



Original Research Article

Predicting chemical shelf life of mozzarella cheese submitted to irregular refrigeration practices by Nuclear Magnetic Resonance spectroscopy and statistical analysis



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ARTICLE INFO

Keywords:

Irregular refrigeration
Mozzarella cheese
Degradation marker
Chemometrics
Principal response curve
NMR

ABSTRACT

The complex mixture found in mozzarella cheese favors microorganism proliferation that causes spoilage. Appropriate refrigeration during storage is one step in the supply chain that is critical to guarantee food quality. However, the impact of the absence of night-time refrigeration, practiced by some commercial establishments, in mozzarella chemical profile has not yet been investigated. To this end, during the evening the simulation of improper refrigeration was carried out using an uncovered Styrofoam box containing ice, allowing the system to reach the thermal balance with the environment. The key degradation compounds produced in mozzarella cheese from simulated inadequate refrigeration were identified and quantified using NMR (Nuclear Magnetic Resonance). While the composition and physicochemical analyses were unable to distinguish refrigeration practices, the NMR data revealed that the succinate contents changed significantly between different refrigeration treatments. At the labelled expiry date, inadequate refrigeration had led to an increase in the succinate content of more than 600%. The results suggested a reduction of 4 weeks of the chemical shelf life. Together, NMR and Principal Response Curve analysis are powerful tools to identify mozzarella cheese submitted to different refrigeration conditions.

1. Introduction

Mozzarella cheese is an amply produced food worldwide, with diversified adaptations in the manufacturing process, such as the milk type used (Marino et al., 2019). Due to the commercial and economic importance of cheese, the Food and Agriculture Organization of the United Nations (FAO), among other specific agencies, such as the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA), defined criteria and classifications for cheese (Codex Alimentarius Commission, 2011; MAPA, 2006). In addition to the internal market, importation and exportation also have rigorous criteria for food quality control, mainly because of the high microorganism levels harmful to human health that may be present in products of animal origin (Neill, 2008).

As the mozzarella cheese production and consumption in Brazil and worldwide has increased, concerns with its quality have arisen. Many safety aspects of mozzarella cheese have been extensively investigated in the literature, as the shelf life extension of lacto-fermented mozzarella

through sustainable process operations (Branca, 2018), the behavior of *Listeria monocytogenes* during the traditional manufacture of water-buffalo Mozzarella cheese (Villani et al., 1996) and the effect of the time and temperature of stretching as critical control points for *Listeria monocytogenes* during production of mozzarella cheese (Kim et al., 1998). In this regard, metabolomics or foodomics have contributed much to food quality and identity, by providing a profile of the metabolites or components of a given biological system or food, respectively (Fiehn, 2002). Nuclear Magnetic Resonance (NMR) spectroscopy has a key role in this context, and regarding aging, degradation or cheese composition studies, several examples using NMR are found in the literature. Among which we can cite: the quality and traceability assessment of mozzarella cheese from Campania buffalo milk (Mazzei and Piccolo, 2012), modifications of the volatile and nonvolatile metabolome of goat cheese due non-starter lactic acid bacteria (Battelli et al., 2019), determination of different salts and their proportions as flavor enhancers on in Prato cheese (Silva et al., 2018), evaluation of packing conditions in the soft cheeses degradation of soft cheese

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<https://doi.org/10.1016/j.jfca.2021.104229>

Received 1 July 2021; Received in revised form 6 October 2021; Accepted 11 October 2021

Available online 21 October 2021

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(Lamanna et al., 2008) and analysis of In vitro digestibility of cyclopropane fatty acids in Grana Padano cheese (Lolli et al., 2018). However, the use of this approach to investigate how food quality is affected by different refrigeration conditions remains a challenge to be explored.

As cheese is high complex mixture and it has a high water content it tends to be more perishable and vulnerable to spoilage (Marino et al., 2019), since the wide range of metabolites favor microorganisms growth (Lacivita et al., 2018). Furthermore, depending on handling frequency and its sanitary conditions, there is a high risk of contamination during the cheesemaking process that may affect the final product and lead to pathogenic bacteria and metabolites (El-Gazzar and Marth, 1992), with adverse effects on consumer health. Hence, appropriate cheese refrigeration is crucial to ensure its quality (James and James, 2013; Heard and Shelie, 2016). Nevertheless, the deliberate shut-down of overnight refrigeration shelves leading to improper cooling of potentially hazardous foods has made worldwide news, as an example, a supermarket in the UK that left foods without being properly refrigerated (Riley, 2018). A similar situation occurred in Brazil, resulting in the interdiction of a commercial food distributor (Rabelo, 2012). Ndraha et al. (2017) emphasized the impact of inadequate refrigeration practice on food quality, specifically on meat, dairy, fish, fruit, and vegetable products, and the consequent endangerment to consumer health. In this context, it is crucial to identify foods that are not fulfilling the quality criteria for suitable storage.

Although many aspects of the shelf life of mozzarella cheese have been investigated in the literature (Altieri et al., 2005; Conte et al., 2007; Faccia et al., 2019; Laurienzo et al., 2008), there is still a lack of a study focused on the effect of inadequate refrigeration practice. So, based on the need of establishing chemical markers and their levels for characterization of inadequate refrigeration practices of mozzarella cheese, identification and quantification of key mozzarella cheese degradation compounds produced from simulated inadequate refrigeration were performed here using NMR spectroscopy. Composition and physicochemical analyses commonly used in cheese quality control, such as pH, milk fat, and total protein, were also undertaken in addition to the NMR data (da Silva et al., 2020; Møller et al., 2012; Margalho et al., 2021). Furthermore, a chemical shelf life reduction of mozzarella cheese caused by this undesirable practice was investigated, using NMR to compare marker levels between adequately and inadequately chilled products at the due expiry date.

2. Materials and methods

2.1. Samples

Fresh industrial low-moisture mozzarella cheese samples, prepared using starter culture method and made from whole cow's milk, from the same lot were purchased, the day after they were produced, at a local market. Twenty two vacuum packs of mozzarella cheese were acquired, each one weighting about 150 g, aiming to keep the same type of packaging, because of the importance of this factor to the chemical shelf life of food. Half of these packs were kept under refrigeration conditions at 8 °C, within the manufacturer's recommended temperature range (0–10 °C). The other half was kept refrigerated only during the day (12 h), and during the night (12 h) inadequate refrigeration was simulated by removing them from the refrigeration and allowing the system to reach thermal equilibrium with the environment, using an uncovered Styrofoam box containing ice, just as it would be in open refrigerated shelves commonly found at supermarket. In this last procedure, the room temperature was constantly monitored by a digital laser thermometer and ranged from 22 to 25 °C.

