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Technical note: Improving modeling of coagulation, curd firming, and syneresis of sheep milk

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ABSTRACT

The importance of milk coagulation properties for milk processing, cheese yield, and quality is widely recognized. The use of traditional coagulation traits presents several limitations for testing bovine milk and even more for sheep milk, due to its rapid coagulation and curd firming, and early syneresis of coagulum. The aim of this technical note is to test and improve model fitting for assessing coagulation, curd firming, and syneresis of sheep milk. Using milk samples from 87 Sarda ewes, we performed in duplicate lactodynamographic testing. On each of the 174 analyzed milk aliquots, using 180 observations from each aliquot (one every 15 s for 45 min after rennet addition), we compared 4 different curd firming models as a function of time (CF_t, mm) using a nonlinear procedure. The most accurate and informative results were observed using a modified 4-parameter model, structured as follows: $CF_t = CF_P \times \left(1 - e^{-k_{CF}\left(t - RCT_{eq}\right)}\right) \times e^{k_{SR} \times \left(t - RCT_{eq}\right)}$, where t is time, RCT_{eq} (min) is the gelation time, CF_P (mm) is the potential asymptotical CF at an infinite time, k_{CF} (%/min) is the curd firming rate constant, and k_{SR} (%/min) is the curd syneresis rate constant. To avoid nonconvergence and computational problems due to interrelations among the equation parameters, CF_P was preliminarily defined as a function of maximum observed curd firmness (CF_{max}, mm) recorded during the analysis. For this model, all the modeling equations of individual sheep milk aliquots were converging, with a negligible standard error of the estimates (coefficient of determination >0.99 for all individual sample equations). Repeatability of the modeled parameters was acceptable, also in the presence of curd syneresis during the lactodynamographic analysis.

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Technical Note

Milk coagulation can be measured by a wide range of mechanical, vibrational, ultrasonic, thermal, and optical methods (O'Callaghan et al., 2002; Klandar et al., 2007). Among those, the monitoring with time of curd firmness (**CF**, mm) of renneted milk samples maintained at a fixed temperature is frequently used to assess the coagulation process (Barbano and Lynch, 2006). Although it presents several limitations, the lactodynamography analysis is extensively used to assess coagulation and curd firming process (Annibaldi et al., 1977; McMahon and Brown, 1982), as it is simple and fast, and it could be used to test contemporarily several milk samples (usually 10). In bovine milk, the major limitations of lactodynamography are related to the traditional single point traits measured by the computerized instruments and include (1) the moderate repeatability of all the traits, (2) the high incidence of noncoagulating samples (rennet coagulation time, **RCT**, not measurable within 30 min from rennet addition) and coagulated samples not attaining a curd firmness of 20 mm (**k_{20}**, min), and (3) the low-informative value of the curd firmness traditionally measured at the end of analysis (**a_{30}**, mm). To solve those limits, Bittante (2011) presents a 3-parameter model that used all the CF measures of a sample (one every 15 s) as a function of time (**CF_t**, mm) over the traditional 30-min analysis interval. The 3 estimated parameters are the RCT from the model equation (**RCT_{eq}**, min), the potential asymptotic curd firmness (**CF_P**, mm), and the instant rate constant of curd firming (**k_{CF}**, %/min). That model reduces the limitations of lactodynamography (except for the incidence of noncoagulating samples), as the parameters are estimated using all the information (120 observations/sample) of the analysis. Moreover, the high presence of bovine milk samples not coagulating within 30 min suggests to extend the interval analysis to 60 or even to 90 min after rennet addition (Cipolat-Gotet et al., 2012). The extension of the analysis of bovine milk indicates CF reduction after the attainment of a maximum value, interpreted by Bittante et al. (2013) as a sign of the beginning of syneresis. Hence, those authors propose a new equation model introducing a fourth parameter (**k_{SR}**, %/min), which describes the instant rate constant of syneresis. A limitation of those CF_t models is the presence of milk samples whose equation does not converge. Nonconverging samples (i.e., very late coagulating samples), provide a few number of available observations to permit a precise estimation of modeled parameters. In those cases, the equation cannot estimate modeled parameters or outliers. Sheep milk presents a pattern of coagulation and curd firming very different from bovine milk (Bencini, 2002; Bittante et al., 2014; Ferragina et al., 2017) because the RCT is usually shorter, the curd firming more rapid, the maximum CF greater, and the syneresis appears earlier (often before 30 min after rennet addition). As a consequence, traditional coagulation traits developed on bovine milk are often not so informative for sheep milk. The aim of this technical note was to improve the CF_t model fitting for assessing coagulation, curd firming, and syneresis and to test these improvements to model individual sheep milk samples.

