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Effects of calf rennet, and microbial and plant coagulants on rheological properties of milk for Grana Padano PDO cheese production

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ABSTRACT

The effects of calf rennet, and microbial and plant coagulants on milk coagulation properties (MCP) measured through lactodynamographic analysis were investigated. Each coagulant was tested at 7 different dilutions: one reference dilution, 3 lower dilutions (−30%, −20%, and −10% IMCU L^{-1} milk) and 3 greater dilutions (+10%, +20%, and +30% IMCU L^{-1} milk). Sources of variation of rennet coagulation time (RCT, min), curd-firming time (k_{20}, min), and curd firmness (a_{30}, mm) were investigated through a linear model that included the effects of coagulant, dilution, and their interaction. All the effects were highly significant in explaining the variability of the studied traits (P <0.001). In particular, calf rennet resulted in shorter k_{20} and greater a_{30} than microbial and plant coagulants. Dilution effect was linearly associated with MCP, whereby a decreasing amount of coagulant led to a lengthening of RCT and k_{20}, whereas a less clear association was observed for a_{30}.

1. Introduction

The efficiency of cheese manufacturing processes and the quality of the resulting products are widely determined by milk composition and ability to coagulate after the addition of clotting agent (Johnson, Chen, & Jaeggi, 2001). The most informative traits associated with the coagulation process are assessed through lactodynamographic analysis (Malacarne et al., 2014) and can be summarised in 3 milk coagulation properties (MCP), namely (i) rennet coagulation time (RCT, min), which is the time between the addition of the clotting agent and the beginning of coagulation, (ii) curd-firming time (k_{20}, min), which is the time required to achieve 20 mm of firmness, and (iii) curd firmness (a_{30}, mm), which is the final firmness of the coagulum 30 min after the addition of the clotting agent (Manuelian, Boselli, Vigolo, Giangolini, & De Marchi, 2020). In particular, when considering lactodynamographic analysis, a_{30} relates to the physical movement of the pendula within the instrument as influenced by progressive curd aggregation, thus being measured in mm, whereas in the context of rheological analyses, firmness is evaluated in compressive terms, thus being expressed in *g* or N. In general, shorter RCT and k_{20}, and greater a_{30} are desired as they are associated with more efficient coagulation process and greater cheese yield, which in turn are related to adequate milk casein content (Emmons, Ernstrom, Lacroix, & Verret, 1990) and organic calcium (Franzoi, Niero, Penasa, Cassandro, & De Marchi, 2018), and low somatic cell count (Ikonen, Morri, Tyrisevä, Ruottinen, & Ojala, 2004).

Different types of milk coagulants are available on the market. In general, their enzymatic activity is aimed to the hydrolysis of Phe_{105}—Met_{106} bond of κ-casein, which is necessary to induce milk syneresis and curd formation through casein micelles aggregation (Egito et al., 2007; Liburdi, Spinelli, Benucci, Lombardelli, & Esti, 2018; Manuelian et al., 2020). In the Italian context, the production of the most widespread PDO cheeses adopt calf rennet, which contains chymosin and pepsin enzymes (Moschopoulou, 2011). The production of calf rennet is characterised by relatively high costs (Gomes et al., 2019; Liu et al., 2021) and possible ethical concerns, due to the fact that calves are slaughtered at young age since chymosin content decreases with animal growth (Shah, Mir, & Paray, 2014).

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Ultimately, various factors associated with calf rennet such as high price, dietary habits and/or ethical issues (e.g., vegetarians and vegans), and cultural and religious concerns have encouraged the development of alternative milk clotting agents (Galán, Prados, Pino, Tejada, & Fernández-Salguero, 2008). In this view, microbial coagulants represent a practical alternative, being commonly adopted in cheese production. In particular, *Aspergillus niger* var. *awamori* fungus (Ustunol & Hicks, 1990) and *Rhizomucor miehei* fungus (Silveira, Oliveira, Ribeiro, Monti, & Contiero, 2005) are grown by submerged fermentation on vegetable substrates to produce alternative coagulants to calf rennet. The adoption of microbial coagulants may have a significant influence on the development of sought-after cheese flavours and textures, which indeed represent an interesting research field (Alves, Merheb-Dini, Gomes, Da Silva, & Gigante, 2013).