Although the interval between the mozzarella cheese production (September 24th) and the expiry date (November 24th), printed by the manufacturer, was of 8 weeks, the analyses were performed for 11 weeks in order to monitor the metabolic dynamics of the cheese even after the expiration date. Every week, two aliquots from each pack under

different refrigeration conditions were prepared and split for composition, physicochemical and NMR analyses. These aliquots were took from the middle and the outside of the cheese and then homogenized.

2.2. Sample preparation

Composition (milk fat, total protein), physicochemical (pH) and NMR analyses from the same aliquot were done weekly, on the same day, by the Brazilian Food Research Center (CPA), according to Regulation 68 of the MAPA (MAPA, 2006).

The chemicals used for the NMR analysis were acquired from Cambridge Isotope Laboratories (TEWKSBURY, MA): deuterium oxide with deuterium at 99.9% (d₂O) used as solvent, and 3-(trimethylsilyl)propionic-2,2,3,3-d₄ acid sodium salt (tsp-d₄) with deuterium at 98.0% used as internal standard.

The cheese pieces were cut, homogenized, and pulverized in a mortar using liquid nitrogen. To obtain the crude sample extract, the methodology proposed by Lamanna et al. (2008) was modified. Briefly, 1 g was transferred to a 15 mL Falcon tube together with 2 mL of D₂O and sonicated for 20 min, followed by centrifugation (2800 × g, 20 min) at 24 °C. After centrifugation, the mixture was separated in three phases: a solid phase at the bottom of the tube with a white color, a colorless liquid phase at the middle of the tube, and a less dense phase at the top of the tube with characteristics of fat.

Aliquots of 1.8 mL of the intermediate phase were collected and again centrifuged under the same previous conditions. Finally, 1.5 mL of the newly formed intermediate phase constituted the crude extract for NMR analysis. To obtain the NMR spectra, a 5-mm resonance tube was used, loading 200.0 µL of 0.1% (w/v) solution of TSP-d₄ in D₂O and 400.0 µL of crude extract. NMR analyses of the samples from both refrigeration conditions were performed weekly on the same day.

2.3. Experimental design of the NMR analysis

NMR spectra were acquired on a Bruker Avance III 500 spectrometer with an 11.75 T standard bore magnet and using a 5-mm triple resonance broadband inverse (TBI) probe with field gradient in the z-direction and a controlled probe temperature of 25 °C. For the ¹H NMR experiments with water suppression, some of the acquisition parameters were 65,536 points in the time domain (TD), pulse sequence noesypr1d (RD(CW)-90°-t-90°-MIX(CW)-t-90°-FID, *t* = 4 µs), relaxation delay of 5 s, 256 scans, acquisition time of 2.62 s, mixing time (MIX) of 10 ms and spectral window was of 20 ppm. The spectra were processed by applying an automatic phase and baseline correction adjustment. The fine phase and baseline adjustments were manually performed, and the same integrated regions for quantification were employed in all spectra.

The quantification was performed by the manual integration of the areas of the signals, in order to use the same integrated regions for all spectra. The area of the internal standard signal was calibrated to one, as a reference to the other signals. The chemical shifts used for integration were: 7.41 ppm for phenylalanine, 6.88 ppm for tyrosine, 4.58 ppm for galactose, 2.46 ppm for succinate, 2.37 ppm for glutamate, 1.93 ppm for acetate and 1.04 ppm for valine.

The two-dimensional (2D) NMR experiments were used to aid in the assignment of the metabolites. The 2D ¹H JRES (*J*-Resolved) NMR experiment was performed using a TD of 32,768, time acquisition of 1.31 s, relaxation delay of 2 s and spectral window of 20 ppm. For data processing, the baseline correction was done in dimension F2 and, later, the tilt algorithm of 45° was applied so that all spin systems with *J*-coupling in F1 dimension were orthogonal to chemical shifts in dimension F2. Subsequently, the baseline was corrected again, and the spectrum symmetrized. The parameters used for the 2D HSQC (heteronuclear single quantum correlation spectroscopy) analysis were 52 scans, TD of 4096 and spectral window of 20 ppm for the ¹H channel, while in the ¹³C dimension, the TD was of 256 and the spectral window was 300 ppm. For the INEPT block, the delay was 1.72 ms

(corresponding to an average $^1J_{CH}$ coupling constant of 145 Hz). It was also used for the HSQC, a module with a continuous wave for double suppression of both water and CH_2 moieties of fatty acids.

2.4. Statistical analyses

Multiple comparisons among averages were performed by rankings-based conjoint analysis on rank using SAS GLM analyses (Statistical Analysis System, version 6.12; SAS Institute Inc., Cary, NC, USA, 1996), with refrigeration (two levels) and storage time (seven levels) as factors. Whenever necessary, the degrees of freedom were corrected according to Satterthwaite procedure (Satterthwaite, 1946; Li and Redden, 2015). Prior to analysis, the data were checked for normal distribution and homogeneity of variance using the Shapiro–Wilk and Bartlett's tests, respectively. Results are shown as mean values, and $p < 0.05$ was regarded as significant.

To perform multivariate analysis, Principal Response Curve (PRC) analysis was applied to investigate the effects of metabolites (and pH variations) and their alterations in time (Van den Brink and Ter Braak, 1999). In PRC, sampling weeks were used as the categorical covariable, and the interaction between sampling time and treatment (inadequate cooling) was used as the explanatory variable. The analysis produces a diagram that shows the time gradient and the first canonical axis of metabolite (and pH) differences between the treatment and a control, here attributed to adequate cooling. Monte Carlo permutation tests were performed to evaluate if the PRC explained a significant part of the variance of the treatment in relation to control in all the time series (999 permutations), as well as to verify if the treatment results in one metabolite significantly altered in each sampling time (199 permutations). PRCs were calculated on $\ln(x + 100,000)$ -transformed and centered data before analysis. The multivariate analyses were conducted in Canoco.

3. Results and discussion

3.1. Assignment of the NMR mozzarella cheese signals

Mozzarella, like other cheese varieties, is a complex mixture with high water content, which makes it susceptible to degradation and spoilage, resulting in metabolite levels that compromise its quality (Møller et al., 2012; Marino et al., 2019). The compounds in mozzarella cheese samples were identified from the 2D HSQC, 2D 1H JRES and 1H NMR spectra, which were interpreted using the Human Metabolome Database – HMDB database (Wishart et al., 2018) and compared to literature data (Mazzei and Piccolo, 2012; Blaise et al., 2007; Gianferri et al., 2007; Mannina et al., 2012; Mozzi et al., 2013; Shintu and Caldarelli, 2005), as summarized in Table 1.