To perform the present study, we used data from the database previously described in Pazzola et al. (2014). Among the animals sampled for that study (1,121 ewes from 23 flocks of Sarda sheep located in the island of Sardinia, Italy), 2 to 13 ewes from 18 farms were selected for a total of 87 ewes. Selected ewes were from the second to the seventh month of lactation (DIM 150 ± 47 d) and had a daily milk yield of 1.53 ± 0.78 kg/d. After the collection, milk samples were divided into 2 aliquots (50 mL each) and kept at 4°C (without preservative). Traditional single-point traits (RCT, k_{20}, a_{30}, a_{45}, and a_{60}) and all the CF measures (240 for each milk aliquot, 1 every 15 s) were measured using a lactodynamograph (Formagraph, Foss Italia, Padova, Italy) and prolonging the test length until 60 min. Two aliquots of each individual milk sample (10 mL each aliquot) were heated at 35°C and mixed with 200 μL of the calf rennet solution [Hansen Naturen Plus 215 (Pacovis Amrein AG, Bern, Switzerland), with 80 ± 5% chymosin and 20 ± 5% pepsin and 215 international milk clotting units/mL; diluted to 1.2% (wt/ vol) in distilled water for the achievement of 0.0513 international milk clotting units/mL of milk]. For all the milk aliquots (n = 174), RCT and k_{20} were achievable within the first 45 min of the lactodynamographic analysis. Therefore, to compare different CF_t models, only the first 45 min (180 observations per aliquot) were used to estimate model parameters, as this is considered an adequate time interval to attain syneresis in individual ewe milk samples (Vacca et al., 2015). We tested 4 different nonlinear models for curd firming (CF_t, mm). The first model (**$3Par_{fixed}$**) was the 3-parameter asymptotic model proposed by Bittante (2011):

$$CF_t = CF_P \times \left(1 - e^{-k_{CF}\left(t - RCT_{eq}\right)}\right),$$

where CF_t is CF at time t (mm); CF_P is the asymptotical potential value of CF at an infinite time (mm); k_{CF} is the curd firming rate constant (%/min) and describes the shape of the curve from coagulation time to infinity (velocity of curd firming); and RCT_{eq} has the same meaning as the traditional RCT trait. A second model (**$3Par_{variable}$**) had the same equation as above but was based on a different and variable number of observations (≤180). In this second model, the observed maximum CF value (**CF_{max}**, mm) for each analyzed aliquot was used to measure the corresponding time after rennet addition (**t_{max}**, min, ≤45 min). Only the CF observations between the rennet addition and t_{max} were used for the estimation of the parameters of $3Par_{variable}$ model. In the case of aliquots presenting tendency to asymptote, the first value of CF_{max} was used if more than one maximum value was recorded during the single analysis. In this way, the potential decreasing phase of the CF_t curve was excluded from calculations, the number of observations was retained, and the corresponding degrees of freedom were different among the analyzed aliquots. The third was a 4-parameter model (**$4Par_{free}$**) previously proposed for bovine milk by Bittante et al. (2013), characterized by prolonging the duration of the lactodynamographic test and including the aforementioned parameters together with k_{SR} (%/min), the instant rate constant of syneresis, as follows:

$$CF_t = CF_P \times \left(1 - e^{-k_{CF}\left(t - RCT_{eq}\right)}\right) \times e^{k_{SR} \times \left(t - RCT_{eq}\right)}.$$

