Survival analysis as a statistical methodology for analyzing factors that affect milk coagulation time in Holstein-Friesian and Brown Swiss cows

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ABSTRACT

The aim of this study was to propose the survival analysis technique as a statistical approach for the analysis of rennet coagulation time (RCT) able to make use of coagulating and noncoagulating (NC) milk information in order to estimate potential sources of variations that affects RCT. A total of 1,025 Italian Holstein-Friesian cows (HF; progeny of 54 sires) and 1,234 Brown Swiss cows (BS; progeny of 58 sires) reared in 34 and 38 herds, respectively, were milk-sampled once. Rennet coagulation time was analyzed with a semiparametric proportional hazard model (i.e., a Cox model), with the NC samples considered as censored records. Furthermore, a different censoring scenario, with a new end point at 18 min, was considered after the rearrangement of the time space originally used for the observation of RCT. The percentage of NC samples was almost 10% for HF and 3.5% for BS cows in the 31 min set, whereas it increased to 44 and 24.9%, respectively, in the 18 min set. Estimated hazard ratios indicated that the most important factors affecting the coagulation process were herd, days in milk, casein number, and milk acidity (expressed in terms of titratable acidity) for both HF and BS, whereas the SCS was relevant only for BS. The survival model seems to be particularly suitable for this analysis, as it can properly account for censored and uncensored records and appropriately use all available information. Moreover, this methodology allows us to rearrange the time space used for the observation of RCT and to define alternative traits (i.e., RCT with an end point at 18 min). Our restriction of the time space and the increased percentage of censored records did not highlight any substantial differences in terms of the risk of coagulating with respect to the traditional 31 min testing time. Although further research is needed to investigate the effect of these sources of variation on cheese yield, our results indicate that casein number,

acidity, and SCS may be used as indicator traits for enhancing the technological properties of bovine milk.

Key words: milk coagulation time, survival analysis, noncoagulating milk, dairy cattle

INTRODUCTION

Milk coagulation properties (MCP) are essential factors in cheese making. Milk that exhibits a short coagulation time, a short curd-firming time, and a firm curd is expected to give more cheese of a desirable composition compared with milk with unfavorable properties. Milk coagulation properties can be assessed with milk coagulation meters, which measure the rennet coagulation time (RCT), curd firmness, and curd-firming time for 31 min after the rennet is added to the milk (Anibaldi et al., 1977).

Several studies have investigated the variation and genetic aspects of MCP in dairy cattle populations using different field conditions and models, as comprehensively reviewed by Bittante et al. (2012). In many studies, samples of noncoagulated (NC) milk (i.e., milk that failed to form any curd within the 31-min testing period after the addition of rennet) were not included in the statistical analysis because of missing MCP information (Ikonen et al., 1999; Cecchinato et al., 2009; Vallas et al., 2010; Cecchinato et al., 2012b). Alternatively, NC samples might be included when the MCP were not measured directly, but rather as a binary trait indicating whether coagulation occurred or not (Tyrisev et al., 2004). Both approaches involve the loss of information: in the first case, samples of milk with very low quality for cheese making are omitted from the analysis, whereas, in the second case, milk samples exhibiting curd formation at different times (e.g., 10 or 30 min after rennet addition) are treated alike, neglecting variations in the RCT of coagulated samples (Cecchinato et al., 2011).

Current instruments are limited to observing coagulation for up to 31 min from the addition of rennet. Rennet coagulation time measures comprise longitudinal data on the occurrence of coagulation; MCP assessment produces censored records (when no coagulation

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occurs) and uncensored records that include information on RCT. Survival analysis (Cox, 1972; Prentice and Gloeckler, 1978; Ducrocq et al., 1988) is the most suitable statistical methodology for the analysis of such data because of its ability to properly account for censored and uncensored records and appropriately use all available information (Casellas et al., 2006; Tarrés et al., 2010). Moreover, this methodology allows rearrangement of the time space used for the observation of RCT, as well as the definition of alternative traits. For instance, after MCP assessment, the time space might be restricted to 18 min after rennet addition, meaning that milk samples with RCT greater than 18 min would be reclassified as NC milk. Such an approach has been proposed by Cecchinato and Carnier (2011b) for the estimation of genetic variation and heritability. However, no prior study has investigated environmental source of variation of RCT including NC milk information in the statistical analysis.