Firstly, the metabolites were identified, and then their integrals were inspected in spectra of cheeses samples stored for 11 weeks. Table 1 indicates the week number in which each compound was identified, highlighting metabolites that appeared or disappeared during the course of inadequate refrigeration or proper refrigeration. Fig. 1 depicts the changes in the metabolite levels from inadequately refrigerated cheese samples presented in Table 1.

3.2. Relative content of compounds in mozzarella cheese

There are several substances that may cause unwanted taste of cheese due to proteolytic activity (McSweeney and Sousa, 2000). Among them, phenylalanine, tyrosine, leucine, valine and lysine, which characterize the bitter taste of cheese, were identified in the mozzarella cheese and their contents changed throughout the adequate refrigeration period. After adequate refrigeration for 8 weeks, when cheese attained the expiry date, the contents of these compounds were considered as the reference of food spoilage and then compared with contents found in samples subject to inadequate refrigeration. As the

Table 1

Mozzarella cheese 1H and ^{13}C NMR signal assignments with its chemical shifts (δ), coupling constants (J_{HH}), multiplicity (multi.) and intervals (weeks) in which the signals were observed for inadequate refrigeration (IR) and proper refrigeration (PR).

Compounds	δ 1H (multi., J Hz)	δ ^{13}C	Group	Weeks
Formate	8.45 (s)	Not detected	CH	1–11 (IR, PR)
Phenylalanine	7.41 (m)	132.08	CH _{ring}	1–11 (IR, PR)
	7.32 (m)	132.23	CH _{ring}	
	3.98 (m)	58.97	CH	
Tyrosine	3.27(m)	39.37	CH	1–11 (IR, PR)
	7.18 (m)	133.70	CH _{ring}	
	6.88 (m)	118.81	CH _{ring}	
N-acetyl tyrosine	3.93 (m)	59.26	CH	1–11 (IR, PR)
	7.13 (m)	133.66	CH _{ring}	
	6.83 (m)	118.49	CH _{ring}	
Galactose	4.44 (m)	59.39	CH	1–5 (PR)
	5.26 (d, 3.7)	95.07	CH $^{\alpha}$ -anomeric	
	4.58 (d, 7.9)	99.25	CH $^{\beta}$ -anomeric	
Lactate	4.12 (q, 6.9)	71.56	CH	1–11 (IR, PR)
Glycerol	1.34 (d, 6.8)	22.80	CH ₃	2–11 (PR)
	3.76 (m)	75.16	CH	
	3.64 (m)	65.50	CH ₂	
	3.56 (m)	65.50	CH ₂	
Lysine	3.01 (t, 7.6)	42.13	CH ₂	7–11 (PR)
			CH ₂	4–11 (IR)
Citrate	2.71 (d _{AB} , 16.4)	47.57	CH ₂	1–11 (PR)
	2.52 (d _{AB} , 16.4)	47.57	CH ₂	1–8 (IR)
Succinate	2.46 (s)	35.77	CH ₂	1–11 (IR, PR)
Glutamate	3.76 (dd, 7.2 and 4.6)	54.06	CH	1–11 (IR, PR)
	2.37 (m)	27.03	CH ₂	
Butyrate	2.19 (t, 7.3)	41.75	CH ₂	7–11 (PR)
	1.56 (sex, 7.3)	Not detected	CH ₂	6–11 (IR)
	0.89 (t, 7.3)	Not detected	CH ₃	
Acetate	1.93 (s)	25.66	CH ₃	1–11 (IR, PR)
Leucine	0.96 (d, 6.4)	Not detected	CH ₃	1–11 (IR, PR)
	0.95 (d, 6.6)	Not detected	CH ₃	
Ethanol	1.18 (t, 7.1)	Not detected	CH ₃	5–11 (PR)
		Not detected		2–11 (IR)
Valine	3.59 (m)	63.21	CH	1–11 (IR, PR)
	1.04 (d, 7.0)	23.06	CH ₃	
	0.99 (d, 7.0)	17.34	CH ₃	

numbers obtained from the quantification were very small, in order to make easy the comparative analysis of the data of the different conditions and weeks, the average areas values of the triplicates, acquired at zero-week, were used as a reference and set to 100%. The absolute quantification of the metabolites is available as supplementary material in Table 1S.

A significant change in the metabolites content was observed only every two weeks, except for the galactose at week 5, and therefore this was the main interval used in our analyses. Relative to zero time, the following contents were found at 8 weeks of adequate refrigeration: 182.7% phenylalanine, 129.5% tyrosine, 71.0% valine, and 152.9% glutamate. These contents were similar to those found at 4 weeks (133.7% tyrosine and 86.0% valine) and 6 weeks (187.7% phenylalanine and 156.0% glutamate) of inadequate refrigeration. McSweeney and Sousa (2000) observed that the environmental conditions favor rapid growth of lactic acid bacteria, as observed from 4 weeks in this study. As soon as this occurs, the proteolysis degree is significantly

