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Effects of animal versus vegetal rennet on milk coagulation traits in Mediterranean buffalo bulk milk

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ABSTRACT

Given consumer interest in Mozzarella di latte di Bufala and other cheeses, and the growing interest of the cheese industry in offering products adequate for lactovegetarian consumers, this study aimed to compare clotting capacity of vegetal and animal rennet in buffalo milk. Milk coagulation properties of 1,261 buffalo bulk milk samples collected during milk quality testing were assessed by lactodynamography using commercial animal (75% chymosin and 25% bovine pepsin) and vegetal (*Cynara cardunculus*) rennets. Chemical composition of milk samples was predicted by MilkoScan (Foss Analytics, Hillerød, Denmark) calibrated with specific buffalo standards. Rennet effect (animal versus vegetal) was statistically analyzed with a paired *t*-test. Fat, protein, and lactose contents of milk samples were 7.94%, 4.52%, and 4.80%, respectively. A similar variability of milk coagulation properties was observed with both rennets, with the exception of greater variability of curd firmness at 30 min after the addition of vegetal rennet compared with animal rennet (73 and 26%, respectively). On average, when using plant rennet, milk started to coagulate and reached the 20-mm coagulum 12 ± 0.22 min and 1.9 ± 0.20 min, respectively, later than with animal rennet. Thirty minutes after rennet addition, curds were almost twice as firm in animal as in vegetal rennet (difference of 23.92 ± 0.66 mm). However, curd firmness at 60 min was only 1.21 ± 0.39 mm thicker with vegetal than with animal rennet. Moreover, when using animal rennet, 99.52% of samples started coagulating within the first 30 min of analysis, whereas only 70.42% did so when using vegetal rennet. We conclude that vegetal rennet has the capacity to coagulate buffalo milk, achieving a similar curd firmness to that of animal rennet at 60 min. Further studies are needed to evaluate the sensory characteristics and consumer acceptability of Mozzarella di latte di Bufala processed with vegetal rennet.

Key words: cheese, Mozzarella, plant extract, renneting, vegetarian

INTRODUCTION

Buffalo (*Bubalus bubalis*) milk is the second most produced milk after bovine milk, representing 15% of worldwide production in 2017 (FAOSTAT, 2019). Buffalo milk production in Italy has increased considerably in the last 50 yr and represents approximately 95% of the total buffalo milk produced in the European Union (FAOSTAT, 2019). In Italy, buffalo milk is mainly made into Mozzarella di Bufala Campana PDO (protected designation of origin), although the manufacturing of other cheese types and dairy products has been investigated (Borghese, 2005; Addeo et al., 2007). The steady increase of buffalo milk production could be ascribed to the higher price of buffalo milk (about 1.50 €/L in Italy; CLAL, 2019b) compared with cow milk (about 0.37 €/L in Italy; CLAL, 2019a) and to the worldwide increase of consumption of Mozzarella (Borghese, 2013).

Milk coagulation is an important step in cheese manufacturing; thus, the renneting properties of milk are crucial aspects informing milk processability for the cheesemaking process. The most relevant milk coagulation properties (**MCP**) are rennet coagulation time (**RCT**), curd firming time ($\mathbf{k_{20}}$), and curd firmness 30 min after rennet addition to milk ($\mathbf{a_{30}}$; McMahon and Brown, 1982); these traits are commonly assessed through lactodynamography. An extended curd firmness (60 min after rennet addition to milk, $\mathbf{a_{60}}$) has also been determined in milk samples when using vegetal rennet (Liburdi et al., 2019). Mozzarella di Bufala Campana PDO is produced with animal rennet from the abomasum of calves (Ministero delle Politiche Agricole e Forestali, 2003), containing chymosin and pepsin that coagulate the milk. However, alternatives to animal rennet in cheese production have been stud-

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ied, such as enzymes of microbial origin, recombinant proteases synthesized by genetically modified microorganisms, and plant proteases (Jacob et al., 2011). Among plants, cardosin proteases from *Cynara* spp. are the most used for cheese manufacturing (Roseiro et al., 2003). The dairy industry's growing interest in using vegetal coagulants for cheese production is due to the global increase in cheese demand and, at the same time, the decreasing supply of calf rennet (Jacob et al., 2011; Bathmanathan et al., 2019), increasing numbers of lactovegetarian consumers, religious considerations (kosher and halal diets), and negative consumer perceptions of the use of genetically modified microorganisms (Roseiro et al., 2003).

Although plant rennet is used for the elaboration of some traditional cheeses in Mediterranean countries, Southern Europe, and West Africa, its use is very limited at industry level due to the high bitterness and lower cheese yield obtained compared with calf rennet (Ben Amira et al., 2017a). Nevertheless, the increasing production of buffalo milk could make room for the development of new and distinguishable products. To our knowledge, there is currently no Mozzarella-type cheese on the market made from buffalo milk with vegetal rennet, and scarce information exists regarding the capability of vegetal rennet to coagulate buffalo milk. Thus, the aim of this study was to investigate the clotting properties of buffalo bulk milk using animal or vegetal rennet.

