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Declaration of Competing Interest

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Highlights

- Coagulation ability of milk after pasteurization depends on raw milk composition
- More acidic raw milk shows better cheese-making properties after pasteurization
- Raw milk β-lactoglobulin unfavorably affects curd firming time of pasteurized milk
- Rennet coagulation time of pasteurized milk can be predicted from raw milk spectra

Abstract

Milk coagulation properties (MCP) worsens after heat treatment, however the specific mechanisms responsible have been scarcely explored. In this study, 100 milk samples were available to i) identify the raw milk characteristics responsible for unfavorable changes in MCP after pasteurization and ii) develop infrared prediction models for pasteurized milk MCP using spectra of raw samples. The loss in coagulation ability due to pasteurization was lower when raw milk had optimal MCP, higher acidity, greater protein content and lower β-lactoglobulin content. For the four MCP, the trait measured before pasteurization (raw milk) was the most important variable influencing the corresponding trait after heating. For example, rennet coagulation time (RCT), κ-casein, protein, lactose and pH of raw milk significantly affected pasteurized milk RCT (P<0.001). For curd firmness, each unit (mm) corresponded to 58.65 g/100 g κ-casein. In general, raw milk β-lactoglobulin unfavorably affected pasteurized milk MCP (e.g., the estimate of curd firming time was 81.39 g/100 g). Results suggested that only the prediction model of RCT (pasteurized milk) achieved an exploitable coefficient of determination in cross-validation (0.66). Our outcomes are relevant for dairy plants manufacturing cheese from pasteurized standardized milk and could support producers' decision-making.

Keywords: rennet coagulation time; casein; whey protein; heat treatment; cheese; dairy industry

1. Introduction

Pasteurization is a common practice in the dairy industry, and is primarily intended for the reduction of the milk pathogenic bacteria load, which has to be below the admissible level. Heat treatment translates into an increased shelf-life of milk and limits proliferation and activity of microorganisms detrimental for cheese processing. Common milk pasteurization techniques comprise heating at either 63°C for 30 min (low-temperature long-time, LTLT), 72°C for 15 s (high-temperature short-time, HTST) or any other equivalent thermal treatment (Stumbo, 1973; Liu et al., 2020). Such temperatures were selected to achieve a 5-log reduction in the presence of the heat resistant pathogen *Coxiella brunetii* detectable in raw milk (Kelly et al., 2005; FIL-IDF, 2019). Cheeses commercially available can be produced from either unpasteurized or pasteurized milk. Some of those entitled with the Protected Designation of Origin label, like Grana Padano and Parmigiano Reggiano (Mammi et al., 2018; Buonaiuto et al., 2021; Cavallini et al., 2021), are produced from the former, whereas Cheddar, mozzarella pasta filata and American soft artisanal cheeses are produced from the latter (Knoll, 2005).

Heating, including the pasteurization process, is known to alter milk composition and impair the technological traits (Anema et al., 2007; Blecker et al., 2012; Britten & Giroux, 2022; Hyslop, 2003; Lucey, 1995), causing a deterioration of the milk coagulation properties (MCP). Various MCP have been described in the literature, but rennet coagulation time (RCT, min), curd firmness (a_{30}, mm) and curd firming time (k_{20}, min) are known to be the most important for describing the milk cheese-ability. The deterioration of MCP observed in heat-treated milk is likely due to the denaturation of β-lactoglobulin (β-LG) and its subsequent complexation with κ-casein (κ-CN) through a sulphydril-disulphide interaction (Fox et al., 2017). In this way, rennet enzymes are sterically prevented from hydrolyzing κ-CN (Dalgleish, 1993; Guinee, 2021). In addition, β-LG binds to para-κ-CN cysteine residues during hydrolysis, reducing the capability of casein micelles to aggregate (Creamer et al., 2004). Heat treatments are also responsible for the demineralization of casein micelles, which furthermore minimizes the aggregation capability (Fox, 1981; Touhami et

al., 2022). As such, less favorable MCP, e.g. longer RCT and weaker curd, are expected when dealing with pasteurized rather than raw milk (Yu et al., 2009).

The HTST pasteurization has a greater impact on MCP than LTLT. In fact, the extent of the detrimental effects on both the enzymatic and non-enzymatic phases of rennet-induced coagulation is determined by the intensity of heating. For instance, even short (15 s) treatments at a temperature greater than 75°C s cause notable damages to the cheese-making properties of milk (Fox et al., 2017).

The interest to further improve milk MCP has grown in recent years, both within the scientific community and among manufacturers. Phenomics can be considered a large scale acquisition of novel phenotypes to be studied and validated for several purposes, including for the definition of new breeding programs (Cole et al., 2020). However, the validation path requires collection of reference data for new phenotypes, which can be extremely expensive and cumbersome. Clotting parameters like RCT, a_{30} and k_{20} need to be determined through the reference analysis, lactodynamography, ideally using milk samples from both bulk tank and individual cows (Kübarsepp et al., 2005). Lactodynamography consists in the analysis of milk thromboelastography and provides various descriptors of coagulation speed and curd syneresis. Mid-infrared spectroscopy (MIRS) has proven very useful in the collection of data of interest in dairy species, including cattle. Beyond determining milk-related traits, spectral data can be exploited to assess and monitor the cows' health status. Nowadays, MIRS is the routine technology employed in DHI programs, as it is fast, easy to implement and relatively inexpensive. In addition, spectral data can be stored for later retrospective analyses (Gengler et al., 2016). Predictive models have been proposed in the past for a large scale acquisition of MCP data in cattle, sheep, goat and buffalo; in some countries, like Italy, such models are used to establish detailed milk payment systems or to estimate animals' breeding value (Cassandro et al., 2008; De Marchi et al., 2014; El Jabri et al., 2019). All above-mentioned predictive models rely exclusively on raw milk spectra and reference MCP data. However, a considerable amount of dairy industries manufacture cheeses from