Plant coagulants are another possible alternative to calf rennet (Roseiro, Barbosa, Ames, & Wilbey, 2003). In recent years, requests for cheeses produced with plant coagulants has increased (Zikiou & Zidoune, 2019). Plant coagulant can be extracted from *Cynara cardunculus* which contains cardosin, a protease enzyme possessing high milk clotting activity. Flowers of *C. cardunculus* contain aspartic proteases such as cardosin A and cardosin B, which are similar to chymosin and pepsin in terms of activity and specificity towards milk casein. Similar to calf chymosin, cardosin has the ability to hydrolyse the Phe_{105}—Met_{106} bond of κ-casein, which is necessary to induce milk syneresis (Liburdi et al., 2018; Manuelian et al., 2020). The use of plant coagulants at industrial level is still limited, mainly due to the resulting bitter flavours in ripened cheeses (Almeida & Simões, 2018). In general, the major drawback of most plant coagulants is the development of an increased bitterness and the appearance of texture defects during cheese ripening (Egito et al., 2007).

With this background, the aim of the present study was to assess the effect of calf rennet, and microbial and plant coagulants on rheological properties of milk for Grana Padano PDO cheese production measured through lactodynamographic analysis.

2. Materials and methods

2.1. Experimental design

The experimental design was set up to assess the effect of 4 coagulants characterised by specific international milk clotting units (IMCU) per L of milk on MCP: (i) calf rennet (95% chymosin and 5% pepsin; 1000 IMCU mL^{-1} rennet; reference dilution 45 IMCU L^{-1} milk); (ii) microbial coagulant from *Asp. niger* var. *awamori* (100% chymosin; 1000 IMCU mL^{-1} coagulant; reference dilution 35 IMCU L^{-1} milk); (iii) microbial coagulant from *Rhizomucor miehei* (100% mucor pepsin; 750 IMCU mL^{-1} of coagulant; reference dilution 45 IMCU L^{-1} milk); (iv) plant coagulant from *C. cardunculus* (100% cardosin; 55 IMCU mL^{-1} coagulant; reference dilution 28 IMCU L^{-1} milk).

Milk and whey starter samples were collected across 4 consecutive days (Day 1, Day 2, Day 3, and Day 4) in a dairy company located in Veneto Region. On Day 1, one aliquot of 1 L of milk was collected directly from a single vat intended for Grana Padano Protected Designation of Origin (PDO) cheese production, along with one aliquot of 0.10 L of whey starter, i.e., cooked whey from previous cheese-making. Immediately after collection, milk and whey starter were transported at 4 °C to the Department of Agronomy, Food, Natural resources, Animals and Environment of the University of Padova (Legnaro, Italy) for milk gross composition and lactodynamographic analysis. Calf rennet was tested according to 7 different dilutions prepared in distilled water: 3 out of the 7 dilutions were prepared using less coagulant (−30%, −20%, and −10% IMCU L^{-1} milk) than the reference dilution; 1 out of the 7 dilutions was prepared according to the reference dilution reported in manufacturer's instructions; the remaining 3 dilutions were prepared using more coagulant (+10%, +20%, and +30% IMCU L^{-1} milk) than the reference dilutions. Six technical replicates were performed for each dilution.

The experimental protocol described above was repeated on Day 2, Day 3, and Day 4 to test coagulants from *Asp. niger* var. *awamori*, *R. miehei*, and *C. cardunculus*, respectively, leading to a total of 4 milk samples and 4 cooked whey samples which were used to determine milk gross composition and to perform 168 lactodynamographic analyses. The experimental design is schematically reported in Table 1.

2.2. Milk gross composition and lactodynamographic analysis

Milk samples were warmed at 37 °C, gently mixed by inversion to promote solid homogenisation, and analysed for gross composition (fat, protein, casein, and lactose, %), pH and titratable acidity (°SH 50 mL^{-1}) using MilkoScan FT3 (Foss, Hillerød, Denmark).

Soon after, lactodynamographic analysis was performed according to the protocol proposed by McMahon and Brown (1982). Briefly, the previously warmed milk was inverted again to promote fat and solids homogenisation; 9.5 mL of milk were dispensed in each of 10 wells of Formagraph 3.0 (Maspress, Firenze, Italy) and warmed again, up to 37 °C; 0.3 mL of whey starter and 0.2 mL of diluted coagulant were added to the warmed milk, and the resulting solution was gently mixed with a plastic spatula; the lactodynamographic analysis started immediately after the previous step, lasted 30 min, and recorded RCT (min), k_{20} (min), and a_{30} (mm).