The aim of this study was to illustrate the use of survival analysis techniques for the analysis of RCT, and to investigate sources of variation of RCT in individual Holstein-Friesian (**HF**) and Brown Swiss (**BS**) cows using coagulating and NC milk information.

MATERIALS AND METHODS

Field Data

Data were obtained from 2 studies, one aimed at estimating genetic parameters for MCP in Italian HF cows (Cassandro et al., 2008) and one seeking to evaluate the use of MCP predictions obtained by midinfrared spectroscopy as indicator traits in breeding programs for enhanced MCP in Italian BS cows (Cecchinato et al., 2009). A total of 1,025 HF and 1,234 BS cows were sampled once. Cows were daughters of 54 HF and 58 BS AI sires and were distributed in 34 and 38 herds, respectively, located in northern Italy. Within 3 h of sampling, individual 10-mL milk samples were assessed for MCP using a computerized renneting meter (Polo Trade, Monselice, Italy). Samples were heated to 35°C and mixed with 200 μ L of rennet (Hansen standard 190 with 63% chymosin and 37% pepsin; Pacovis Amrein AG, Bern, Switzerland) diluted to 1.6% with distilled water. Milk coagulation properties were measured for 31 min after the addition of rennet, and milk samples that failed to form any curd within that period were classified as NC milk.

In the same laboratory, fat, protein, CN contents (Combi Foss 6000 FC, Foss Electric A/S), pH, titratable acidity (expressed in Soxhlet-Henkel degrees; Crison Compact D, Crison Instruments SA, Alella, Spain), and SCC (Cell Fossomatic 250, Foss Electric A/S) were

determined. The SCC values were log-transformed to SCS, as suggested by Ali and Shook (1980).

Trait Definition

Of the MCP traits, only measures of RCT (min) were of interest for this study. In practical cheese making, milk that coagulates more than 18 min after rennet addition is considered unsatisfactory. Thus, after an initial assessment at 31 min, all statistical analyses were repeated using 18 min as a new end point for classifying milk samples as coagulating or NC milk. This increased the fraction of NC samples in both HF and BS data sets, but did not affect the RCT of coagulated samples. Throughout the paper, the original data set is referred to as **SET31**, whereas that obtained with the 18-min end point is called **SET18**.

Statistical Analysis

Survival Analysis. Because the use of survival analysis to study MCP is new, we must introduce some specific terminology. The term “coagulation function” will be used for a function, $C(t)$, that is equivalent to the survivor function in the context of longevity or failure-time studies. Hence, the coagulation function indicates the fraction of milk samples that, at a given time t , has not yet exhibited coagulation. Likewise, the function describing the “hazard” of coagulation at a given time, t (i.e., the instantaneous rate of milk coagulation conditional on noncoagulation until time t), will be referred to as the “instantaneous coagulation rate function” and denoted as $cr(t)$.

The coagulation function for the general population was preliminarily estimated by the Kaplan-Meier method (Kaplan and Meier, 1958). The linear regression of $\ln\{-[C(t)]\}$ on $\ln(t)$, where $C(t)$ is the Kaplan-Meier estimated coagulation function, was used to check the suitability of the assumption of a Weibull distribution for the baseline instantaneous coagulation rate function, $cr_0(t)$ (Ducrocq et al., 1988). Because the relationship between $\ln\{-[C(t)]\}$ and $\ln(t)$ was not linear, the assumption of a Weibull distribution function for the baseline $cr_0(t)$ was rejected and the instantaneous coagulation rate was modeled using a proportional hazard model (Cox, 1972) of the general form:

$$cr(t) = cr_0(t)\exp(\mathbf{x}'\boldsymbol{\beta} + \mathbf{w}'\mathbf{h}),$$

where $cr(t)$ is the instantaneous coagulation rate function at time t ; $cr_0(t)$ is the baseline instantaneous coagulation rate function; $\boldsymbol{\beta}$ is an unknown vector of fixed regression coefficients for a set of systematic effects, $\mathbf{x}'$ is a vector of indicator variables for systematic ef-

fects; **h** is an unknown vector of regression coefficients for random effects due to the herd; and **w'** is a vector of the indicator variables for herd effects. Because the distribution function for the baseline $cr_0(t)$ was left completely unspecified, the resulting model was a semiparametric proportional hazard model (i.e., a Cox model).