pasteurized milk and knowing the potential coagulative performance of the pasteurized milk in advance, i.e., before heating (raw), would thus allow them to optimize milk standardization and processing. The objective of the present study was to understand which components in raw milk influence the MCP of the pasteurized milk. Particularly, we aim to i) quantitatively determine compositional traits of raw milk, including the detailed protein profile, that are reported to be involved in the MCP loss after pasteurization, ii) identify the main variable responsible for the deterioration of MCP when milk is subjected to heat treatment, and iii) develop MIRS prediction equations for pasteurized milk MCP using the spectra collected on the untreated (raw) samples.

2. Material and methods

2.1. Sampling and milk composition analysis

Milk was collected from 100 cows at different lactation stages, i.e. from 5 to 410 days after calving, by trained personnel during the morning milking. Cows with lactations from 1 to 8 were represented. Cows belonged to Simmental (40), Jersey (30), Holstein (20) and Rendena (10) breeds and were reared in 4 single-breed farms located in Northern Italy under intensive or semi-intensive farming conditions. Sampling took place between July and December 2021.

The tubes used for milk collection contained 0.05% (w/w) of preservative (Bronopol; 2-bromo-2-nitropropan-1,3-diol; Knoll Pharmaceuticals, Nottingham, UK) to prevent microbial spoilage. After filling, samples were transported (4°C) to the Department of Agronomy, Food, Natural resources, Animals and Environment of the University of Padua (Legnaro, Italy) within 2 h. For each sample, various aliquots were obtained: 50 mL was sent to the milk laboratory of Breeders Association of Veneto Region (ARAV, Padua, Italy) for MIRS spectra collection and gross composition determination; 10 mL was used to determine raw milk MCP; 10 mL underwent heat treatment to subsequently assess MCP in the pasteurized matrix; and 0.5 mL was kept for the determination of raw milk protein fractions via HPLC. The MIRS device MilkoScan FT7 (FOSS A/S, Hillerød, Denmark) provided information on content of fat, protein, lactose, and casein and

pH. According to the ISO 21543:2020 (ISO, 2020), samples were warmed at 37°C and homogenized by gentle inversion before analysis. The sample intake was set to 5 mL and the time required for a single analysis was 6 s. The somatic cell count (SCC) was also determined using a Fossomatic 7 DC (FOSS A/S, Hillerød, Denmark) following the ISO 13366-2:2006 (ISO, 2006) and was mathematically converted into somatic cell score (SCS) to normalize the distribution of the data.

2.2. Milk coagulation properties

Assessment of MCP at 60 min was performed in parallel for the raw and the pasteurized aliquot of milk through lactodynamographic analysis (MaPe System, Firenze, Italy). The LTLT pasteurization was carried out based on Fox et al. (2015), i.e. 63°C for 30 min in a water bath under mild agitation (Yu et al., 2009). The protocol proposed by Vigolo et al. (2022) was followed for the lactodynamographic analysis; in brief, milk was dispensed in the wells according to the scheme depicted in Fig. 1 and the whole plate was thereafter heated (35°C). 200 µL of a commercial calf rennet solution (Naturen Plus 215, Chr Hansen, Hørsholm, Denmark) diluted in distilled water (1.2:100 v/v) was added to each well to induce the coagulation. Measurements were taken for 60 min after rennet addition and the traits recorded included RCT, a_{30}, k_{20} and the curd firmness measured at 2 times the RCT (a2r). By definition, RCT is the time between the addition of rennet and coagulation initiation, k_{20} is the time necessary to reach a 20 mm firmness of the curd, and a_{30} measures the consistency of the curd at 30 min of analysis.

2.3. Analysis of protein fractions

Quantification of α-CN s2, α-CN s1, β-CN, κ-CN, α-LA and β-LG was performed on a small representative aliquot of raw milk by using the HPLC station Agilent 1260 Infinity II LC (Agilent Technologies, Santa Clara, CA) equipped with a quaternary pump (Agilent 1260 Infinity II, G7111B), a diode array Detector (Agilent 1260 Infinity II, G7115A), a column thermostat (Agilent 1260 Infinity II, G7116A), and an auto-sampler (Agilent 1260 Infinity II, G7129A). A reversed-