2.3. Statistical analysis

Repeatability of RCT, k_{20}, and a_{30} was calculated as the relative standard deviation (RSD_r) of 6 consecutive measurements within each coagulant and for each dilution (Niero et al., 2019).

Sources of variation of MCP were investigated through the GLM procedure of SAS (SAS Institute Inc., Cary, NC) according to the following linear model:

Table 1
Experimental design.[a]

Coagulant	Dilution (replicates)							Total
	−30%	−20%	−10%	Reference	+10%	+20%	+30%	
Calf rennet	Day 1 (6)	Day 1 (6)	Day 1 (6)	Day 1 (6)	Day 1 (6)	Day 1 (6)	Day 1 (6)	42
Aspergillus niger var. *awamori*	Day 2 (6)	Day 2 (6)	Day 2 (6)	Day 2 (6)	Day 2 (6)	Day 2 (6)	Day 2 (6)	42
Rhizomucor miehei	Day 3 (6)	Day 3 (6)	Day 3 (6)	Day 3 (6)	Day 3 (6)	Day 3 (6)	Day 3 (6)	42
Cynara cardunculus	Day 4 (6)	Day 4 (6)	Day 4 (6)	Day 4 (6)	Day 4 (6)	Day 4 (6)	Day 4 (6)	42

[a] Within a day, each coagulant (calf rennet, *Aspergillus niger* var. *awamori*, *Rhizomucor miehei*, and *Cynara cardunculus*) was tested at seven dilutions (−30%, −20%, −10%, reference, +10%, +20%, +30% IMCU L^{-1} milk). Six technical replicates were performed within each coagulant and dilution.

$$y_{ijk} = \mu + C_i + D_j + (C \times D)_{ij} + e_{ijk},$$

where y_{ijk} is the dependent variable (RCT, k_{20}, or a_{30}); C_i is the fixed effect of the ith coagulant (i = calf rennet, *Asp. niger* var. *awamori*, *R. miehei*, *C. cardunculus*); D_j is the fixed effect of the jth dilution (j = −30%, −20%, −10%, reference, +10%, +20%, +30% IMCU L^{-1} milk); $(C \times D)_{ij}$ is the first order interaction between the ith coagulant and the jth dilution; and e_{ijk} is the residual ~N(0, σ_e^2), where σ_e^2 is the residual variance. A multiple comparison of the means was performed for the fixed effects using Bonferroni's test (P <0.05).

3. Results and discussion

3.1. Descriptive statistics of milk gross composition and coagulation properties

Descriptive statistics for fat, protein, casein, lactose, pH and titratable acidity of milk samples used for lactodynamographic analyses are reported in Table 2. Fat, protein, and casein content averaged 2.85, 3.35, and 2.73%, respectively. The relatively low fat content is due to natural creaming followed by partial milk skimming that is adopted for Grana Padano PDO manufacturing (Specification of Grana Padano PDO, 2022). Fat-to-casein ratio averaged 1.04, which indeed falls within the range prescribed by Grana Padano PDO disciplinary (Specification of Grana Padano PDO, 2022). Milk gross composition of the present study reflects that reported by Chiarin, Niero, Cassandro, De Marchi, and Penasa (2023) who assessed the effectiveness of different spectroscopy techniques for traceability purposes of Grana Padano PDO cheese. Milk pH averaged 6.51, being close to that reported by Pretto, De Marchi, Penasa, and Cassandro (2013) in a study on the effect of milk composition and coagulation traits on Grana Padano cheese yield. At the same time, such pH is lower compared with that reported by other authors (Niero et al., 2020), probably due to the addition of aged whey adopted in the present study, as prescribed for Grana Padano cheese manufacturing. The coefficient of variation ranged from 0.08% for lactose to 0.68% for titratable acidity.