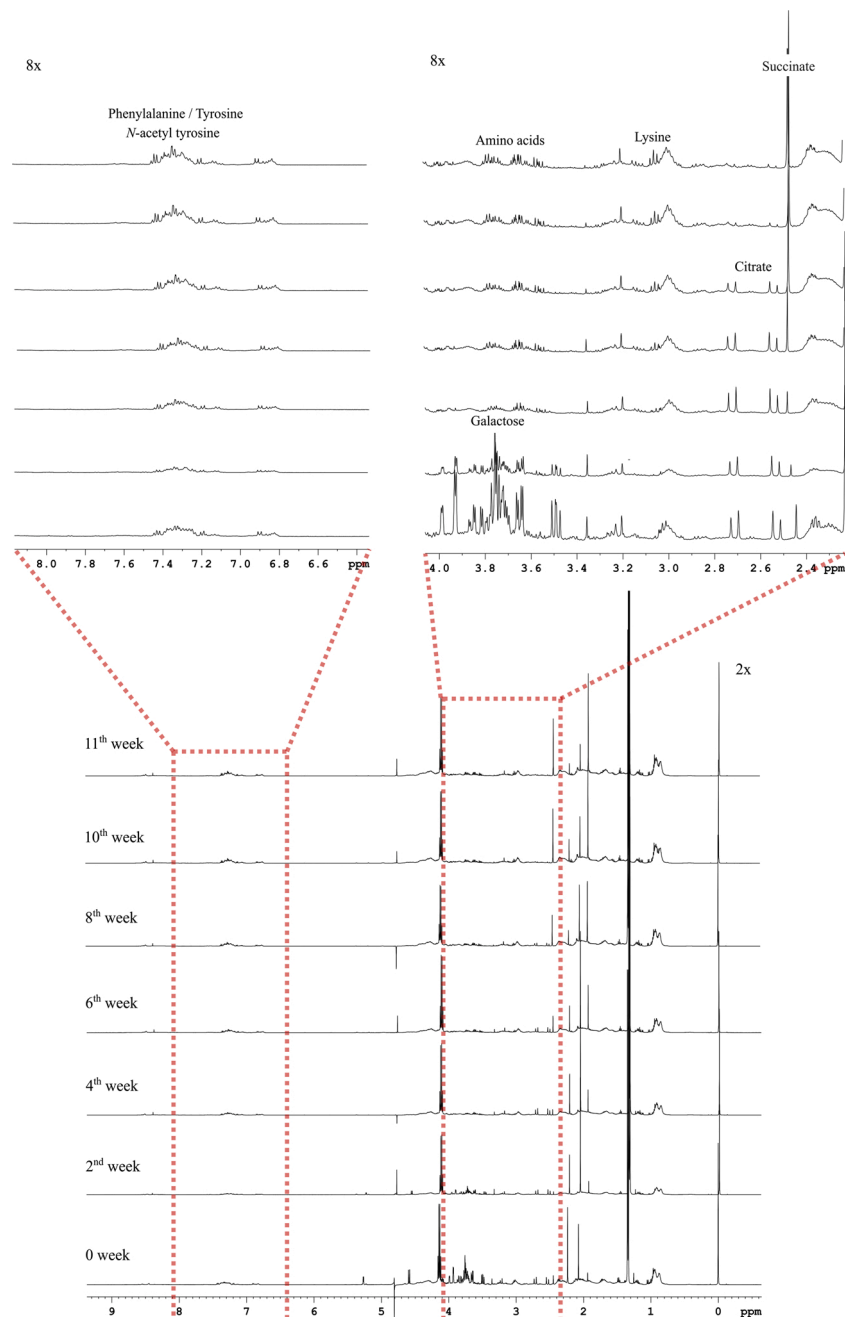


Fig. 1. Chemical profile of cheese samples submitted to inadequate refrigeration for up to 11 weeks. All spectra are on a same scale and show the evolution of the ^1H NMR profiles every 2 weeks of cheese of storage. In the expanded region, there is a decrease in galactose levels, followed by an increase in amino acids, mainly lysine. Around 2.6 ppm, the decrease in citrate levels and increase of the succinate signal can also be observed.

increased, resulting in higher amino acid contents in inadequately refrigerated cheese samples at 4 weeks compared with those stored under adequate refrigeration.

The presence of galactose, due to lactose hydrolysis in the cheese-making process (Dudley and Steele, 2005), was identified even in the spectra of fresh cheese (zero week) and the samples stored for 1 weeks under appropriate refrigeration, although its content changed significantly at week 5 when its level decreased 53.1% relative to the zero-week level (Fig. 2A). After week 5 the galactose signs were no longer observed in the ^1H NMR spectra from properly refrigerated samples. Such a similar decay of 51.7% was already observed in the second week for the cheese samples kept overnight outside the refrigerator, at room temperature ranging from 22 to 25 °C. Thus, galactose began to degrade about 3 weeks early upon this undesirable storage

practice and were not reported from week 3 forward. For this reason only the data until week 4 are presented for the galactose in Fig. 2A.

Lactate metabolism occurs by the bacterial action on lactic acid, which can then be oxidized to acetate, an important flavor compound in cheese (McSweeney et al., 2017). Fig. 2B shows the acetate contents relative to zero week, for which we could observe a cumulative increase of 228.8% at 11 weeks sampling period for the samples that remained in adequate refrigeration, and 716.0% for improperly chilled samples. Thereby, the acetate content increase was much greater in samples under inadequate cooling than in those correctly stored.

Dudley and Steele (2005) showed that succinate is one of the main products of citrate metabolism, being also known as a compound that affects the flavor of foods (Steele and Dudley, 1999), and Pisano et al. (2016) founded the succinate as one of the key compounds to statistical

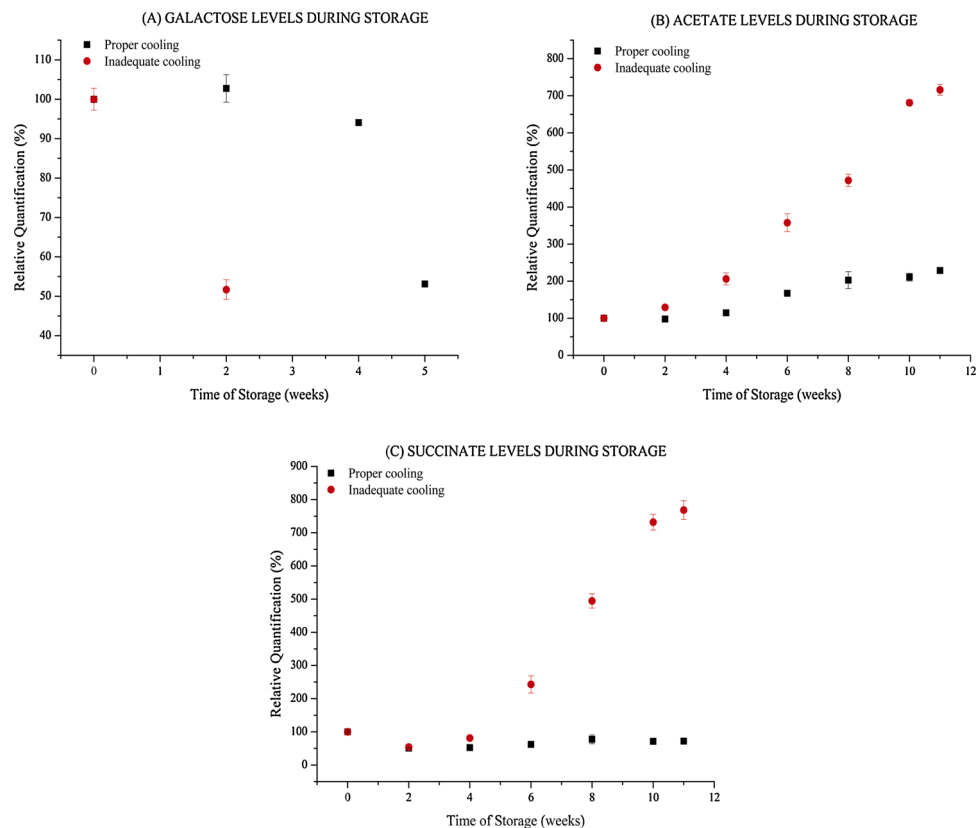


Fig. 2. Percentages of galactose (A), acetate (B) and succinate (C) levels during storage relative to the zero-week levels. Each measurement corresponds to the mean with standard error ($n = 3$).