phase analytical column C8 (Zorbax 300SB-C8 RP, Agilent Technologies) preceded by a pre-column (300SB-C8 Guard Cartridges 4.6 × 12.5 mm, 4/PK, Agilent Technologies), was used for separation. Before injection, the samples were prepared as described in Bobe et al. (1998): briefly, 500 μL of milk were added to an aqueous solution of guanidine (Gdn) HCl (6 M GdnHCl, 0.1 m bisTris buffer, 5.37 mm sodium citrate, and 19.5 mm DTT) in a 1:1 ratio (v/v). Each sample was shaken for 10 s, incubated at room temperature for 1 h, and thereafter centrifuged at 13,000 *g* for 10 min at room temperature. The aqueous phase was diluted in the proportion 1:3 (v/v) with a solution containing 4.5 M GdnHCl in water, acetonitrile and trifluoroacetic acid (100:900:1). The chromatographic conditions were those described by Bonfatti et al. (2008), i.e. gradient elution was carried out with a mixture of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). Separations were performed with the following gradients: linear gradient from 33 to 35% B in 5 min, from 35 to 37% B in 4 min, from 37 to 40% B in 9 min, from 40 to 41% B in 4 min, isocratic elution at 41% B for 5.5 min, linear gradient from 41 to 43% B in 0.5 min, and from 43 to 45% B in 8 min. Before the injection of the subsequent sample, the column was re-equilibrated at 33% B for 8 min. The flow rate was 0.5 mL/min, the column temperature was kept at 45°C, the detection was made at a wavelength of 214 nm and the injection volume was 5 μL (Bonfatti et al., 2008). Agilent OpenLab 2 CDS software (Agilent Technologies, Santa Clara, SA) was used for data acquisition and analysis. The identification of single protein fractions was carried out using external standards of α-CN, β-CN, κ-CN, α-LA (Merck, Darmstadt, DE) and β-LG (BOC Sciences, NY, USA), and the quantification of each chromatographic peak was obtained with 5-point calibration curves (coefficient of determination ≥ 0.99).

2.4 Statistical analysis

Variables determining MCP of pasteurized milk were identified using the GLMSELECT procedure of SAS software v. 9.4 (SAS Institute Inc., Cary, NC). The explanatory variables imputed for RCT, a_{30}, k_{20} and a2r were: the correspondent MCP measured in the raw milk (both the first and

181 the second power), gross composition traits and all the protein fractions expressed as g/100 g of
182 total protein. The stepwise selection algorithm was the chosen selection method, with the Akaike
183 Information Criterion (AIC) used as selection/exclusion criterion; the selection criterion of the final
184 model was the adjusted coefficient of determination. Selection algorithm was refitted 1,000 times
185 on 80% of randomly selected samples and the variables selected in the final model were those
186 included in at least 10% of refitted models. The final output consisted in: intercept, average and SD
187 of variables regression estimates, first and third quartile of estimates, and variable inclusion rate, i.e.
188 rate of variables appearing in refitted models. Finally, for each MCP trait, the selected variables
189 were refitted to the dependent variable (RCT, a_{30}, k_{20} or a2r of the pasteurized samples) using a
190 multiple linear regression through the REG procedure of SAS, in order to assess the significance of
191 each covariate (selected variable), the estimates, and the standard error of estimates.
192
193 *2.5 Spectral collection and chemometric analysis*
194 Although milk spectra were collected in the window between 900 and 5,000 cm^{-1}, every 3.858
195 cm^{-1}, prediction models were developed using only part of the whole spectral region. In fact, only
196 wavelengths belonging to intervals that the manufacturer refers to as "good spectrum" (FOSS A/S,
197 Hillerød, DK) were kept to exclude regions associated with water-related noise and poor signal-to-
198 noise ratio. 450 spectral variables in the intervals 964.5 to 1,562.5 cm^{-1}, 1,720.7 to 2,291.7 cm^{-1}
199 and 2,415.1 to 2,970.7 cm^{-1} were available for each sample.
200 In order to improve the linear relationship between the spectra and reference values, statistical
201 procedures and mathematical pretreatments were applied to the milk spectrum. Prediction equations
202 were built using a modified partial least squares regression analysis (WinISI III v. 1.60; Foss and
203 Infrasoft International LLC, State College, PA) through a 5-fold cross-validation. Several
204 combinations in terms of both scattering correction (no correction, None; detrend, Det; standard
205 normal variate, SNV; SNV + Det; and standard multiplicative scatter correction, MSC) and
206 mathematical treatment (0,0,1,1; 1,4,4,1; 1,8,8,1; 2,5,5,1; and 2,10,10,1) were tested. The 4 digits

defining the mathematical treatment indicate: number of the derivative, gap used for derivative calculation, data points in the first smoothing, and data points in the second smoothing, respectively. The number of latent variables included in the model were selected according to van der Voet (1994).

Before each regression, spectral data points were evaluated for global Mahalanobis distance (GH) and those with GH > 3 were excluded. Hereafter, potential outliers were removed using the T-outlier test (Soyeurt et al., 2012) available in the WinISI software (Foss, Hillerød, Denmark), by setting the critical value to 3. Both the modified partial least squares regression and the outlier determination were iterated three times and the best prediction equation was chosen based on the standard error of cross-validation (SE_{CV}). The standard error (SE_C) and the coefficient of determination in calibration (R^2_C) as well as the coefficient of determination (R^2) in cross-validation (R^2_{CV}) were reported to evaluate the model performance.