Table 2
Descriptive statistics of gross milk composition, rennet coagulation time (RCT), curd-firming time (k_{20}), and curd firmness (a_{30}).[a]

Trait	n	Mean	SD	CV (%)
Milk composition				
Fat (%)	4	2.85	0.010	0.35
Protein (%)	4	3.35	0.004	0.12
Casein (%)	4	2.73	0.004	0.15
Lactose (%)	4	4.82	0.004	0.08
pH	4	6.51	0.02	0.31
Titratable acidity (°SH 50 mL^{-1})	4	2.94	0.02	0.68
Milk coagulation properties				
RCT (min)				
Calf rennet	42	9.63	1.85	19.21
Aspergillus niger var. *awamori*	42	14.24	3.20	22.47
Rhizomucor miehei	42	8.62	1.83	21.23
Cynara cardunculus	42	11.21	1.65	14.72
k_{20} (min)				
Calf rennet	42	2.82	0.44	15.60
Aspergillus niger var. *awamori*	42	3.06	0.59	19.28
Rhizomucor miehei	42	3.44	0.83	24.13
Cynara cardunculus	42	3.22	0.36	11.18
a_{30} (mm)				
Calf rennet	42	48.39	2.92	6.03
Aspergillus niger var. *awamori*	42	45.13	4.85	10.75
Rhizomucor miehei	42	46.32	4.26	9.83
Cynara cardunculus	42	47.20	2.19	4.64

[a] Abbreviations are: SD, standard deviation; CV, coefficient of variation.

Such a very low variability was expected, since samples used in the present study consisted of standardised vat milks intended to produce a specific cheese (Grana Padano PDO) and collected on a short time span (4 consecutive days). The low variability is an important condition to achieve adequate method repeatability and to highlight the effects of type of coagulant and dilution, excluding other sources of variation as much as possible. Milk coagulation properties were characterised by greater variation than milk gross composition (Table 2), mainly because of the architecture of the experimental design which considered 7 different dilutions within each type of coagulant.

3.2. Significance of fixed effects

Results from the analysis of variance of MCP are reported in Table 3. The type of coagulant, the dilution of coagulant, and their interaction were highly significant (P <0.001) in explaining the variability of MCP. The coefficient of determination (R^2) ranged from 0.81 (a_{30}) to 0.98 (RCT) suggesting that a large part of the phenotypic variance of the studied traits was explained by fixed effects included in the model. The goodness of fitting of the model is confirmed also by the low root mean square error (RMSE) of MCP (Table 3). Considerably lower R^2 (0.15−0.17 for RCT and a_{30}, respectively) and greater RMSE (4.77 and 11.81 for RCT and a_{30}, respectively) were reported by Niero, Penasa, De Marchi, Visentin, and Cassandro (2015) who investigated MCP of Burlina local cattle breed. Such divergence is likely due to the fact that the present study was performed under controlled laboratory conditions, while Niero et al. (2015) analysed the variation of MCP on the basis of a longitudinal experimental design.

3.3. Effect of type of coagulant

Least squares means of RCT, k_{20}, and a_{30} for calf rennet, *Asp. niger* var. *awamori*, *R. miehei*, and *C. cardunculus* coagulants are depicted in Fig. 1. As expected, calf rennet can be regarded as the most effective coagulant, with the second best RCT (9.63 min), and the best k_{20} (2.82 min) and a_{30} (48.39 mm). Rennet coagulation time and k_{20} were generally shorter compared with those reported in literature (De Marchi et al., 2009; Pretto et al., 2013), likely due to some distinctive features of the present study, which included (i) the adoption of a controlled experimental design instead of longitudinal or observational approaches, (ii) the use of standardised and partially skimmed vat milk instead of individual raw milk or bulk raw milk, and (iii) the use of milk added with whey starter instead of milk only.

Among alternative coagulants, *Asp. niger* var. *awamori* showed the worst performances for RCT (14.24 min) and a_{30} (45.13 mm). Similar RCT (12.30 min) and a_{30} (48.90 mm) were observed by Kappeler et al. (2006) who tested the clotting ability of chymosin produced by *Asp. niger* var. *awamori* on bovine raw milk. In the present study, *R. miehei* had the shortest RCT (8.62 min) and the longest k_{20} (3.44 min; Fig. 1). Slightly longer RCT was reported by Sheehan, O'Sullivan, and Guinee (2004) who used coagulant obtained from *R. miehei* for mozzarella cheese production.