Selection of Fixed Effects. Before survival analysis, we categorized the potential sources of variation that could influence the instantaneous coagulation rates of each HF or BS cow. We did not know the form of the relationship between these variables and the hazard, and thus did not make any assumption in this regard. Before categorization, milk yield (**MY**), casein number (casein:protein ratio), fat, titratable acidity expressed as Soxhlet-Henkel degrees ($SH^\circ/50$ mL; **SH**), and SCS were standardized to a mean of zero and a standard deviation of 1. All standardized traits were grouped into 4 classes (class 1 = SD <−1; class 2 = SD −1 to −0.33; class 3 = SD −0.33 to 0.33; and class 4 = SD 0.33 to 1). Days in milk was classified into 6 bimonthly classes from 5 to 300 DIM; 1 class for records collected after 300 DIM. The parity effect was assessed in 4 groups of first, second, third, and 4 or more. Test-day effects were confounded with herd effects because all cows of each herd were sampled once and in the same test day.

A preliminary analysis was carried out to identify fixed effects that were statistically significant at $P < 0.05$. The nonsignificant effects were then individually tested with the group of significant fixed effects to determine whether any of those effects became significant. To test the proportional hazards assumption, time-dependent factors (i.e., interaction terms between the time-independent effects and the function of time) were defined. The inclusion of these interaction terms did not significantly increase ($P > 0.05$) the likelihood for any of the models analyzed (results not shown); thus, the proportionality hypothesis was not rejected. All analyses were carried out using the Survival Kit software, version 3.12 (Ducrocq and Solkner, 1994).

Figure 1. Coagulation function [C(t)] estimated by the Kaplan-Meier over 18 min (indicated with the vertical black line) and 31 min testing time for Holstein-Friesian and Brown Swiss cows.

RESULTS AND DISCUSSION

Descriptive Statistics and Coagulation Function

Descriptive statistics for RCT and the NC samples are reported in Table 1. For SET31, the average RCT of coagulated milk samples was lower for BS than HF. The results confirmed the superiority of the BS, as previously reported by Bittante et al. (2012). The between-breed difference in RCT found in the present study was almost identical to the average differences found in 8 other direct comparisons (Bittante et al., 2012). The between-breed difference in the average RCT of coagulating samples decreased when we considered samples with $RCT \geq 18$ min as NC milk (SET18). As expected, setting the end point for coagulation to 18 min reduced the variation of RCT among the coagulated samples. The variation in RCT (i.e., the standard deviation) for SET31 was twice that for SET18. Because sample collection was carried out in different periods and herds, the effects due to breeds, herds, and testing conditions might be confounded.

Table 1. Descriptive statistics for coagulation time (RCT) and fraction of noncoagulated samples

Item ¹	Holstein-Friesian	Brown Swiss
Samples that coagulated within 18 min		
Fraction, %	56.0	75.1
RCT mean, min	13.9	13.4
RCT SD, min	2.3	2.3
Samples that coagulated within 31 min		
Fraction, %	90.3	96.5
RCT mean, min	16.9	15.0
RCT SD, min	4.7	4.0

¹Time origin is the addition of rennet to milk.

The coagulation functions for HF and BS, as estimated by the Kaplan-Meier method, are presented in Figure 1. The proportion of censored records in SET31 were 10 and 4% for HF and BS, respectively, whereas those values were 44 and 25% in SET18, respectively. The incidence of NC samples from BS cows was lower than that obtained from the same breed by Cipolat-Gotet et al. (2012), but the previous authors used a lower rennet concentration and mechanical lactodynamographs that differed from the equipment used in the present study. They also obtained different patterns of coagulation time, depending on whether they used a mechanical or optical lactodynamograph. Using the same 2 instruments, Cecchinato et al. (2013) found

similar genetic parameters of RCT and other coagulation traits (curd-firming rate and curd firmness 30 and 45 min after rennet addition). Moreover, RCT estimation and NC occurrence were found to be affected by modeling of curd firmness (Bittante, 2011; Bittante et al., 2013) and the reproducibility of the technique used (Dal Zotto et al., 2008).