3. Results & discussion

3.1. Overview of milk traits

Descriptive statistics of all milk parameters available are summarized in Table 1. Overall, the average and SD of gross composition traits and pH were in line with multi-breed studies carried out in Italy (Gottardo et al., 2017; Benedet et al., 2020) and other countries (Visentin et al., 2017; Frizzarin et al., 2021). The SCS averaged 2.38 and was characterized by a large coefficient of variation (81.11 %), in accordance with previous studies (Costa et al., 2019; Franzoi et al., 2020). The minimum, median and maximum SCC were 5 000, 55 500 and 6 077 000 cells/μL, respectively, suggesting that collected samples were representative of different udder health conditions (Gill et al., 1990; Franzoi et al., 2020). Casein fractions and concentration of whey proteins (g/100 g of total protein) revealed that the total protein content was mostly given by two fractions, the α-CN s1 and the β-CN fractions (Holt et al., 2013). These were also characterized by the lowest variability compared to the other fractions, with a CV of 6.13 and 10.65%, respectively.

Such low phenotypic variability is in agreement with Sanchez et al. (2019) who investigated protein fractions predicted via MIRS. In that study, MIRS models used for protein fractions were characterized by a moderate to good accuracy, with R^2_V ranging from 0.59 to 0.92. The contribution of α-CN s1 (33%) and β-CN (31%) to total protein content of Sanchez et al. (2019) is similar to the contribution seen in the present study: 27% and 30%, respectively. The same can be said for the contribution (15.5%) of the two whey proteins, which was equal to 16.5% in Sanchez et al. (2019).

Considering protein titers expressed in relation to volume (mg/mL of milk), average values reported in Table 1 are similar to those of Niero et al. (2016) who investigated casein fractions of 114 cows belonging to Holstein, Brown Swiss and Jersey breed. The amount of whey proteins, however, was slightly higher compared to previous studies; indeed, β-LG and α-LA averaged 3.71 and 1.30 mg/mL in Simmental cows (De Marchi et al., 2009) and 2.7 and 1.1 mg/mL in Jersey cows (Eskildsen et al., 2016). Nevertheless, results in Table 1 are similar to those reported by Frizzarin et al. (2021) for Irish cows: α-CN s2 (3.67 g/L, CV = 26%), α-CN s1 (14.09 g/L, CV = 17%), β-CN (12.80 g/L, CV = 17%), κ-CN (5.77 g/L, CV = 25%), α-LA (1.12 g/L, CV = 27%) and β-LG variant A (2.49 g/L, CV = 47%) and variant B (2.45 g/L, CV = 69%). In that study, the authors assessed the milk protein profile of cows belonging to various breeds via HPLC and determined MCP using the Formagraph (FOSS A/S, Hillerød, Denmark). As regards the MCP, Frizzarin et al. (2021) obtained descriptive statistics similar to the current study, with a mean equal to 20.81 min, 5.82 min and 32.24 mm for RCT, k_{20} and a_{30}, respectively. Moreover, Costa et al. (2019b) and Niero et al. (2021) reported similar statistics for MIRS-predicted traits related to coagulation ability. For instance, in Niero et al. (2021) the RCT averaged 21.64 min using multi-breed data. In the paper of Costa et al. (2019b), RCT and k_{20} of Holstein cows averaged 23.25 and 6.07 min, respectively. In that case, CV of MCP were smaller than those observed in the present study, which may likely be due to the larger sample size (>120,000 records) and to the presence of just a single breed. On the other hand, a_{30} differed from previous results obtained by Costa et al. (2019a), De Marchi et al (2007) and Niero et al. (2021) using traits predicted via MIRS, but were instead highly similar to results

reported by Frizzarin et al. (2021) and Zendri et al. (2017) for traits measured with the reference instrument. Using a database of samples from Holstein, Brown Swiss, Simmental, Rendena and Alpine Grey breeds reared in 15 farms located in Italian mountain areas, Zendri et al. (2017) reported an average a_{30} of 33.6 mm. Overall, slight discrepancies between the present study and literature are attributable to the limited amount of data (100 samples) and to the relative contribution of each breed to the database. Milk a2r has often been measured with the Formoptic, a device that provides the measurement in Firmness Index (FI); El Jabri et al. (2019) found values ranging from 13.35 to 29.04 FI, with a mean of 22.86 FI (CV = 11.74%). In the paper of Sanchez et al. (2019), the a2r was predicted through an equation developed using Formoptic measurements as reference data (R^2_V = 0.69) and obtained an a2r average of18.9 FI with a SD of 1.80.

The MCP of pasteurized milk were less favorable compared to those of raw milk (Table 1; Fig. 2). However, for some samples it was not possible to determine all MCP within the time frame (60 min) of the analysis. The deterioration observed could therefore somehow have been underestimated. In particular, some samples did not reach a curd firmness of 30 mm within the 60 min resulting in missing data points for the $a_{30.}$ On average, the absolute difference between raw and pasteurized samples was 7.19 min and 6.54 min for RCT and k_{20}, respectively. Moreover, pasteurization had an adverse effect on firmness-related traits, with a decrease of 13.02 mm and 10.31 mm for a_{30} and a2r, respectively. Casiraghi et al. (1989) reported longer RCT and slower coagulation rate for pasteurized milk and retentates derived from ultrafiltration, compared to their raw counterpart. Most of the studies that demonstrated a deterioration of MCP after heat treatment were conducted using higher temperatures and/or longer durations compared to conventional and commercial pasteurization treatments used in the field. Consequently, their results are not suitable for a direct comparison with findings reported in the present study (Ustunol & Brown, 1985; Anema et al., 2007). Blecker et al. (2012) demonstrated that milk heated to 60°C for 20 min had longer gelation time (0.7 min and 1.6 min at 30 and 40°C gelation temperature, respectively)

compared to raw milk. Moreover, compared to raw milk, the maximum firming rate of heated milk was 13 and 47% lower at 30°C and 40°C gelation temperature, respectively (Blecker et al., 2012).