C. cardunculus had intermediate coagulation properties with RCT, k_{20}, and a_{30} of 11.21 min, 3.22 min, and 47.20 mm, respectively. On average, when using plant coagulant, milk started coagulating 1.58 min later than milk with calf rennet (P <0.05). Manuelian et al. (2020) reported longer RCT (+12 min) for Mediterranean buffalo bulk milk added with *C. cardunculus* than with calf rennet. Even greater delays were reported by Liburdi, Boselli, Giangolini, Amatiste, and Esti (2019) who compared clotting time induced by calf rennet (12.22 min) with clotting time induced by *Ficus carica* (22.90 min) and *C. cardunculus* (42.30 min).

Table 3
F-values and significance of fixed effects included in the analysis of variance for rennet coagulation time (RCT), curd-firming time (k_{20}), and curd firmness (a_{30}).[a]

Milk coagulation property	Coagulant (C)	Dilution (D)	C × D	R^2	RMSE
RCT (min)	924.96***	425.45***	15.63***	0.98	0.52
k_{20} (min)	60.06***	147.15***	8.87***	0.90	0.22
a_{30} (mm)	23.34***	52.32***	11.26***	0.81	1.85

[a] Abbreviation: RMSE, root mean square error; ***P <0.001.

3.4. Effect of dilution

Least squares means of RCT, k_{20}, and a_{30} for different dilutions of coagulant are depicted in Fig. 2. As expected, RCT linearly decreased with greater dilutions of coagulant ($R^2 = 0.97$), ranging from 14.43 min for the lowest dilutions (−30% IMCU L^{-1} milk) to 8.29 min for the highest concentration (+30% IMCU L^{-1} milk). Landfeld, Novotna, and Houska (2002) reported a linear trend ($R^2 = 0.88$) using six different volumes of diluted calf rennet (20, 30, 40, 50, 60, and 70 mL) in 1 L of milk. In particular, those authors obtained the longest and the shortest RCT (18.85 and 6.93 min, respectively) at the lowest and the greatest rennet volume (20 and 70 mL, respectively). The shortening of RCT was observed also when increasing the concentration of *R. miehei* (Abbas, Foda, Kassem, Bayomi, & Moharam, 2013). Curd-firming time exhibited a similar decreasing trend ($R^2 = 0.81$), even if a curve flattening was observed for +10%, +20%, and +30% IMCU L^{-1} milk. As expected, an opposite trend was observed for a_{30} ($R^2 = 0.41$)