Noncoagulation of milk is a common problem in many dairy cattle breeds (Van Hooydonk et al., 1986; Malossini et al., 1996; Kübarsepp et al. 2005). As reported by Ikonen et al. (1999), noncoagulation of milk is a problem in Finnish Ayrshire, with some cows producing NC milk at almost every sampling. Tyrisevä et al. (2008) performed a genome-wide scan under a selective DNA-

Table 2. Number of records and percentage of censored records in each level of fixed effects included in the survival model

Item	Holstein-Friesian			Brown Swiss		
	n	Censored after (%)		n	Censored after (%)	
		18 min	31 min		18 min	31 min
Parity						
First	286	44.4	9.8	387	27.6	2.84
Second	390	45.6	11.5	381	24.9	2.62
Third and more	349	41.8	9.2	512	22.7	3.52
DIM						
<60	102	26.5	5.9	161	4.9	0.1
60 to 120	145	37.2	8.3	221	22.2	0.9
120 to 180	191	48.7	13.1	278	29.5	5.0
180 to 240	189	50.3	10.6	221	34.4	4.1
240 to 300	142	40.1	9.2	156	24.3	3.8
>300	256	48.9	11.3	243	26.7	3.3
Milk yield, kg/d						
<-1 SD	169	50.3	16.6	198	33.3	8.6
-1 to -0.33 SD	242	47.5	12.4	302	26.2	2.3
-0.33 to 0.33 SD	232	45.7	8.2	352	29.5	2.5
0.33 to 1 SD	203	40.4	5.4	210	22.4	2.4
>1 SD	179	35.2	9.5	218	10.1	0.5
Casein number						
<-1 SD	113	67.3	35.4	165	46.7	11.5
-1 to -0.33 SD	222	54.0	9.0	262	26.7	1.5
-0.33 to 0.33 SD	335	47.5	8.1	393	18.1	1.2
0.33 to 1 SD	226	30.5	4.9	299	18.7	2.0
>1 SD	129	20.9	5.4	161	27.3	3.1
Fat, %						
<-1 SD	116	44.8	15.5	173	30.1	6.9
-1 to -0.33 SD	263	45.6	10.3	255	27.1	4.3
-0.33 to 0.33 SD	305	43.3	10.5	382	26.2	2.4
0.33 to 1 SD	206	47.6	9.7	301	20.9	1.6
>1 SD	135	36.3	5.9	169	20.1	1.2
SH°/50 mL ¹						
<- SD	154	68.8	27.3	168	38.7	12.5
-1 to -0.33 SD	227	50.2	9.2	316	25.6	2.8
-0.33 to 0.33 SD	262	43.5	5.3	334	26.6	1.5
0.33 to 1 SD	227	32.2	7.0	279	19.7	0.7
>1 SD	155	28.4	7.7	183	15.3	1.1
SCS						
<-1 SD	155	27.1	6.4	181	14.4	0.1
-1 to 0.33 SD	264	39.0	7.6	281	19.2	1.4
-0.33 to -0.33 SD	268	46.3	8.2	372	22.0	1.6
0.33 to 1 SD	176	53.4	14.2	259	33.2	2.7
>1 SD	162	54.3	17.3	187	37.4	11.7

¹SH°/50 mL = titratable acidity expressed as Soxhlet-Henkel degrees.

Table 3. Tests for statistical significance of fixed effects based on changes of likelihood statistic obtained with a semiparametric proportional hazard model in Holstein-Friesian and Brown Swiss data sets¹