3.2. Raw milk variables selected

The most informative variables selected by the algorithm to explain the pasteurized milk MCP are reported in Table 2, Table 3, Table 4 and Table 5. For all the MCP, the specific trait itself determined in raw milk was selected by the algorithm as being greatly relevant for determining its corresponding value in the pasteurized samples (Fig. 2).

3.2.1 RCT

The RCT of pasteurized milk was primarily influenced by the RCT measured in raw milk. The proposed regression model resulted in a Lin's concordance correlation coefficient (CCC) of 0.87 (Lin, 1989) with an R^2 of 0.88. The Lin's CCC provides information about the concordance between a predicted and a reference (gold standard) trait. In order of importance, the other influencing variables were lactose content, protein content, pH and κ-CN, with the latter being selected in only 17.8% of the resampling iterations (Table 2). In fact, the effect of κ-CN was not significant in the subsequent fitted regression (P = 0.188). This confirmed findings of previous publications by Marziali & Ng-Kwai-Hang (1986) and Politis & Ng-Kwai-Hang (1988) who demonstrated that κ-CN concentration does not have a significant effect on RCT in raw milk. Nevertheless, literature has shown that that κ-CN and β-LG interact during heating, causing a reduction in the ability of the milk to coagulate. The presence of κ-CN in just 17.8% of the variable selection iterations together with the not significant P-value found in regression (P=0.188; Table 2) may suggest that future studies would benefit from a larger sample size to increase statistical power of the study. This would allow a better understanding of the behavior of κ-CN in pasteurized milk and thus disclose the relationship between κ-CN and RCT.

All the other selected variables showed a significant *P*-value in the multiple linear regression for RCT. The desired values are those in the negative direction, thus raw milk with higher protein content and lower lactose content had shorter/better RCT after pasteurization. This was also demonstrated in Guinee et al. (1996) and is important for cheese producers who rely on pasteurized milk. In fact, findings indicate that proper standardization adjustments of protein content at tank level (raw milk) could compensate the inevitably longer RCT. By studying the effect of processing procedures like heat treatment and mechanical stress on MCP, Casiraghi et al. (1989) observed that pasteurization was the main milk treatment responsible for longer RCT. The lactose content shows low variability in bovine milk, especially in standardized conditions, like bulk tank in industrial dairy plants. Nevertheless, in this study the raw milk lactose content was one of the main determinants of the RCT of pasteurized milk, with greater concentrations being related to longer/worse RCT. In cows, both greater lactose content and better MCP were observed in milk secreted by healthy mammary glands (Costa et al., 2019a, 2019b) which is in contrast with results observed in this study. Although the mechanisms that make lactose relevant in RCT after heating deserve a more thorough investigation, two potential explanations may be considered: i) the outcome is an artefact due to the small sample size and the low variability of lactose content, ii) during heat treatment, the isomerization of lactose -coupled with its interaction with certain milk components- could make the starting raw milk lactose concentration particularly relevant for RCT of heat-treated milk. The latter seems a reliable hypothesis, as specific lactose-derived compounds such as lactulose and furosine are detectable exclusively in heat-treated milk. Lactulose is the product of lactose isomerization, while furosine represents the first stable product of the Maillard reaction (Mendoza et al., 2005; van den Oever and Mayer, 2021). Lactulose, for example, can be used as an indicator of the level of heat treatment to which the milk was subjected (Olano et al., 1989). In addition, the concentration of both lactose and minerals in the starting raw milk directly determines the amount of lactulose produced during the thermal treatment (Olano et al., 1989; van den Oever and Mayer, 2021). Based on Fox et al. (2015), treatments at temperatures greater than

100°C result in lactose degradation along with the liberation of its organic compounds, followed by a decrease in pH. Further investigations may reveal the exact dynamics that link raw milk lactose and pasteurized milk RCT.

Finally, the pH of the raw milk had an effect on the RCT after pasteurization (Table 2). In particular, more acidic raw milk samples were those presenting a shorter RCT after heat treatment. A greater milk acidity is generally in favor of MCP due to the concentration of desirable salts, like Ca^{2+} (Fox et al., 2015) and the intrinsic ability of low pH to increase the heat stability of milk (Miller & Sommer, 1940; Rose, 1962). This is in agreement with Ménard et al. (2005) who demonstrated that raw milk pH is one of the factors responsible for differences in RCT before and after pasteurization of reconstituted milk. In particular, the difference in RCT between raw and pasteurized milk was smaller if the starting milk pH was lower, i.e. more acidic. Having a lower pH at pasteurization stabilizes the κ-CN interaction with casein micelles, reducing migration of κ-CN to the whey phase (Ménard et al., 2005).