MATERIALS AND METHODS

Sample Collection and Reference Analysis

From January to August 2018, a total of 1,302 buffalo bulk milk samples from herds located in Central Italy (Lazio region) were collected after morning milking (representative of morning and previous evening milkings) during routine sampling for a milk quality payment program. Lazio and Campania are the 2 main Italian regions that produce buffalo milk intended for Mozzarella di Bufala PDO elaboration (Ministero delle Politiche Agricole e Forestali, 2003). In Italy, buffalo are reared in intensive conditions, with dairy buffaloes kept loose in paddocks close to the milking room and artificially inseminated preferably from February to March (Borghese, 2013). Animals are usually fed high-energy, high-protein total mixed rations based on maize silage, concentrate, hay, straw, and sometimes by-products (Borghese, 2013).

Samples were transported refrigerated (4°C) within 24 to 36 h from collection to the Experimental Zooprophylactic Institute of Lazio and Tuscany "Mariano Aleandri" (Rome, Italy), the national reference laboratory for dairy product quality in Central Italy. Milk samples were analyzed for chemical composition, citric acid levels, and SCC using standard methods. Briefly, a MilkoScan FT6000 (Foss Analytics, Hillerød, Denmark) calibrated with appropriate buffalo standards was used to predict fat, protein, casein, and lactose percentages, and a Fossomatic FC (Foss Analytics) was used to determine SCC. The SCC × 1,000 was logarithmically ($\mathbf{Log_{10}}$) transformed to normalize distribution (Ali and Shook, 1980). The pH was measured by a potentiometric pH meter (Mettler Delta 345; Mettler Toledo SpA, Novate Milanese, Italy).

Milk coagulation properties of each sample were determined via Formagraph (Foss Analytics) using animal or vegetal rennet. For MCP determination with animal rennet, milk samples (10 mL) were heated to 35°C, and 200 μL of commercial calf rennet (Caglificio Clerici spa-Sacco srl, Cadorago, Italy; 75% chymosin and 25% bovine pepsin; 175 international milk clotting units/mL; strength 1:15.000 in Italian commercial units) diluted to 1% (wt/wt) in distilled water was added to the milk sample. For MCP determination with vegetal rennet, the same procedure was followed, adding to each milk sample 200 μL of commercial Galium vegetal rennet (*Cynara cardunculus*, batch CV60118, Laboratorio Prodor, Bobbio, Italy; 98% cardosin; strength 1:6.000 in Italian commercial units) diluted to 2.5% (wt/wt) in distilled water. The analysis was begun simultaneously with both rennets for each milk sample (2 aliquots of the same milk sample were used, 1 for each rennet), and measurement ended 60 min after rennet addition.

Statistical Analysis

Before statistical analysis, the data set was edited as follows. For milk composition traits (i.e., levels of fat, protein, casein, lactose, citric acid, pH, and $\log_{10}$ SCC), values that deviated more than 3 standard deviations (SD) from the mean of each trait were treated as missing values. For each rennet type, values of RCT and k_{20} $\leq$0 or $\geq$60 min, and a_{30} and a_{60} that deviated more than 3 SD from the mean were treated as missing values. Then, only traits with data recorded for both rennet types were retained. In addition, records without RCT were deleted. Therefore, the final data set for the statistical analysis consisted of 1,261 records.

Rennet effect was analyzed with a paired *t*-test in SAS version 9.4 (SAS Institute Inc., Cary, NC). The difference for the matched pairs (animal vs. vegetal rennet) for each trait followed a normal probability distribution. Data are presented as means and SD or standard error. Significance was declared at $P < 0.05$. Moreover, a new classificatory variable was created based on samples' RCT, stratifying them into 5 classes:

class 1 (RCT < 10 min), class 2 (10 min ≤ RCT < 20 min), class 3 (20 min ≤ RCT < 30 min), class 4 (30 min ≤ RCT < 45 min), and class 5 (45 min ≤ RCT < 60 min).