Effect	df	Holstein-Friesian				Brown Swiss			
		SET18		SET31		SET18		SET31	
		Seq.	Last	Seq.	Last	Seq.	Last	Seq.	Last
DIM	5	27.35***	34.64***	18.04**	33.65***	71.15***	62.74***	80.22***	70.58***
Parity	2	3.08	31.48***	3.06	31.65**	1.20	18.09***	0.33	18.55***
Milk yield, kg/d	4	4.83	5.76	11.67*	8.82	24.66***	7.21	33.41***	11.38*
Casein number	4	74.46***	25.18***	97.56***	36.12***	45.68***	35.29***	57.06***	47.91***
Fat, %	4	6.08	9.31	5.68	10.49*	9.79	6.02	19.61***	8.28
SH ² /50 mL ²	4	82.46***	90.01***	75.08***	79.25***	62.33***	113.38***	78.23***	147.4**
SCS	4	11.69*	4.92	10.50*	1.35	36.27***	18.51**	51.02***	14.58**
Herd	33–37	217***	217***	228.4***	228.4***	229***	229***	266***	266***

¹Seq. = effects were included in the model in sequential order, the numbers expressing the increase of $-2 \times \log$ -likelihood after including the effect in the model; Last = the full model was compared with models excluding one effect at a time, the numbers indicating the loss of $-2 \times \log$ -likelihood after excluding the effect from the model; SET31 = original data set with end point defined at 31 min; SET18 = data set obtained after the rearrangement of the time space at 18 min.

²SH²/50 mL = titratable acidity expressed as Soxhlet-Henkel degrees.

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

pooling method to locate genomic regions associated with the noncoagulation of milk and detected regions, located on BTA 2 and BTA18, significantly associated with the noncoagulation of milk.

Risk Factors Affecting Rennet Coagulation Time

Number of records and percentage of censored records in each level of fixed effects included in the survival model are presented in Table 2. The results of our likelihood ratio test for the statistical significances of fixed effects are summarized in Table 3. Days in milk, casein number, SH, and herd effects were significantly related to the instantaneous coagulation rate when they were entered sequentially in the model or were excluded from the full model one at a time, giving similar results and showing very little redundancy when explaining variation of the investigated trait. The SCS was much more strongly related to the instantaneous coagulation rate for BS than HF. Parity, MY, and fat had smaller effects on RCT than the other individual effects. We observed different patterns for SET31 versus SET18 due to the different percentages of censored records. These results are consistent with our expectations based on the percentages of censored records with respect to the levels of fixed effects (Table 2).

Estimated hazard ratios (**HR**) for the fixed effects included in the survival model are presented in Figure 2. To ensure that we would get a set of meaningful, easily interpreted estimable effects, we imposed a specific constraint upon the effects to be estimated: the first level of each factor included in the model has been imposed. The HR differed between the herds; this arose from several sources of variation, including management and feeding practices (data not shown).

The DIM was a relevant effect for coagulation ($P < 0.001$), as milk from early-lactation cows exhibited the most favorable RCT for both HF and BS. For both breeds, the HR decreased rapidly until midlactation, and then recovered thereafter. This recovery was more pronounced for HF than for BS. The HR decreased from the first class (reference value) to about 0.4 for SET18 and SET31 of BS cows and SET18 of HF cows, whereas SET31 of HF cows decreased to about 0.5. At midlactation, therefore, the risk of coagulating decreased to around 50 to 60% less risk than what was seen for the first class. With respect to the beginning of lactation, the risk of coagulation showed very similar patterns in SET18 and SET31 for BS, whereas it was slightly higher after 31 min in HF. The variation of RCT across DIM could be related to changes in the physical and chemical characteristics of milk during lactation; differences were seen in its basic components, micelle structure, and salt equilibrium, leading to differences in its technological and physicochemical properties.

Parity significantly affected the instantaneous coagulation rate ($P < 0.001$), with milk from primiparous cows having less risk of coagulating compared with pluriparous cows. This is in agreement with Schaar et al. (1984), who reported that MCP improved with parity in bovine milk, but contrasts with other studies that failed to find any significant association between parity and MCP (Lindstrom et al., 1984; Davoli et al., 1990). The improvement of coagulation with number of calvings was more evident for HF than BS.

At the same parity, DIM, and milk composition, cows with higher MY exhibited a better coagulation pattern than lower producers, although the effect was significant only for BS. Though milk protein content was not associated with the occurrence of coagulation and fat had

Figure 2. Estimated hazard ratios (HR) and 95% CI of HR for fixed effects included in the Cox model for Holstein-Friesian and Brown Swiss cows considering the original set of data (SET31) and the data obtained after the rearrangement of the time space (SET18). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. SH°/50 mL = titratable acidity expressed as Soxhlet-Henkel degrees.