3.2.2 Other MCP

According to Table 3, the a_{30} of pasteurized milk can be predicted from various raw milk traits, namely a_{30} (squared), κ-CN, α-CN s1, β-CN, whey proteins, lactose, SCS and pH. The model of a_{30} was characterized by a Lin's CCC of 0.84 and an R^2 of 0.72. Although the most important variables in terms of inclusion rate were a_{30} (squared) and whey proteins, the multiple linear regression revealed that only the former was a significant covariate factor for the targeted trait. Similarly, only a few variables among the total selected showed a significant effect on the k_{20} in regression (Table 4). In order of inclusion rate, the k_{20}-related variables were protein content, α-CN s2, α-LA and β-LG (Lin's CCC = 0.73, R^2 = 0.58). Similarly, raw milk β-LG had a significant and negative effect also on the a2r (Table 5) and the other significant covariate found for this MCP was the squared raw milk a2r (Lin's CCC = 0.83, R^2 = 0.70). The undesired effect of raw milk β-LG on the coagulation ability of pasteurized milk has previously been discussed by Kannan & Jennes (1961) and current

results support the hypothesis that β-LG exerts its detrimental effect by binding to para-κ-CN cysteine residues, reducing casein micelles aggregation rate (Creamer et al., 2004). Overall, whey proteins measured in raw milk were important for all the MCP, suggesting that high or low concentrations determine a difference in the coagulation ability. As an example, elevated concentrations of α-LA in the raw matrix were associated with a more desirable a_{30} (Table 3) and k_{20} (Table 4) after pasteurization. As regards the β-LG, a lower concentration in the raw sample resulted in a better a2r (Table 5) with an inclusion rate of 91.4%; concurrently, β-LG was also selected as an explaining variable for a_{30} (Table 3) and k_{20} (Table 4) in the negative direction.

In general, for all MCP, the selected variables highlight the importance of supplying dairy factories with raw milk of good technological aptitude.

3.3.MIRS prediction

Table 6 shows the prediction performance of MIRS for MCP using spectra collected on the raw matrix, and the scatter plots of reference and the predicted values for both raw and pasteurized milk are reported in Fig. 3. Based on the outcomes, the coagulation ability of pasteurized milk can be predicted with an accuracy sufficient for screening purposes (Grelet et al., 2020). The only exception is given by k_{20}, which was not predictable using the raw milk spectral data, having an R^2_{CV} of 0.26. In both milks, RCT was the trait with the best R^2_{CV}, equaling to 0.64 and 0.66 prior and post pasteurization, respectively. Overall, the MIRS predictive ability for pasteurized milk MCP mirrors the predictive ability of the raw milk (Table 6). On a routine basis, MCP prediction equations are used for raw milk in Italy and their accuracy only allows for a rough screening (Visentin et al., 2016). In Visentin et al. (2016), lower accuracies were achieved: R^2_{CV} of 0.55, 0.56 and 0.59 for k_{20}, a_{30} and RCT, respectively. On the other hand, by using lactodynamography as the gold standard, De Marchi et al. (2013) reported greater R^2_{CV} compared to the present study: 0.72 (k_{20}), 0.70 (a_{30}) and 0.76 (RCT).

386 Given the accuracies presented in Table 6, predicted and reference values were expected to show
387 a diverging distribution. As reported in previous research (e.g., Costa et al., 2021), an MIRS-
388 predicted trait can be scarcely correlated with its reference at the phenotypic level. This is
389 particularly true for difficult-to-measure phenotypes like MCP, whose prediction accuracy tends to
390 be moderate to low.
391
392 **4. Conclusion**
393 Regression coupled with prior variable selection allowed for the identification of raw milk traits
394 responsible for the reduction in pasteurized milk coagulation ability. Findings revealed that raw
395 milk technological properties, pH, total protein content and detailed protein fractions were
396 important factors to consider when assessing the detrimental effect of heating on MCP. The raw
397 milk delivered to the dairy factory by farmers must be of high quality to preserve the technological
398 ability and maintain favorable MCP after pasteurization. Out of the four MCP traits, only RCT had
399 reliable prediction accuracy (coefficient of determination in cross-validation = 0.66) and can thus be
400 predicted in advance using the raw milk spectra. This study provides new insights into the
401 deterioration of cheese-making properties of milk following heat treatment. Such novel insights are
402 of great interest for dairy plants manufacturing cheese from pasteurized milk as having knowledge
403 of the MCP of pasteurized milk in advance is useful for defining proper strategies and to support
404 decision-making about the incoming raw milk. In perspective, efforts should be made to develop
405 more robust prediction models using bulk milk data ,considering both HTST and LTLT
406 pasteurization protocols available.
407
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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this study.

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Table 1

Descriptive statistics[a] of milk composition traits, protein profile, and coagulation properties.