discrimination of varieties of mozzarella cheese. Fig. 2C shows a significant escalation in succinate levels if cheese is not appropriately stored, with increases from 54.1% to 768.5% from week 2 to 11, and a corresponding lower increase of 50.9–71.9% in adequately refrigerated cheese. Such a large difference in succinic acid levels highlights this constituent as another important marker for differentiating cooling conditions. In general, the substantial production of this acid is common during cheese metabolism since it can be produced not only from citrate but also from lactate (Dudley and Steele, 2005), both which are some of the main mozzarella cheese components.

In summary, the main potential markers for distinguishing the mozzarella cheese subjected to different refrigeration conditions were succinate, acetate, and lactate, with more accentuated differentiation for acetate and succinate. In addition, incorrect refrigeration practice decreases the validity of this cheese type in about 4 weeks. The same compounds identified in cheese kept under adequate refrigeration were

also identified in samples submitted to inadequate refrigeration, but both their relative and absolute amounts were indicative of early degradation of mozzarella cheese due to inadequate refrigeration. Cheese submitted to inadequate refrigeration presented high succinate and acetate contents at 4 weeks storage, which were comparable to those levels found in cheese maintained under appropriate refrigeration only at 8 weeks storage.

3.3. Composition, physicochemical and NMR data statistical analyses

The data of adequate and inadequate refrigeration and sampling time were used as factors for the two-way ANOVA shown in Table 2. These data were collected every 2 weeks, except after week 10 when these data were also evaluated at 11 weeks sampling period.

Results for the total protein showed that most of the changes in contents occurred throughout inadequate refrigeration, regardless of

Table 2

Mean values^a of the pH, milk fat, and total protein present in mozzarella cheese stored under adequate and inadequate refrigeration conditions. The data were acquired in triplicate.

Parameter	Refrigeration	Storage time (weeks)								Average of refrigeration
		0	2	4	6	8	10	11		
pH	Adequate	5.36 Aab	5.61 Aa	5.73 Aa	5.10 Ac	5.13 Bc	5.24 Abc	5.17 Ac		
	Inadequate	5.34 Aa	5.36 Aa	5.33 Ba	5.07 Ab	5.33 Aa	5.22 Ab	5.23 Aab		
Total protein (g/100 g)	Adequate	25.34	23.87	24.50	24.00	25.23	23.79	23.34	24.30 A	
	Inadequate	24.17	23.23	25.02	24.06	24.68	23.62	23.30	24.01 B	
	Average of time	24.76 a	23.55 bc	24.76 a	24.03 ab	24.95 a	23.70 bc	23.32 c		
Milk fat (g/100 g)	Adequate	24.87 Abc	24.77 Ac	25.88 Aa	25.72 Aa	25.27 Aabc	25.88 Aa	25.71 Aab		
	Inadequate	25.43 Aab	24.83 Aabc	25.53 Ba	24.64 Bbc	23.98 Bc	25.07 Babc	25.63 Aa		
Succinate (mg/L)	Adequate	0.038 Aa	0.019 Ad	0.020 Ad	0.024 Bcd	0.029 Bb	0.027 Bbc	0.027 Bbc		
	Inadequate	0.037 Acd	0.020 Ae	0.030 Bd	0.089 Abc	0.181 Aab	0.268 Aab	0.281 Aa		

^a Based on original data. Averages followed by the same uppercase letter in the columns and by the same lowercase letter in the rows did not share significant differences at 5% probability by Tukey’s test. Main significant differences are highlighted in bold.

time. However, a distinct influence was observed only for the final sampling times, regardless of refrigeration, whereby the contents decreasing significantly over time from 8 weeks. The influence of the combination of both factors (refrigeration–time interaction) was observed for milk fat contents, which were lower after 4 weeks in inadequate refrigeration compared with adequate refrigeration. In contrast, the pH values showed a significant decrease (week 4) during adequate cooling, while for inadequate refrigeration, the pH showed an oscillating trend throughout the experiment, with significantly higher and lower levels for most sampling times. Nonetheless, in respect of NMR analysis, Fig. 1 showed that there was no significant change of the chemical shifts due to such pH changes, besides the fact that we used NMR buckets, which compensates the effect of these small NMR variations in the statistical analyses.

These composition and physicochemical parameters, such as total protein, milk fat, and pH, of cheese are related to its quality (Cortez et al., 2008), constituting the main composition and physicochemical parameters required by MAPA for cheese quality certification in Brazil.

Aiming to evaluate if the differences between composition, physicochemical and succinate levels are altered along sampling time, PRC was performed between adequate (control) and inadequate refrigeration (treatment) for the 11 weeks sampling period (Fig. 3). The results indicated that 56.0% of the total variation of the data could be explained by the treatment effect, of which 88.9% is shown in the first PRC diagram ($F = 538.1$, $p = 0.001$, 999 permutations). The differences among the sampling weeks presented a relatively smaller contribution to the total variance (41.4%). The variable that most influenced the first PRC axis was the succinate levels (highest weighting). Variables with weightings from -0.5 to $+0.5$ either show no response or a response that is unrelated to the pattern shown by the PRC (Van den Brink and Ter Braak, 1999). Thus, total protein, milk fat, and pH did not contribute to the differentiation between samples.

When the data were restricted to each sampling time, a significant difference was observed between weeks 2 and 5, besides a discrete significant difference ($0.060 < p > 0.080$) between weeks 3 and 4. From 4 weeks sampling period, the succinate level increased sharply in cheese under inadequate refrigeration (treatment) relative to the control. These differences may indicate citrate and/or lactate conversion during cheese metabolism modulated by bacterial action in inadequately cooled samples (Rodrigues et al., 2011; McSweeney et al., 2017; Dudley and Steele, 2005).