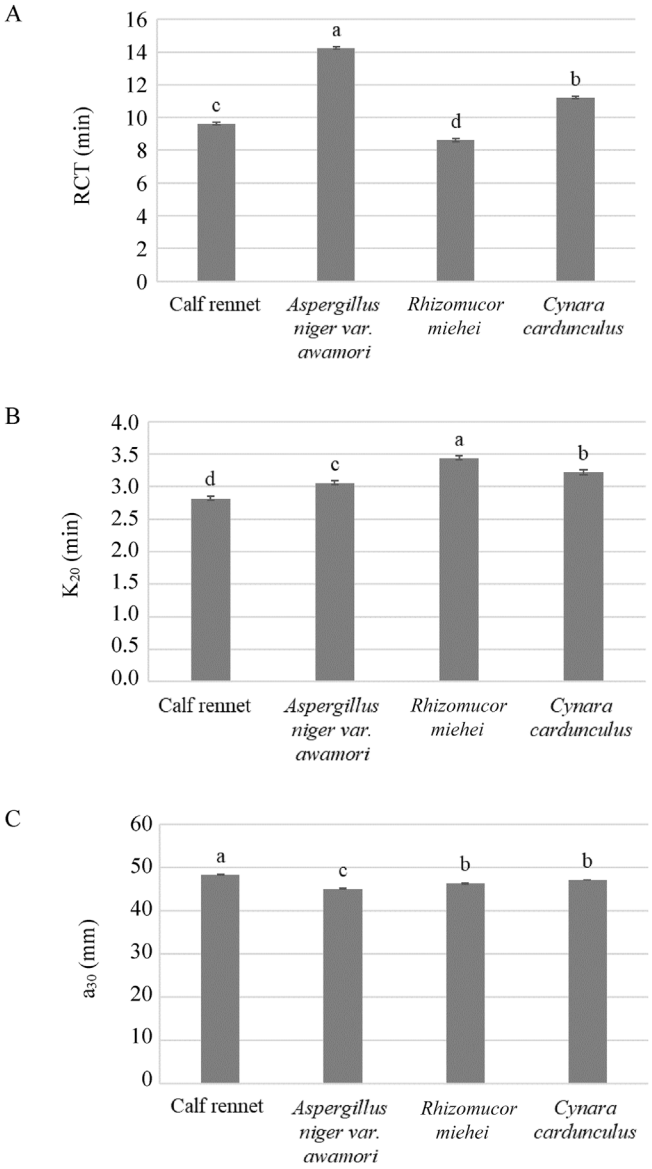

Fig. 1. Least squares means of A) rennet coagulation time (RCT, min), B) curd-firming time (k_{20}, min), and C) curd firmness (a_{30}, mm) for calf rennet, *Aspergillus niger* var. *awamori*, *Rhizomucor miehei*, and *Cynara cardunculus* coagulants. Different letters indicate significantly different estimates (P <0.05).

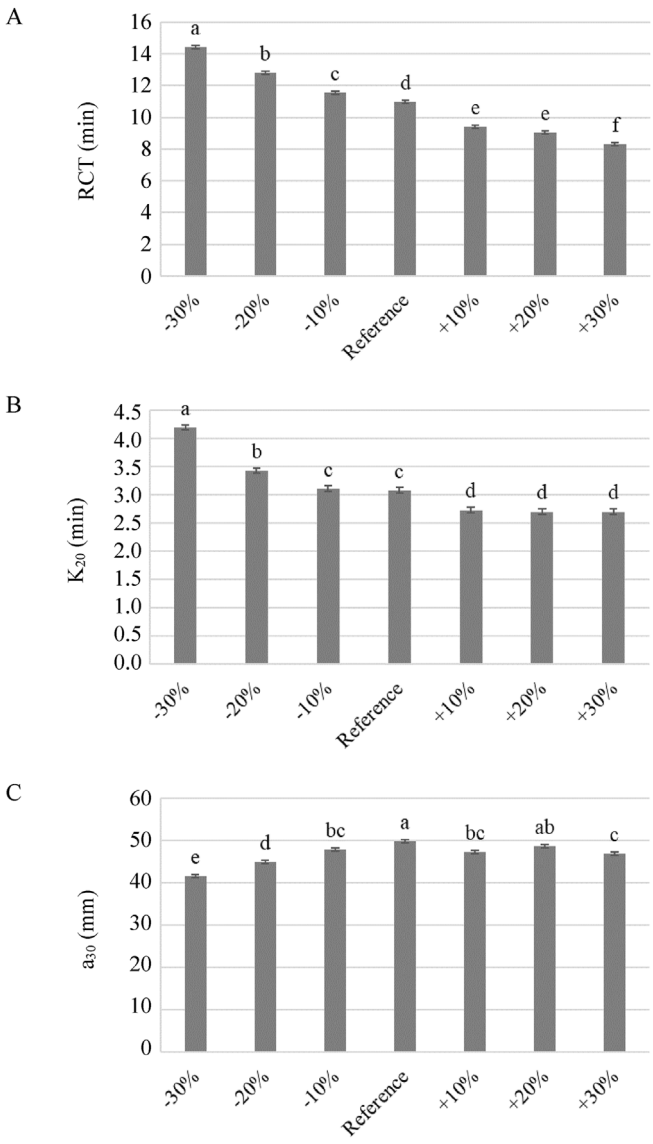

Fig. 2. Least squares means of A) rennet coagulation time (RCT, min), B) curd-firming time (k_{20}, min), and C) curd firmness (a_{30}, mm) for different dilutions of coagulant (−30%, −20%, −10%, reference, +10%, +20%, and +30% IMCU L^{-1} of milk). Different letters indicate significantly different estimates (P <0.05).

which linearly increased from the lowest dilution (41.65 mm) to the reference dilution (49.94 mm) and had an erratic trend thereafter.

3.5. Interaction between coagulant and dilution

Least squares means of MCP for the interaction between milk coagulant and dilutions are reported in Table 4. In respect to RCT, calf rennet had statistically different estimates (P <0.01) between reference (9.20 min) and −10% dilution (10.52 min). On the other hand, RCT measured within each alternative coagulant (*Asp. niger* var. *awamori*, *R. miehei*, and *C. cardunculus*) did not differ significantly between the reference dilution and −10% IMCU L^{-1} milk. Also, k_{20} measured within all tested coagulants did not differ significantly between reference and −10% IMCU L^{-1} milk. As for a_{30}, only *C. cardunculus* coagulant showed significantly different estimates (P <0.05) between reference (51.12 mm) and −10% IMCU L^{-1} milk (46.22 mm). Overall, results of the present study suggest that the use of alternative coagulants at slightly lower dilutions (−10% IMCU L^{-1} milk) does not alter significantly the MCP. This can translate in cost savings due to lower coagulant consumption and less bitter flavours in ripened cheese products (Galán, Cabezas, & Fernández-Salguero, 2012). As expected, all tested coagulants showed improved MCP at their greatest concentration (+30% IMCU L^{-1} milk).