Trait[b]	N	Mean	SD	CV, %	Minimum	Maximum
Raw milk						
Gross composition						
Fat (%)	100	4.65	1.51	32.52	2.24	11.23
Protein (%)	100	3.63	0.44	12.26	2.78	5.08
Lactose (%)	100	4.73	0.21	4.45	4.03	5.23
SCS	100	2.38	1.93	81.11	-1.32	8.93
pH	100	6.57	0.08	1.26	6.24	6.79
Protein fractions (mg/mL)						
α-CN_{s2}	100	5.76	1.41	24.48	3.25	11.37
α-CN_{s1}	100	11.82	1.98	16.78	8.30	19.04
β-CN	100	13.25	2.80	21.10	1.61	23.25
κ-CN	100	6.25	1.33	21.24	3.19	10.61
α-LA	100	1.71	0.26	15.32	1.23	2.50
β-LG	100	5.01	1.09	21.67	2.00	7.62
Protein fractions (g/100g)						
α-CN_{s2}	100	13.18	2.72	20.60	7.49	30.31
α-CN_{s1}	100	27.00	1.65	6.13	23.67	35.83
β-CN	100	30.11	3.21	10.65	4.28	35.03
κ-CN	100	14.25	1.85	12.96	7.41	18.29
α-LA	100	3.95	0.62	15.76	2.62	5.72
β-LG	100	11.55	2.31	19.97	4.47	16.98
Coagulation properties						
RCT (min)	94	20.46	8.53	41.68	6.75	50.63
k_{20} (min)	87	6.41	3.88	60.52	1.75	21.50
a_{30} (mm)	83	30.86	13.22	42.83	1.00	51.90
a2r (mm)	78	37.65	7.32	19.43	17.64	51.00
Pasteurized milk						
Coagulation properties						
RCT (min)	92	27.65	12.38	44.75	8.50	59.00
k_{20} (min)	65	12.95	7.83	60.47	2.13	36.00
a_{30} (mm)	62	17.84	11.31	63.38	1.00	47.00
a2r (mm)	61	27.34	8.30	30.37	12.70	43.10

[a] N: number of samples; SD: standard deviation; CV: coefficient of variation. [b]SCS: somatic cell score, α-CN_{s1}: α-casein s1; α-CN_{s2}: α-casein s2; β-CN: β-casein; κ-CN: κ-casein; α-LA: α-lactalbumin; β-LG: β-lactoglobulin; RCT: rennet coagulation time; k_{20}: curd-firming rate; a_{30}: curd firmness; a2r: curd firmness at two times the rennet coagulation time.

Table 2

Variables selected by the stepwise algorithm explaining variability of rennet coagulation time of pasteurized milk and multiple linear regression output.

Trait[a]	Stepwise variable selection							Multiple linear regression		
	Estimate[b]					Effect inclusion rate (%)	r[c]	Estimate	SE[d]	*P*-value
	Average	SD	q1	median	q3					
Intercept	143.80	21.36	131.89	144.01	156.49	100.0		143.69	39.75	<0.001
Raw milk										
RCT (min)	1.38	0.04	1.36	1.38	1.41	100.0	0.91*	1.39	0.06	<0.001
κ-CN (g/100g)	-34.18	12.70	-42.28	-33.69	-25.43	17.8	0.02	-34.31	25.86	0.188
Protein (%)	-4.06	0.50	-4.36	-4.06	-3.74	99.9	-0.06	-4.07	1.09	<0.001
Lactose (%)	10.58	1.64	9.52	10.53	11.68	100.0	-0.03	10.54	2.66	<0.001
pH	-26.58	3.64	-28.95	-26.68	-24.41	99.9	-0.22*	-26.52	6.32	<0.001

[a] RCT: rennet coagulation time; κ-CN: κ-casein.[b] SD: standard deviation; q1: first quartile; q3: third quartile. [c] r: Pearson correlation (*P<0.05) with pasteurized milk RCT. [d] SE: standard error of the estimate.

Table 3

Variables selected by stepwise algorithm explaining the variability of curd firmness of pasteurized milk and multiple linear regression output.

Trait[a]	Stepwise variable selection							Multiple linear regression		
	Estimate[b]					Effect inclusion rate (%)	r[c]	Estimate	SE[d]	*P*-value
	Average	SD	q1	median	q3					
Intercept	-115.72	49.06	-147.43	-115.17	-85.81	100.0		-114.33	89.73	0.208
Raw milk										
a_{30*} a_{30} (mm^2)	0.01	0.00	0.01	0.01	0.01	99.5	0.80*	0.01	0.00	<0.001
κ-CN (g/100g)	60.21	33.33	39.83	59.98	79.90	25.4	-0.09	58.65	54.94	0.291
α-CN_{s1} (g/100g)	44.53	37.81	19.14	42.16	69.05	21.1	0.19	43.89	71.75	0.543
β-CN (g/100g)	-39.43	29.36	-57.73	-38.74	-20.24	12.9	0.09	-38.93	61.09	0.527
α-LA (g/100g)	282.24	131.36	210.44	284.40	363.79	50.5	-0.02	280.77	181.19	0.127
β-LG (g/100g)	-85.60	36.70	-108.84	-84.16	-62.13	47.8	-0.22	-84.41	58.21	0.153
Lactose (%)	-7.95	3.11	-9.92	-7.79	-5.84	11.4	0.02	-8.01	5.85	0.177
SCS	-0.37	0.29	-0.52	-0.33	-0.17	16.4	-0.09	-0.38	0.47	0.426
pH	21.58	6.91	16.72	21.66	26.41	30.6	0.08	21.45	14.43	0.143

[a] a_{30}: curd firmness; α-CN_{s1}: α-casein s1; β-CN: β-casein; α-LA: α-lactalbumin; β-LG: β-lactoglobulin; SCS: somatic cell score.[b] SD: standard deviation; q1: first quartile; q3: third quartile. [c] r: Pearson correlation (*P<0.05) with pasteurized milk a_{30}; SE: standard error of the estimate.