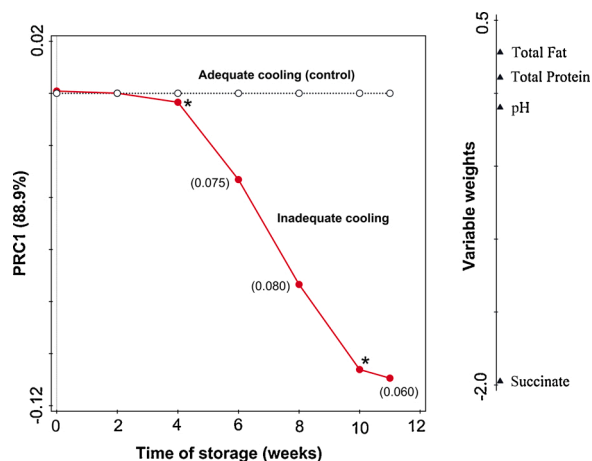


Fig. 3. First component of the PRC showing the differences in measured composition and physicochemical parameters and succinate contents between inadequate and adequate (control) refrigeration along sampling times (weeks). Variable weightings can be interpreted as a relative contribution of individual contents to the response given in the diagram. For each sampling time, the Monte Carlo permutation test (199 permutations) was performed for comparison between cooling processes. * ($p < 0.050$).

The PRC was also conducted to find patterns in the changes of the metabolites by ^1H NMR. The results (Fig. 4) revealed that 28.8% of the total variance was explained by the improper cooling effect (treatment) and 67.1% by differences between sampling dates (time). The first canonical axis in the PRC captured a significant part (61.8%) of the variance explained by treatment ($F = 118.9$, $p = 0.001$, 999 permutations). On sampling weeks 2, 4, and 5, the treatment differed significantly from the control ($p < 0.047$, 199 permutations). Lactate, acetate, and succinate were mainly responsible for the divergence between the treatment and control.

From the above discussion, it was possible to highlight succinate as an important marker of either adequate or inadequate cheese refrigeration. As the data in Table 2 indicate, the inadequately cooled sample reached a succinate content of 0.181 mg/L at week 8 (due expiry date) that was more than 624% higher than the content of the adequately cooled sample at 8th week of storage (0.029 mg/L). Furthermore, the succinate content for the inadequately chilled sample at week 4 reached approximately the same level of the corresponding sample of adequate refrigeration at week 8 (0.030 and 0.029 mg/L, respectively), suggesting a 4-week reduction of the chemical shelf life of the inadequately cooled cheese sample. In addition, it was found that the succinate levels did not change significantly in the samples stored under suitable refrigeration.

4. Conclusions

In summary, the main potential markers for distinguishing the mozzarella cheese subjected to different refrigeration conditions were succinate, acetate, and lactate, with more accentuated differentiation for acetate and succinate. The same compounds identified in cheese kept under adequate refrigeration were also identified in samples submitted to inadequate refrigeration, but both their relative and absolute amounts were indicative of early degradation of mozzarella cheese due to inadequate refrigeration. From the absolute quantification of succinate, it was observed a level of 0.089 mg/L under inadequate refrigeration condition, which can be a potential metabolite to be monitored in future studies, in order to confirm if it is really a biomarker of the inappropriate practices explored here. Conversely, the identification of different cheese refrigeration conditions was not possible by analyzing the composition and physicochemical parameters commonly used in cheese quality control. Based on the chemical profile changes monitored by ^1H NMR experiments, it was possible to infer that the food quality

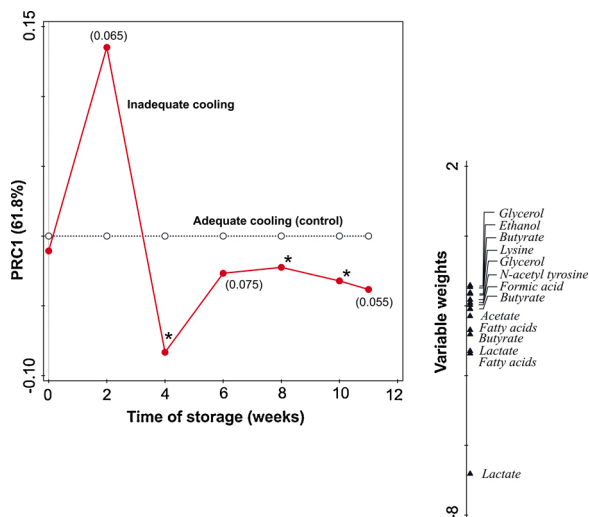


Fig. 4. PRC plot of the differences in relative contents of the metabolites by ^1H NMR between inadequate and adequate (control) refrigeration along sampling times (weeks). For each sampling time, the Monte Carlo permutation test (199 permutations) was performed for comparison between cooling processes. * ($p < 0.050$).

may have been affected by different cooling conditions since the mild taste of this cheese type is associated with low enzymatic activity levels. The combined ^1H NMR data and statistical analyses represented a potential approach to both evaluate the quality and predict the chemical shelf life of mozzarella cheese.

Authors' contribution

Flávia Carneiro Gonçalves: conceptualization, methodology, formal analysis, writing – original draft preparation, writing – reviewing and editing. Vitor Mendes de Oliveira: validation, writing – review & editing. Felipe Terra Martins: visualization, writing – reviewing and editing. Luciano Morais Lião: resources, conceptualization. Pedro Henrique Ferri: software, validation. Luiz Henrique Keng Queiroz Júnior: supervision, writing – reviewing and editing.

Conflict of interest

None declared.

Acknowledgements

The authors are grateful to the Federal University of Goiás for structural support as well as FINEP, FAPEG, CNPq, and CAPES for continued financial support and scholarships.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jfca.2021.104229>.