Figure 2 (Continued). Estimated hazard ratios (HR) and 95% CI of HR for fixed effects included in the Cox model for Holstein-Friesian and Brown Swiss cows considering the original set of data (SET31) and the data obtained after the rearrangement of the time space (SET18). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. SH°/50 mL = titratable acidity expressed as Soxhlet-Henkel degrees.

only a minor effect, the casein number had a marked influence on the instantaneous coagulation rates for both HF and BS ($P < 0.001$). The HR increased 2-fold or more as the casein number classes increased in both breeds, with very similar patterns seen in SET18 and SET31. This means that cows that produced milk with less whey protein and nonprotein compounds compared with caseins had the most favorable RCT. A good RCT is usually associated with good curd firmness, and RCT and curd firmness are known to influence cheese yield and quality (Aleandri et al., 1989; Ng-Kwai-Hang et al., 1989). As protein content was not associated with coagulation risk in either breed, it is not clear if the favorable effect of casein number is due to the higher proportion of casein or the lower proportion of whey protein and nonprotein compounds.

The SCS exhibited a significant effect on the instantaneous coagulation rate for BS cows ($P < 0.001$). In this breed, a lower SCS was correlated with a higher risk of coagulation, and the risk pattern among SCS classes was almost linear. Cows with clinical mastitis were not sampled so SCS could reflect subclinical mastitis. The near-linear pattern of coagulation pattern is

interesting, as it does not appear to reflect a subpopulation of at-risk animals, but rather a continuous effect between 2 biological extremes. In fact, the first class (SCS < 0.26) had almost 30% more risk of coagulating than the last class (SCS > 4.54). This pattern is in agreement with previous results obtained by Politis and Ng-Kwai-Hang (1988), who reported that variation in SCC had a marked effect on MCP, and suggested that increased SCC may be associated with increased plasmin activity. The accumulation of plasmin degradation products might affect the coagulation process, as these components interfere with the aggregating micelles responsible for curd formation (Politis and Ng-Kwai-Hang, 1988). Plasmin can rapidly cleave β -CN into γ -CN and smaller polypeptides (Auld et al., 1996), and it can also degrade α_{s1} -CN and α_{s2} -CN (Le Bars and Gripon, 1993; McSweeney et al., 1993). Increased plasmin activity is a major factor in the impairment of coagulation features and casein degradation in milk (Lucey, 1996; Srinivasan and Lucey, 2002). As reported by Zachos et al. (1992), increased plasmin activity leads to functional changes in milk proteins, altered milk coagulation, and decreased cheese yield.

Consistent with the findings of several studies (Ikonen et al., 1999; Cassandro et al., 2008; Cecchinato et al., 2011), the probability of coagulating increased with increasing SH, eventually reaching HR >4 for the last class of SH in both breeds (i.e., SH >3.67 and >3.73 for HF and BS, respectively). A recent review of the genetics and modeling of MCP reported that the milk-related traits of pH and SH had the highest phenotypic and genetic correlations with RCT (Bittante et al., 2012), whereas the complex relationships between MCP and other milk traits were recently highlighted by Macciotta et al. (2012).

CONCLUSIONS

Models that incorporate information from NC samples into analyses of milk renneting properties have been proposed as alternatives to the standard linear model in an effort to avoid losing highly informative samples and obtaining biased results. The survival model seems to be most suitable for this purpose because it can properly account for censored and uncensored records and appropriately use all of the available information. Moreover, this methodology allowed us to rearrange the time space used for the observation of RCT, and to define alternative traits (i.e., an RCT end point of 18 min). Restricting the time space and increasing the percentage of censored records did not yield any substantial differences in terms of the risk of coagulation compared with the traditional 31 min testing time. The most important factors affecting coagulation for both breeds were herd, DMI, casein number, and acidity. For BS cows, SCS also had a significant effect. In conclusion, although further research is needed to investigate the effect of these sources of variation on cheese yield, our results indicate that these factors may be used as indicator traits for enhancing the technological properties of bovine milk.

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