As regard RCT measured at reference dilution, calf rennet and *R. miehei* had the best performances, with estimates of 9.20 and 8.56 min, respectively. It is worth noting that, within a single dilution, all coagulants have been tested under (almost) the same experimental conditions in terms of operator, starting milk, and time span. Also, all tested coagulants were characterised by the same enzymatic activity in terms of the hydrolysis of Phe_{105}−Met_{106} bond of κ-casein. Therefore, differences observed at the same concentration are likely due to different affinity of clotting enzymes toward casein fraction substrate, which is the greatest in the case of bovine chymosin (Andrén, 2021). As an example, even if the specific activity of cardosin B in clotting bovine milk is 6 fold the activity of cardosin A, the overall activity of plant coagulant is estimated to be 20 times lower than that of chymosin (Silva, Allmere, Malcata, & Andrén, 2003).

3.6. Repeatability of milk coagulation properties

For each dilution factor within each coagulant, repeatability of RCT, k_{20}, and a_{30} was calculated as RSD_r of 6 consecutive measurements (Table 5). The lower (greater) the RSD_r, the greater (lower) the repeatability of the method. In the present study, RSD_r ranged from 1.19% (*C. cardunculus* at +10% dilution) to 9.52% (*Asp. niger* var. *awamori* at +30% dilution) for RCT, from 3.10% (*R. miehei* at reference dilution) to 9.20% (*Asp. niger* var. *awamori* at −10%

Table 4
Least squares means of rennet coagulation time (RCT, min), curd-firming time (k_{20}, min), and curd firmness (a_{30}, mm) for the interaction between milk coagulant and dilution.[a]

Milk coagulation property	Coagulant	Dilution (IMCU L^{-1} milk)						
		−30%	−20%	−10%	Reference	+10%	+20%	+30%
RCT (min)	Calf rennet	12.40^{aC}	11.70^{aB}	10.52^{bB}	9.20^{cC}	8.08^{cdC}	7.91^{dB}	7.62^{dC}
	Aspergillus niger var. *awamori*	19.50^{aA}	17.06^{bA}	15.18^{cA}	14.71^{cA}	11.81^{dA}	11.22^{dA}	10.23^{eA}
	Rhizomucor miehei	11.71^{aC}	10.41^{bC}	8.81^{cC}	8.56^{cC}	7.58^{cdC}	6.81^{deC}	6.48^{eC}
	Cynara cardunculus	14.12^{aB}	12.12^{bB}	11.64^{bB}	11.38^{bB}	10.23^{cB}	10.16^{cA}	8.83^{dB}
k_{20} (min)	Calf rennet	3.54^{aC}	3.13^{abB}	2.98^{abA}	2.83^{abcA}	2.46^{cdB}	2.48^{cdB}	2.33^{dB}
	Aspergillus niger var. *awamori*	4.33^{aB}	3.10^{bB}	2.94^{bcA}	2.98^{bcA}	2.67^{cB}	2.56^{cAB}	2.79^{cA}
	Rhizomucor miehei	5.04^{aA}	4.10^{bA}	3.29^{cA}	3.29^{cA}	2.77^{dB}	2.75^{dAB}	2.81^{dA}
	Cynara cardunculus	3.83^{aC}	3.37^{abB}	3.25^{bA}	3.21^{bA}	3.00^{bA}	3.00^{bA}	2.87^{bA}
a_{30} (mm)	Calf rennet	48.88^{bA}	46.39^{bA}	49.98^{aA}	50.96^{aA}	46.55^{bA}	52.76^{aA}	45.24^{bB}
	Aspergillus niger var. *awamori*	35.12^{cB}	43.77^{bA}	47.86^{aA}	48.02^{aA}	47.04^{aA}	48.02^{aB}	46.06^{aB}
	Rhizomucor miehei	38.55^{cB}	43.94^{bA}	47.53^{aA}	49.65^{aA}	49.49^{aA}	46.06^{aB}	49.00^{aA}
	Cynara cardunculus	46.06^{bA}	45.57^{bA}	46.22^{bA}	51.12^{aA}	46.39^{bA}	47.86^{abB}	47.20^{abAB}

[a] Within rows and columns, different lowercase and uppercase superscript letters, respectively, indicate statistically different estimates (P <0.05).