References

- Altieri, C., Scrocco, C., Sinigaglia, M., Del Nobile, M., 2005. Use of chitosan to prolong mozzarella cheese shelf life. *J. Dairy Sci.* 88, 2683–2688. [https://doi.org/10.3168/jds.S0022-0302\(05\)72946-5](https://doi.org/10.3168/jds.S0022-0302(05)72946-5).
- Battelli, G., Scano, P., Albano, C., Cagliani, L.R., Brasca, M., Consonni, R., 2019. Modifications of the volatile and nonvolatile metabolome of goat cheese due to adjunct of non-starter lactic acid bacteria. *LWT – Food Sci. Technol.* 116, 108576.
- Blaise, B.J., Giacomotto, J., Elena, B., Dumas, M.-E., Toulhoat, P., Ségalat, L., Emsley, L., 2007. Metabotyping of *Caenorhabditis elegans* reveals latent phenotypes. *Proc. Natl. Acad. Sci. U.S.A.* 104, 19808–19812. <https://doi.org/10.1073/pnas.0707393104>.
- Branca, M.L., 2018. Shelf-Life Extension of Lacto-Fermented Mozzarella Through Sustainable Process Operations (Ph.D. thesis). Università degli studi della Tuscia, Viterbo, IT.
- Codex Alimentarius Commission, 2011. Codex Alimentarius. Milk and milk products, 2nd ed. FAO/WHO Press, Rome.
- Conte, A., Scrocco, C., Sinigaglia, M., Del Nobile, M., 2007. Innovative active packaging systems to prolong the shelf life of mozzarella cheese. *J. Dairy Sci.* 90, 2126–2131. <https://doi.org/10.3168/jds.2006-709>.
- Cortez, M.A.S., Furtado, M.M., Gigante, M.L., Kindstedt, P.S., 2008. Effect of pH on characteristics of low-moisture mozzarella cheese during refrigerated storage. *J. Food Sci.* 73, S443–S448. <https://doi.org/10.1111/j.1750-3841.2008.00943.x>.
- Dudley, E.G., Steele, J.L., 2005. Succinate production and citrate catabolism by Cheddar cheese nonstarter lactobacilli. *J. Appl. Microbiol.* 98, 14–23. <https://doi.org/10.1111/j.1365-2672.2004.02440.x>.
- El-Gazzar, F.E., Marth, E.H., 1992. Salmonellae, salmonellosis, and dairy foods: a review. *J. Dairy Sci.* 75, 2327–2343. [https://doi.org/10.3168/jds.S0022-0302\(92\)77993-4](https://doi.org/10.3168/jds.S0022-0302(92)77993-4).
- Faccia, M., Gambacorta, G., Natrella, G., Caponio, F., 2019. Shelf life extension of Italian mozzarella by use of calcium lactate buffered brine. *Food Control* 100, 287–291. <https://doi.org/10.1016/j.foodcont.2019.02.002>.
- Fiehn, O., 2002. Metabolomics – the link between genotypes and phenotypes. *Plant Mol. Biol.* 48, 155–171. <https://doi.org/10.1023/A:1013713905833>.
- Gianferri, R., Maioli, M., Delfini, M., Brosio, E., 2007. A low-resolution and high-resolution nuclear magnetic resonance integrated approach to investigate the physical structure and metabolic profile of Mozzarella di Bufala Campana cheese. *Int. Dairy J.* 17, 167–176. <https://doi.org/10.1016/j.idairyj.2006.02.006>.
- Heard, B.R., Shelie, A.M., 2016. Critical research needed to examine the environmental impacts of expanded refrigeration on the food system. *Environ. Sci. Technol.* 50, 12060–12071. <https://doi.org/10.1021/acs.est.6b02740>.
- James, S.J., James, C., 2013. Food technologies: Chilling. In: Motarjemi, Y. (Ed.), *Encyclopedia of Food Safety*, Vol. 3. Academic Press, Waltham, MA, USA, pp. 140–148. <https://doi.org/10.1016/B978-0-12-378612-8.00265-1>.
- Kim, J., Schmidt, K.A., Phebus, R.K., Jeon, I.J., 1998. Time and temperature of stretching as critical control points for *Listeria monocytogenes* during production of mozzarella cheese. *J. Food Protect.* 61, 116–118. <https://doi.org/10.4315/0362-028X-61.1.116>.
- Lacivita, V., Conte, A., Musavian, H.S., Krebs, N.H., Zambrini, V.A., Del Nobile, M.A., 2018. Steam-ultrasound combined treatment: a promising technology to significantly control mozzarella cheese quality. *LWT – Food Sci. Technol.* 93, 450–455.
- Lamanna, R., Piscioneri, I., Romanelli, V., Sharma, N., 2008. A preliminary study of soft cheese degradation in different packaging conditions by ^1H -NMR. *Magn. Reson. Chem.* 46, 828–831. <https://doi.org/10.1002/mrc.2258>.
- Laurienzo, P., Malinconico, M., Mazzarella, G., Petitto, F., Piciocchi, N., Stefanile, R., Volpe, M., 2008. Water buffalo mozzarella cheese stored in polysaccharide-based gels: correlation between prolongation of the shelf-life and physicochemical parameters. *J. Dairy Sci.* 91, 1317–1324. <https://doi.org/10.3168/jds.2007-0482>.
- Li, P., Redden, D.T., 2015. Comparing denominator degrees of freedom approximations for the generalized linear mixed model in analyzing binary outcome in small sample cluster-randomized trials. *BMC Med. Res. Methodol.* 15, 1–12.
- Lolli, V., Dall'Asta, M., Del Rio, D., Caligiani, A., 2018. In vitro digestibility of cyclopropane fatty acids in Grana Padano cheese: a study combining ^1H NMR and GC-MS techniques. *J. Food Eng.* 237, 226–230. <https://doi.org/10.1016/j.jfoodeng.2018.05.034>.
- Mannina, L., Sobolev, A.P., Viel, S., 2012. Liquid state ^1H high field nmr in food analysis. *Prog. Nucl. Magn. Reson. Spectrosc.* 66, 1–39. <https://doi.org/10.1016/j.pnmrs.2012.02.001>.
- MAPA, 2006. Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa N° 68 – 12/12/2006 (Accessed 13 April 2019). <http://extranet.agricultura.gov.br/sislegis-consulta/consultarLegislacao.do?operacao=visualizar&id=17472>.
- Margallo, L.P., Kamimura, B.A., Pimentel, T.C., Balthazar, C.F., Araujo, J.V., Silva, R., Conte-Junior, C.A., Raices, R.S., Cruz, A.G., Sant'Ana, A.S., 2021. A large survey of the fatty acid profile and gross composition of Brazilian artisanal cheeses. *J. Food Compos. Anal.* 101, 103955. <https://doi.org/10.1016/j.jfca.2021.103955>.
- Marino, M., de Wittenau, G.D., Sacca, E., Cattonaro, F., Spadotto, A., Innocente, N., Radovic, S., Piasentier, E., Marroni, F., 2019. Metagenomic profiles of different types of Italian high-moisture Mozzarella cheese. *Food Microbiol.* 79, 123–131. <https://doi.org/10.1016/j.fm.2018.12.007>.
- Mazzei, P., Piccolo, A., 2012. ^1H HRMAS-NMR metabolomic to assess quality and traceability of mozzarella cheese from Campania buffalo milk. *Food Chem.* 132, 1620–1627. <https://doi.org/10.1016/j.foodchem.2011.11.142>.
- McSweeney, P.L.H., Fox, P.F., Cotter, P.D., Everett, D.W., 2017. *Cheese: Chemistry. In: McSweeney, P.L.H., Fox, P.F., Cotter, P.D., Everett, D.W. (Eds.), Physics and Microbiology*, 4th ed. Academic Press, London, UK.
- McSweeney, P.L.H., Sousa, M.J., 2000. Biochemical pathways for the production of flavour compounds in cheeses during ripening: a review. *Le Lait* 80, 293–324. <https://doi.org/10.1051/lait:2000127>. arXiv:013.
- Møller, S.M., Hansen, T.B., Andersen, U., Lillevang, S.K., Rasmussen, A., Bertram, H.C., 2012. Water properties in cream cheeses with variations in pH, fat, and salt content and correlation to microbial survival. *J. Agric. Food Chem.* 60, 1635–1644. <https://doi.org/10.1021/jf204371v>.
- Mozzi, F., Ortiz, M.E., Bleckwedel, J., De Vuyst, L., Pescuma, M., 2013. Metabolomics as a tool for the comprehensive understanding of fermented and functional foods with lactic acid bacteria. *Food Res. Int.* 54, 1152–1161. <https://doi.org/10.1016/j.foodres.2012.11.010>.
- Ndraha, N., Hsiao, H.I., Vlajic, J., Yang, M.F., Lin, H.T.V., 2017. Time-temperature abuse in the food cold chain: review of issues, challenges, and recommendations. *Food Control* 89, 12–21. <https://doi.org/10.1016/j.foodcont.2018.01.027>.
- Neill, M.A., 2008. Food safety in the industrialized world. In: Mayer, K.H., Pizer, H. (Eds.), *The Social Ecology of Infectious Diseases*. Academic Press, San Diego, CA, USA, pp. 215–240. <https://doi.org/10.1016/B978-0-12370466-5.50013-3>.
- Pisano, M.B., Scano, P., Murgia, A., Cosentino, S., Caboni, P., 2016. Metabolomics and microbiological profile of Italian mozzarella cheese produced with buffalo and cow milk. *Food Chem.* 192, 618–624. <https://doi.org/10.1016/j.foodchem.2015.07.061>.
- Rabelo, M., 2012. Decon: Distribuidora que armazenava produtos vencidos e de forma irregular é interdita (Accessed 13 April 2019). <https://www.policiacivil.go.gov.br/noticias/policia-civil-distribuidora-que-armazenava-produtos-vencidos-e-de-forma-irregular-e-interdita-pela-decon.html>.
- Riley, E., 2018. Iceland Is Accused of Failure to Ensure Chilled Food Is Stored Properly and Safe to Eat After It Was Left to Stand for Up To 14 Hours After Delivery Without Being Refrigerated (Accessed 13 April 2019). <https://www.dailymail.co.uk/news/article-5630479/Iceland-accused-failing-store-chilled-food-properly-BBC-probe.html>.
- Rodrigues, D., Santos, C.H., Rocha-Santos, T.A.P., Gomes, A.M., Goodfellow, B.J., Freitas, A.C., 2011. Metabolic profiling of potential probiotic or synbiotic cheeses by nuclear magnetic resonance (nmr) spectroscopy. *J. Agric. Food Chem.* 59, 4955–4961. doi:1021/jf104605r.
- Satterthwaite, F.E., 1946. An approximate distribution of estimates of variance components. *Biomet. Bull.* 2, 110–114. <https://doi.org/10.2307/3002019>.
- Shintu, L., Caldarelli, S., 2005. High-resolution MAS NMR and chemometrics: characterization of the ripening of Parmigiano Reggiano cheese. *J. Agric. Food Chem.* 53, 4026–4031. <https://doi.org/10.1021/jf048141y>.
- da Silva, D.F., Tziouri, D., Ipsen, R., Hougaard, A.B., 2020. Towards the manufacture of camembert cheese powder: characteristics of cheese feeds without emulsifying salts. *LWT – Food Sci. Technol.* 127, 109412.
- Silva, H.L., Balthazar, C.F., Esmerino, E.A., Neto, R.P., Rocha, R.S., Moraes, J., Cavalcanti, R.N., Franco, R.M., Tavares, M.I.B., Santos, J.S., Granato, D., Costa, R.G., Freitas, M.Q., Silva, M.C., Raices, R.S., Senaka Ranadheera, C., Nazzaro, F., Mortazavian, A.M., Cruz, A.G., 2018. Partial substitution of NaCl by KCl and addition of flavor enhancers on probiotic Prato cheese: a study covering