The $4Par_{free}$ model was proposed as a large percentage of individual milk samples showed a decreasing phase of CF_t, sometimes within 30 min after rennet addition (Vacca et al., 2015). In the first part of the curve, the effect of k_{CF} prevails over k_{SR}, and CF_t tends to increase until t_{max}. At t_{max} the effects of k_{CF} and k_{SR} are equal and opposite in sign. At t_{max}, the effects of these 2 traits are equal and opposite. After t_{max}, the effect of syneresis becomes more forceful than k_{CF}, and CF_t curve tends to decrease toward a null value. The decrease of CF is apparent and is due to the increasing amount of whey in the cuvette that allows the curd to float freely so that the pendulum of the instrument finds a decreased resistance to movement. As the rate constant k_{SR} is associated with this part of the curve (from t_{max} to the end of testing), it describes the effect of the corresponding expulsion of whey. The 4Par$_{free}$ model estimates all the 4 parameters directly from all the 180 CF available measures. The **4Par$_{forced}$** model was derived from 4Par$_{free}$ and was proposed to limit convergence and estimation problems: in this case, CF_P was calculated by multiplying CF_{max} with 1.12. This multiplicative coefficient was obtained from the linear regression between CF_P and CF_{max} values, estimated in a preliminary analysis of the entire data set using 4Par$_{free}$ (Vacca et al., 2015). Then, using the 4Par$_{forced}$ modeling procedure, we estimated directly only 3 parameters (RCT_{eq}, k_{CF}, and k_{SR}) using all 180 CF observations, whereas CF_P was preliminarily estimated as a function of the observed CF_{max}.

For the parameters estimation of the 4 models (3Par$_{fixed}$, 3Par$_{variable}$, 4Par$_{free}$, and 4Par$_{forced}$), we used univariate curvilinear regressions based on all the CF observations available for each aliquot with a nonlinear procedure (PROC NLIN; SAS version 9.4, SAS Institute Inc., Cary, NC). The parameters of each individual equation were estimated by using the Marquardt iterative method (350 iterations and 10^{-5} level of convergence). To test the fitting of the models, individual modeled CF_t traits (2 replicates per ewe), together with traditional traits, were analyzed using a MIXED procedure (SAS Institute Inc.) that included the fixed effect of well (measuring unit of the coagulation meter) and the random effects of herd/date, animal, and residual (called aliquot). Herd/date, animal, and aliquot were assumed to be independently and normally distributed with a mean of zero and variance σ^2_{HD}, σ^2_{animal}, and $\sigma^2_{aliquot}$, respectively. The coefficient of repeatability (**REP**) was estimated as

$$\mathrm{REP} = \frac{\sigma^2_{HD} + \sigma^2_{animal}}{\sigma^2_{HD} + \sigma^2_{animal} + \sigma^2_{aliquot}} \times 100,$$

where σ^2_{HD}, σ^2_{animal}, and $\sigma^2_{aliquot}$ are the variance components for herd/date, animal, and milk sub-sample from the same individual ewe, respectively.

As shown by the descriptive statistics (Table 1), results of traditional and modeled milk coagulation properties are similar or more favorable than previous studies on Sarda sheep milk (Pazzola et al., 2014; Vacca et al., 2015; Manca et al., 2016). Figure 1 presents an example of how models can fit the pattern of lactodynamographic analyses of an individual aliquot of ovine milk. The performance of the models was evaluated on the basis of the percentage of individual milk sample aliquots with convergent equations and by estimating the coefficient of determination of the convergent individual equations. We observed the best results for 3Par$_{variable}$, 4Par$_{free}$, and 4Par$_{forced}$. These model were characterized by coefficients of determination higher than 0.99 and negligible residual standard deviations (Figure 2). In opposite, 3Par$_{fixed}$ showed the lowest coefficients of determination and the highest residual standard deviations (Figure 2); moreover, 3 individual aliquot equations were not convergent. Although 4Par$_{free}$ exhibited good fitting results, 6 out of the 174 aliquots were nonconvergent (data not shown). When a milk sample exhibits a nonconvergent equation, all the predicted modeled traits have to be considered missing. Nonconverging CF_t model equations are more common for late-coagulating samples (long RCT) or with very high k_{CF} and CF_{max}, respectively, and for those samples characterized by absence or nonlinear trend in the syneresis process. Using 4Par$_{free}$, nonconvergent individual milk samples are 7% for bovine (Bittante et al., 2015) and almost 5% for sheep milk (Vacca et al., 2015).

