1 ± 0.3	2.8 ± 0.6	2.5 ± 0.9	5.7 ± 1.0	3.1 ± 0.8	4.5 ± 1.2

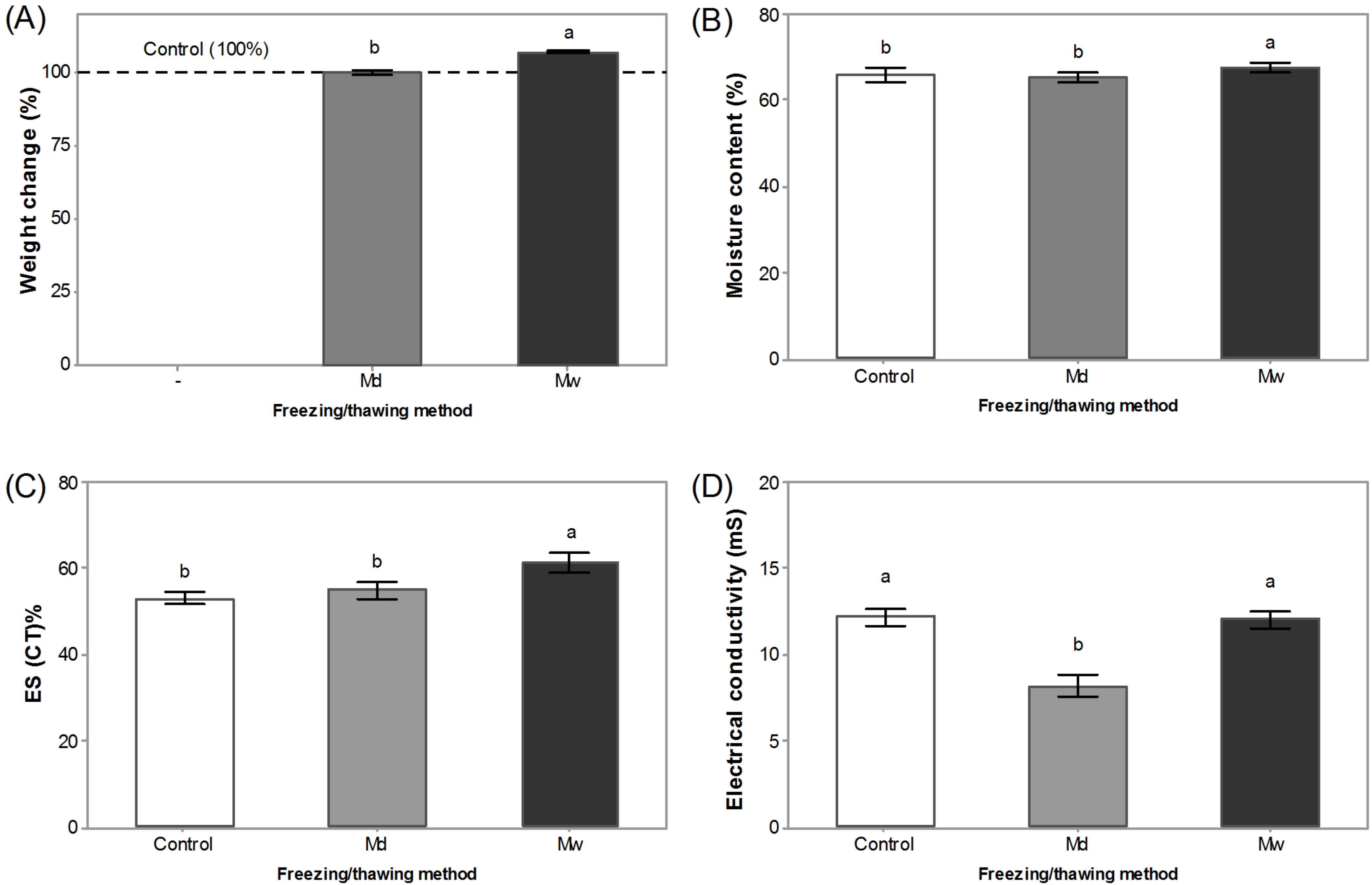
[a-b] Mean values within a column with different superscript letters are significantly different ($P < 0.05$).