- manufacturing, ripening and storage time. *Food Chem.* 248, 192–200. <https://doi.org/10.1016/j.foodchem.2017.12.064>.
- Steele, J.L., Dudley, E.G., 1999. Succinate Production by *Lactobacillus casei*: Pathways Responsible and Development of Strategies to Control Its Accumulation. Technical Report Wisconsin Center for Dairy Research. CDR Annual Report, Madison, WI, USA.
- Van den Brink, P.J., Ter Braak, C.J.F., 1999. Principal response curves: analysis of time-dependent multivariate responses of biological community to stress. *Environ. Toxicol. Chem.: Int. J.* 18, 138–148. <https://doi.org/10.1002/etc.5620180207>.
- Villani, F., Pepe, O., Mauriello, G., Moschetti, G., Sannino, L., Coppola, S., 1996. Behaviour of *Listeria monocytogenes* during the traditional manufacture of water-buffalo mozzarella cheese. *Lett. Appl. Microbiol.* 22, 357–360. <https://doi.org/10.1111/j.1472-765X.1996.tb01178.x>.
- Wishart, D.S., Feunang, Y.D., Marcu, A., Guo, A.C., Liang, K., Vázquez-Fresno, R., Sajed, T., Johnson, D., Li, C., Karu, N., Sayeeda, Z., Lo, E., Assempour, N., Berjanskii, M., Singhal, S., Arndt, D., Liang, Y., Badran, H., Grant, J., Serra-Cayuela, A., Liu, Y., Mandal, R., Neveu, V., Pon, A., Knox, C., Wilson, M., Manach, C., Scalbert, A., 2018. HMDB 4.0: the human metabolome database for 2018. *Nucleic Acids Res.* 46, D608–D617. <https://doi.org/10.1093/nar/gkx1089>.