In the present study, models with only 3 parameters were not able to explain the appropriate pattern of milk samples characterized by a decreasing phase of CF_t curve due to curd syneresis and whey expulsion (Figure 1). Figure 1 also showed that the exclusion of the decreasing part of the curve (3Par$_{variable}$) allowed to obtain a more reliable estimation of CF_P and k_{CF} parameters and to fit the curve until CF_{max}. Among parameters of the models, RCT_{eq} estimates were similar for all models and slightly greater, on average, than the traditional RCT (Table 1). The curvilinear graph shape before and after RCT (no singular point, or cusp, is detectable) for observed data (Figure 1) derived from the lactodynamograph mechanism, which mitigates the curve line by taking a mobile average of CF_t data. Therefore, measured RCT is recorded when CF_t is lower than 1 mm, whereas RCT_{eq} is estimated when CF_t is 0 mm. The 3Par$_{fixed}$ model underestimated CF_P because of the bias induced by the decreasing phase of CF_t curve, which was excluded from computations of parameters from 3Par$_{variable}$. The 3Par$_{variable}$ model was particularly appropriate when the decreasing phase was not evident in the CF_t curve, as in the case of sheep milk samples tested for short periods, or in the case of late-coagulating bovine milk samples, as frequently observed in milk from Holstein Friesian cows (Bittante et al., 2012).

Figure 1 shows that the models including k_{SR} (4Par$_{free}$ and 4Par$_{forced}$) were able to describe the curves presenting decreasing phases. The differences between observed and predicted CF measurements for these 2 models can be defined as negligible because of the moderate repeatability and reproducibility that characterizes the lactodynamographic analysis (Stocco et al., 2015). However, regardless of the fitting of the 4Par models, the limitation of k_{SR} is mainly attributable to the descending phase of the curve, which reflects the expulsion of the whey (and not a direct measure of CF), and it has to be considered only an indirect measure of syneresis. The two 4Par models presented higher CF_P estimates than 3-parameter models because the effect of syneresis caused a partial disguising of the actual CF. This is in accordance with data recorded from bovine milk (Bittante et al., 2013). As expected, the k_{CF} rate constant of both the 4-parameter models was smaller than those of the 3-parameter models because of the greater CF_P. Results of ANOVA (Table 1) were used to evaluate the reproducibility among different wells, sampling date/herds, individual ewes, and the repeatability of modeled traits measured between the 2 aliquots of each milk sample. The RCT, and k_{CF} and k_{SR} from the $4Par_{free}$ model, were not affected by the well effect. Contrary to traditional RCT, RCT_{eq} was significantly affected by well. This was not attributable to the effect of the wells on this trait, but to its much lower residual (aliquot) variance. Indeed, the aliquot RMS is about halved in modeled than in single point RCT, so that its variance is about one-fourth and then the F-value of the well is about 4 times higher for RCT_{eq} (Table 1). Also for the 2

Table 1. Descriptive statistics, ANOVA, and repeatability of traditional single point milk coagulation properties (MCP) and curd firming models as a function of time (CF_t) model parameters estimated for each milk sample aliquot according to $3Par_{fixed}$, $3Par_{variable}$, $4Par_{free}$, and $4Par_{forced}$ models[1]

	Descriptive statistics			ANOVA, effect: F[2]	ANOVA, effect: Random (RMS)[3]			Variance,[4] %		
Trait	N	Mean	SD	Well	HD	Animal	Aliquot	HD	Animal	Repeatability
Traditional MCP										
RCT, min	174	9.05	3.90	0.9	1.91	3.24	0.78	25	71	95.8
k_{20}, min	174	2.03	0.83	2.7**	0.00	0.74	0.36	0	81	81.1
a_{30}, mm	174	48.73	12.00	5.4***	7.19	6.39	6.17	40	31	70.9
a_{45}, mm	174	44.33	13.95	4.6***	9.35	4.59	8.36	49	12	60.8
a_{60}, mm	174	40.96	15.78	4.0***	9.51	4.63	10.73	40	9	49.3
CF_t models										
RCT_{eq}, min										
$3Par_{fixed}$	171	10.28	3.81	3.7***	1.81	3.27	0.35	23	76	99.2
$3Par_{variable}$	174	9.98	3.83	3.4**	1.89	3.23	0.36	25	74	99.1
$4Par_{free}$	168	9.75	3.51	3.5**	1.64	3.12	0.36	21	78	99.0
$4Par_{forced}$	174	9.97	3.83	3.2**	1.84	3.27	0.42	24	75	98.8
CF_P, mm										
$3Par_{fixed}$	171	49.50	10.70	14.5***	7.33	5.70	3.73	54	32	86.1
$3Par_{variable}$	174	54.30	7.51	20.5***	3.82	5.17	2.61	30	56	85.9
$4Par_{free}$	168	61.71	13.09	3.2**	5.86	6.59	9.89	20	25	44.3
$4Par_{forced}$	174	59.73	9.63	16.9***	5.39	6.28	3.50	36	49	84.8
k_{CF}, % × min^{-1}										
$3Par_{fixed}$	171	49.46	25.13	2.2*	17.3	11.1	11.6	54	22	75.8
$3Par_{variable}$	174	31.48	7.00	3.2**	3.36	5.41	2.78	23	61	84.0
$4Par_{free}$	168	28.12	8.08	1.7	3.76	5.17	4.96	22	41	62.4
$4Par_{forced}$	174	29.53	10.59	2.1*	7.46	2.81	6.97	50	7	56.6
k_{SR}, % × min^{-1}										
$4Par_{free}$	168	1.02	1.03	1.7	0.55	0.19	0.86	28	3	31.3
$4Par_{forced}$	174	0.93	0.57	2.3*	0.39	0.00	0.42	47	0	46.7
CF_{max}, mm										
$4Par_{free, forced}$	174	53.33	8.60	16.9***	4.81	5.61	3.13	36	49	84.8
t_{max}, min										
$4Par_{free, forced}$	174	27.08	9.58	2.2*	6.34	3.77	5.73	46	16	62.4