Table 5
Relative standard deviation of repeatability (RSD_r, %; n = 6) calculated across different coagulants and dilutions for rennet coagulation time (RCT), curd-firming time (k_{20}), and curd firmness (a_{30}).[a]

Milk coagulation property	Coagulant	Dilution (IMCU L^{-1} milk)						
		−30%	−20%	−10%	Reference	+10%	+20%	+30%
RCT (min)	Calf rennet	2.88	2.92	1.89	2.22	4.03	9.50	5.67
	Aspergillus niger var. *awamori*	5.08	1.88	2.07	2.97	5.16	2.74	9.52
	Rhizomucor miehei	8.57	4.90	3.08	2.88	5.08	6.88	6.64
	Cynara cardunculus	4.16	5.85	7.54	1.20	1.19	1.69	2.46
k_{20} (min)	Calf rennet	6.34	6.11	6.65	4.16	6.27	8.88	3.97
	Aspergillus niger var. *awamori*	7.70	4.75	9.20	4.09	7.07	5.14	7.33
	Rhizomucor miehei	7.78	9.09	7.53	3.10	5.20	5.05	4.68
	Cynara cardunculus	7.87	6.66	8.83	3.18	5.27	5.90	6.16
a_{30} (mm)	Calf rennet	2.78	2.18	2.77	4.21	1.76	2.47	2.13
	Aspergillus niger var. *awamori*	5.42	5.23	5.56	3.16	5.59	6.32	3.01
	Rhizomucor miehei	9.19	3.84	2.16	3.22	4.47	2.69	5.93
	Cynara cardunculus	3.56	2.26	2.82	1.44	3.18	3.53	3.33

[a] Dilutions were −30%, −20%, −10%, reference, +10%, +20%, +30% IMCU L^{-1} milk.

dilution) for k_{20}, and from 1.44% (*C. cardunculus* at reference dilution) to 9.19% (*R. miehei* at −30% dilution) for a_{30}. For all coagulants, the best repeatability was registered at reference dilution, with average RSD_r of 2.32% (RCT), 3.63% (k_{20}), and 3.00% (a_{30}). It is also worth noting that within each trait and among different coagulants, repeatability results were more stable at reference dilution. Dilutions other than the reference were characterised by lower repeatability, probably due to the lack of coagulant (in the case of −10%, −20%, and −30% dilutions) and to the excess of coagulant (in the case of +10%, +20%, and +30% dilutions) which may have led to unspecific reactions.

Overall, RSD_r values reflected satisfactory repeatability, being consistently below 10% for all MCP and within all coagulants and dilutions. Results of the present study are comparable with those reported by Stocco, Cipolat-Gotet, Cecchinato, Calamari, and Bittante (2015), who investigated the effect of different milk technological treatments on the repeatability of MCP. Repeatability of RCT in the present study is comparable with that reported by Dal Zotto et al. (2008) who assessed reproducibility and repeatability of measures of MCP and predictive ability of mid-infrared spectroscopy. The same authors reported lower repeatability for a_{30} than that of the present study.

4. Conclusions

The objective of the present study was to assess the effect of different coagulants at different dilutions on MCP assessed through lactodynamographic analysis. For all tested coagulants, RCT was relatively short, probably due to the addition of whey starter. As expected, calf rennet showed the best results in terms of coagulation performances and a decrease in the amount of coagulant was linearly associated with a lengthening of RCT and k_{20}. Still, dilutions other than the reference were characterised by lower repeatability, likely because of the lack of coagulant and to unspecific reactions (i.e., repeatability results were more stable at reference dilution). Results also suggest that the use of microbial and plant coagulants at slightly lower dilutions does not hamper milk coagulation pattern significantly. This may find advantages in cost savings and reduced appearance of bitter flavours when plant coagulants are adopted for the production of ripened cheese.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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