Figure 1



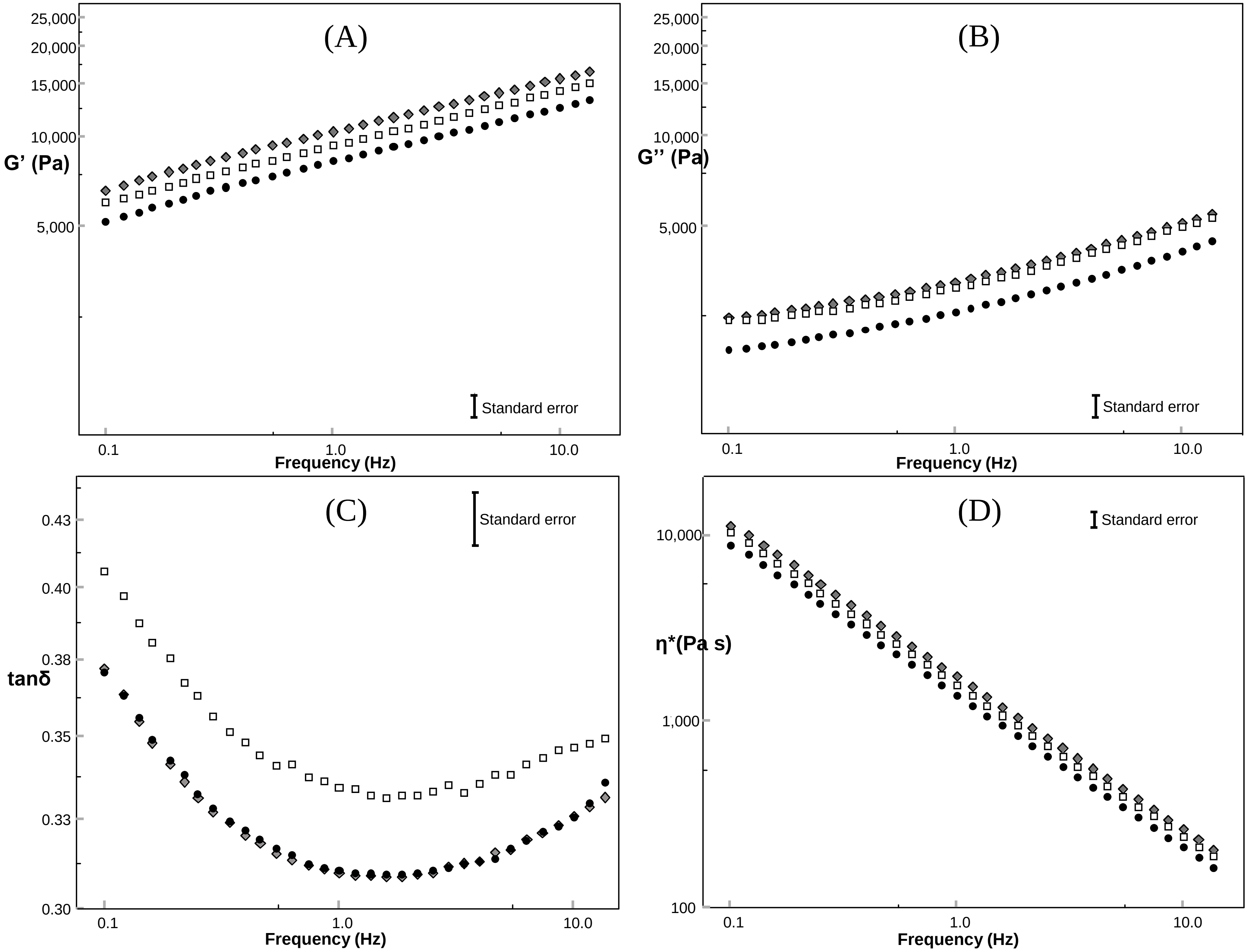
Blocking factor
Freezing/thawing method (main plot)
Freezing condition (1° subplot)
Thawing condition (2° subplot)
Batch of cheese
(1, 2, 3, 4)
n=91
M$_d$ cheeses
(absence of covering liquid)
n=42
M$_w$ cheeses
(presence of covering liquid)
n=42
Control cheeses
n=7
C1 (-40°C, 4.1 ± 0.6 m/s)
n=14
C2 (-30°C, 2.5 ± 0.4 m/s)
n=14
C3 (-25°C, 1.3 ± 0.2 m/s)
n=14
S1 (+4°C, 1.3 ± 0.2 m/s) n=7
S2 (+4°C, 4.1 ± 0.6 m/s) n=7