Table 4

Variables selected by stepwise algorithm explaining the variability of curd firming time of pasteurized milk and multiple linear regression output.

Trait[a]	Stepwise variable selection							Multiple linear regression		
	Estimate[b]					Effect inclusion rate (%)	r[c]	Estimate	SE[d]	*P*-value
	Average	SD	q1	median	q3					
Intercept	13.55	11.16	7.12	14.83	20.66	100.0		14.90	14.25	0.300
Raw milk										
k_{20} (min)	2.50	1.06	1.88	2.47	3.14	60.8	0.63*	2.37	1.92	0.221
k_{20}*k_{20} (min^2)	-0.03	0.09	-0.07	-0.03	0.03	39.3	0.63*	-0.02	0.17	0.906
α-CN_{s2} (g/100g)	101.18	23.33	87.07	101.74	117.06	77.5	0.13	99.20	39.66	0.015
α-LA (g/100g)	-337.48	81.24	-391.87	-341.73	-282.12	73.0	0.05	-341.68	151.27	0.028
β-LG (g/100g)	80.69	19.63	68.55	80.66	93.25	63.6	0.14	81.39	34.34	0.021
Fat (%)	0.47	0.40	0.19	0.39	0.74	17.3	0.12	0.42	0.54	0.435
Protein (%)	-6.49	1.49	-7.52	-6.85	-5.78	93.7	-0.54*	-6.60	1.86	<0.001

[a] k_{20}: curd firming rate; α-CN_{s2}: α-casein s2; α-LA: α-lactalbumin; β-LG: β-lactoglobulin. [b] SD: standard deviation; q1: first quartile; q3: third quartile. [c] r: Pearson correlation (*P<0.05) with pasteurized milk k_{20}. [d] SE: standard error of the estimate.

Table 5

Variables selected by stepwise algorithm explaining the variability of curd firmness at two times the rennet coagulation time of pasteurized milk and multiple linear regression output.

Trait[a]	Stepwise variable selection							Multiple linear regression		
	Estimate[b]					Effect inclusion rate (%)	r[c]	Estimate	SE[d]	*P*-value
	Average	SD	q1	median	q3					
Intercept	27.76	3.91	25.14	27.31	30.24	100.0		27.45	7.38	<0.001
Raw milk										
a2r[*]a2r (mm^2)	0.01	0.00	0.01	0.01	0.01	95.5	0.81*	0.01	<0.01	<0.001
α-CN_{s2} (g/100g)	-41.27	24.05	-55.56	-40.06	-24.89	14.8	-0.11	-40.39	36.65	0.275
β-LG (g/100g)	-87.48	15.18	-97.07	-87.33	-77.38	91.4	-0.21	-87.37	32.49	0.001
Fat (%)	-0.68	0.27	-0.85	-0.66	-0.50	34.4	-0.20	-0.65	0.47	0.168

[a] a2r: curd firmness at two times the rennet coagulation time; α-CN_{s2}: α-casein s2; β-LG: β-lactoglobulin.[b] SD: standard deviation; q1: first quartile; q3: third quartile. [c] r: Pearson correlation (*P<0.05) with pasteurized milk a2r. [d] SE: standard error of the estimate.

Table 6

Spectral treatments and fitting statistics[a] of the Fourier-Transformed mid-infrared spectroscopy calibrations developed for the four coagulation properties.

Trait[b]	Correction	Math treatment[c]	N	SE_C	R^2_C	SE_{CV}	R^2_{CV}
Raw milk							
RCT (min)	SNV	2,5,5,1	83	3.43	0.79	4.53	0.64
k20 (min)	None	2,5,5,1	69	1.20	0.73	1.55	0.54
a30 (mm)	MSC	2,5,5,1	76	6.85	0.74	9.02	0.54
a2r (mm)	D	0,0,1,1	66	3.39	0.76	3.74	0.70
Pasteurized milk							
RCT (min)	None	2,5,5,1	79	5.41	0.79	6.89	0.66
k20 (min)	D	1,8,8,1	52	4.02	0.51	4.89	0.26
a30 (mm)	D	1,4,4,1	49	6.09	0.69	8.04	0.45
a2r (mm)	SNV	1,4,4,1	56	3.64	0.80	4.80	0.65

[a] SNV: standard normal variate; MSC: multiplicative scatter correction; D: detrending; N: number of samples; SE_C: standard error in calibration; R^2_C: coefficient of determination in calibration; SE_{CV}: standard error in cross-validation; R^2_{CV}: coefficient of determination in cross-validation. [b] RCT: rennet coagulation time, k20: curd firming rate, a30: curd firmness, a2r: curd firmness at two times the rennet coagulation time. [c] Digits indicate number of the derivative, gap used for derivative calculation, data points in the first smoothing, and data points in the second smoothing, respectively.

Figures captions

Fig. 1. Representation of the plate used for lactodynamographic analysis of raw (R) and pasteurized (P) milk samples (n = 5 at each run).

Fig. 2. Diagrams obtained from the lactodynamographic analysis of three bovine milk samples A) after and B) before pasteurization.

Fig. 3. Scatter plot of measured and predicted A) rennet coagulation time (RCT), B) curd firming time (k_{20}), C) curd firmness (a_{30}), and D) curd firmness at 2 times RCT (a2r) in raw (●) and pasteurized (■) milk.