[1]RCT = rennet coagulation time; k_{20} = coagulated samples not attaining a curd firmness of 20 mm; a_{30} = curd firmness traditionally measured at the end of analysis; a_{45} = ; a_{60} = ; CF_P = potential asymptotical CF at an infinite time; k_{CF} = the curd firming rate constant; CF_{max} = maximum observed curd firmness; and t_{max} = maximum time after rennet addition. $3Par_{fixed}$ = 3-parameter asymptotic model proposed by Bittante (2011); $3Par_{variable}$ = same equation as $3Par_{fixed}$ but based on a different and variable number of observations (≤180); $4Par_{free}$ = 4-parameter model previously proposed for bovine milk by Bittante et al. (2013); $4Par_{forced}$ = model derived from $4Par_{free}$ and was proposed to limit convergence and estimation problems; $4Par_{free, forced}$ = CF_{max} and t_{max} were estimated using the same procedures for both models.

[2]F = F-value of fixed effect of well.

[3]RMS = root mean squares of random effects [herd day (HD), animal, and aliquot, respectively].

[4]HD and animal variance are expressed by the ratio between the corresponding variance component and the total variance (%).

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

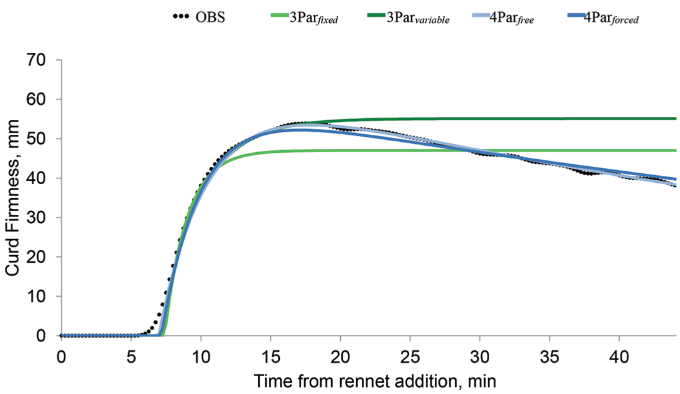

Figure 1. Modeled pattern of curd firmness after rennet addition [curd firming models as a function of time (CF_t) modeling] of an individual sheep milk sample using $3Par_{fixed}$, $3Par_{variable}$, $4Par_{free}$, and $4Par_{forced}$ models. OBS = observed; $3Par_{fixed}$ = 3-parameter asymptotic model proposed by Bittante (2011); $3Par_{variable}$ = same equation as $3Par_{fixed}$ but based on a different and variable number of observations (≤180); $4Par_{free}$ = 4-parameter model previously proposed for bovine milk by Bittante et al. (2013); $4Par_{forced}$ = model derived from $4Par_{free}$ and was proposed to limit convergence and estimation problems. Color version available online.