Figure 2



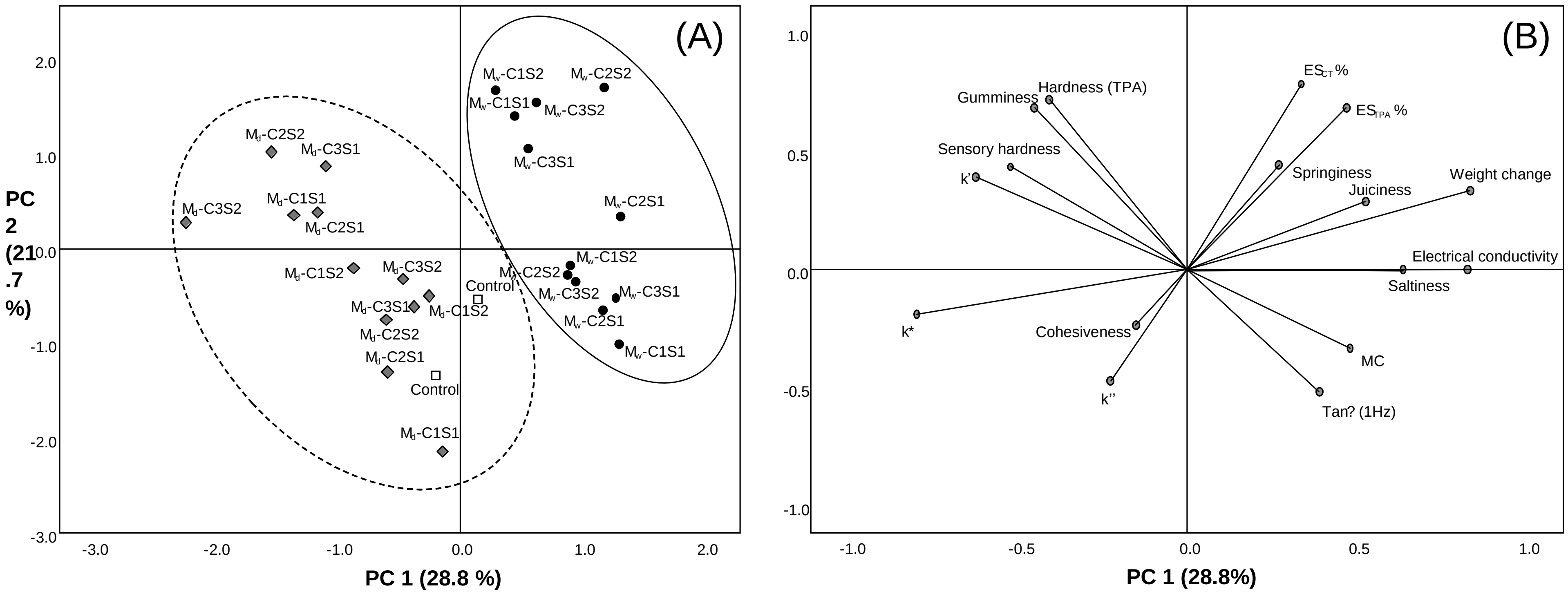
(A)
Control (100%)
b
a
Weight change (%)
100
75
50
25
0
-
Md
Mw
Freezing/thawing method
(B)
80
60
40
20
0
b
b
a
Moisture content (%)
Control
Md
Mw
Freezing/thawing method
(C)
80
60
40
20
0
b
b
a
ES (CT)%
Control
Md
Mw
Freezing/thawing method
(D)
20
15
10
5
0
a
b
a
Electrical conductivity (mS)
Control
Md
Mw
Freezing/thawing method

Figure 3



(A)
G' (Pa)
25,000
20,000
15,000
10,000
5,000
Standard error
0.1
1.0
10.0
Frequency (Hz)
(B)
G'' (Pa)
25,000
20,000
15,000
10,000
5,000
Standard error
0.1
1.0
10.0
Frequency (Hz)
(C)
tanδ
0.43
0.40
0.38
0.35
0.33
0.30
Standard error
0.1
1.0
10.0
Frequency (Hz)
(D)
η*(Pa s)
10,000
1,000
100
Standard error
0.1
1.0
10.0
Frequency (Hz)

Figure 4



(A)
Mw-C1S2
Mw-C2S2
Mw-C1S1
Mw-C3S2
Mw-C3S1
Mw-C2S1
Md-C2S2
Md-C3S1
Md-C1S1
Md-C3S2
Md-C2S1
Md-C1S2
Md-C3S2
Md-C3S1
Md-C1S2
Md-C2S2
Md-C2S1
Md-C1S1
Control
Control
Mw-C1S2
Mw-C2S2
Mw-C3S2
Mw-C3S1
Mw-C2S1
Mw-C1S1
PC 2 (21.7 %)
PC 1 (28.8 %)
(B)
ESCT %
ESTPA %
Gumminess
Hardness (TPA)
Sensory hardness
k'
Springiness
Juiciness
Weight change
Electrical conductivity
Saltiness
k*
Cohesiveness
MC
k''
Tan? (1Hz)
PC 1 (28.8%)

Supplementary Material

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***Credit Author Statement**

Author Contributions

Alinovi Marcello: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing - original draft; **Germano Mucchetti:** Conceptualization, Resources, Writing- review and editing, Supervision.

*Conflict of Interest Form

AUTHOR DECLARATION

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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