constant rates obtained from $4Par_{free}$ model, the aliquot root means square was relatively high. These results were mostly related to the decrease of accuracy and repeatability (Table 1) that characterized CF measures from RCT to the end of lactodynamographic analyses. The effect of wells was particularly evident for the CF_P and CF_{max} traits, confirming the results obtained from bovine milk (Stocco et al., 2017). With regard to random effects, we observed a higher repeatability (REP) for RCT than the other traits. The results in terms of REP among the 4 models were different among modeled parameters. In the case of RCT_{eq}, all the 4 models showed excellent REP values, higher than the traditional RCT (Table 1). Comparing the 3-parameter models, REP was similar for CF_P and greater for $3Par_{variable}$ than $3Par_{fixed}$ in the case of k_{CF}. Among parameters obtained using $4Par_{free}$, the REP value for CF_P and k_{CF} was lower compared with the 3-parameter models, and very low for k_{SR} rate. This was attributable to the interrelations among the 4 parameters. The adoption of a preliminary estimate of CF_P on the basis of CF_{max} ($4Par_{forced}$ model) reduced the interdependency of parameters and allowed improvement of the REP of both CF_P and k_{SR} (Table 1).

In conclusion, the $3Par_{variable}$ model could be a valid tool for representing coagulation and curd-firming processes when the length of the lactodynamographic test was limited, when milk coagulation was delayed, when the CF_t curve did not present a decreasing phase, or even when the eventual decreasing or flat section of the curve was excluded from calculations. The 4-parameter models offered a better fitting of CF_t curves in all the other cases and new indirect information regarding syneresis. In this case, the preliminary definition of CF_P on the basis of CF_{max} reduced the interdependency of modeled parameters, allowed more repeatable and affordable parameters, and the convergence of the individual milk samples equation ($4Par_{forced}$ model). Although practi-

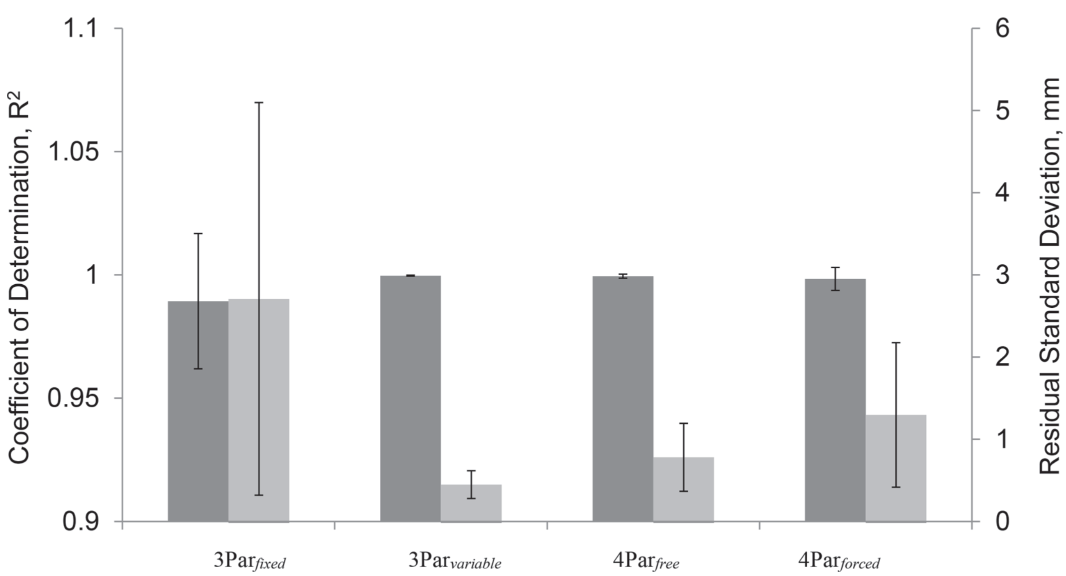

Figure 2. Coefficients of determination (dark bars) and residual SD (light bars) for model fitting results obtained for $3Par_{fixed}$, $3Par_{variable}$, $4Par_{free}$, and $4Par_{forced}$ models. $3Par_{fixed}$ = 3-parameter asymptotic model proposed by Bittante (2011); $3Par_{variable}$ = same equation as $3Par_{fixed}$ but based on a different and variable number of observations (≤180); $4Par_{free}$ = 4-parameter model previously proposed for bovine milk by Bittante et al. (2013); $4Par_{forced}$ = model derived from $4Par_{free}$ and was proposed to limit convergence and estimation problems.

cal application of proposed methods for inline use still has to be evaluated, this new knowledge can be useful to promote lactodynamographic tests along the dairy chain to better characterize individual milk samples at farm level and to improve milk payment systems.

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