RESULTS AND DISCUSSION

Buffalo Bulk Milk Composition

Chemical composition and $\log_{10}$ SCC of buffalo bulk milk are shown in Table 1. Buffalo milk has greater fat, protein, and casein contents and slightly less lactose than cow milk (Guo, 2010). It is also known that buffalo milk has higher Ca, P, and Fe, and lower Na than cow milk (Guo, 2010). These characteristics have important effects on cheesemaking ability that lead to greater cheese yield from buffalo than from cow milk. Fat and protein contents of our samples were similar to the last national statistics available for Italian buffalo milk (A.N.A.S.B., 2019). Several authors have reported fat, protein, casein, lactose, pH, and $\log_{10}$ SCC contents and ranges in bulk milk of Italian Mediterranean buffalo similar to those obtained in the present study (Di Francia et al., 2003; Liotta et al., 2015; Pasquini et al., 2018). The coefficients of variation (**CV**) for milk composition ranged from 3% (lactose) to 8% (fat), in agreement with the variability reported by Liotta et al. (2015) for bulk milk samples.

Milk Coagulation Properties Using Animal Rennet

When using animal rennet, 17 out of 1,302 milk samples (1.3%) did not start to coagulate within the 60 min of analysis. Moreover, 30 out of 1,302 samples (2.3%) did not achieve 20-mm curd firmness within the 60 min of analysis. The percentage of samples with RCT or k_{20} <60 min using animal rennet agrees with the outliers reported by Manuelian et al. (2017), who evaluated MCP in individual buffalo milk samples within 30 min of analysis. However, those authors defined outliers for RCT and k_{20} as values that deviated more than 3 SD from the mean, among the samples that coagulated in less than 30 min. They classified all samples with an RCT >30 min as non-coagulating, which corresponded to 18% of their samples.

Table 2 displays the descriptive statistics of MCP for bulk buffalo milk using animal and vegetal rennets. Considering the results obtained with animal rennet, the CV were 37% for RCT, 92% for k_{20}, 26% for a_{30}, and 28% for a_{60}. The variation within traits observed in the present study contrasted with those reported by Manuelian et al. (2017) from individual buffalo milk samples, in particular for k_{20}. Those authors reported a CV of 45% for RCT, 39% for k_{20}, and 38% for a_{30}. The disagreement between these studies could be due to the type of sample (individual vs. bulk milk), the editing before performing the statistical analysis, and the longer time of analysis in our study (30 vs. 60 min). The RCT with animal rennet were similar to those observed by Manuelian et al. (2017) and Ariota et al. (2007) from individual buffalo milk samples, whereas Cecchinato et al. (2012) reported a slightly shorter time (11.62 min) in individual milk and Liotta et al. (2015) a longer time (21.3 min) in bulk buffalo milk. The average k_{20} in our study was longer than those reported in previous studies for individual buffalo milk (3.25 min, Bartocci et al., 2002; 1.73 min, Ariota et al., 2007; 3.17 min, Manuelian et al., 2017) and bulk milk (3.08 min, Liotta et al., 2015). Curd firmness 30 min after rennet addition was consistent with previous studies in individual buffalo milk samples (47.49 mm, Bartocci et al., 2002; 46.01 mm, Ariota et al., 2007). Nevertheless, some authors have indicated lower a_{30} values in bulk (32.69 mm, Liotta et al., 2015) and individual buffalo milk (39.52 mm, Manuelian et al., 2017). We found a very small difference between curd firmness at 30 and 60 min after addition of animal rennet, which supports the idea that a_{60} is rarely calculated to evaluate MCP when using animal rennet. To our knowledge, this is the first time that a_{60} has been estimated for buffalo milk. Despite all the studies assessing MCP using lactodynamography, direct comparison of the obtained values should be performed carefully because of the differences in animal rennet solution characteristics, such as the proportion of chymosin to pepsin and the degree of dilution (Manuelian et al., 2017).

Table 1. Chemical composition of Mediterranean buffalo bulk milk samples

Trait	n	Mean	SD	Minimum	Maximum
Milk composition, %					
Fat	1,143	7.94	0.63	5.93	9.92
Protein	1,138	4.52	0.23	3.78	5.20
Casein	1,144	3.76	0.23	3.04	4.42
Lactose	1,154	4.80	0.17	3.89	6.10
Citric acid	1,261	0.13	0.02	0.08	0.20
Log_{10} SCC	1,255	5.18	0.25	4.43	5.91
pH	1,141	6.77	0.11	6.43	7.08

Table 2. Milk coagulation properties of Mediterranean buffalo bulk milk and paired *t*-test results comparing animal versus vegetal rennet

	Animal rennet					Vegetal rennet				Mean difference	SE of the difference	*P*-value
Trait[1]	n	Mean	SD	Minimum	Maximum	Mean	SD	Minimum	Maximum			
RCT, min	1,261	13.14	4.87	2.50	53.75	25.19	9.06	2.50	58.25	12.05	0.22	<0.001
k_{20}, min	1,226	4.89	4.49	0.20	39.00	6.79	5.98	0.75	41.25	1.91	0.20	<0.001
a_{30}, mm	962	48.32	12.35	1.62	83.96	24.40	17.77	0.02	72.60	−23.92	0.66	<0.001
a_{60}, mm	1,241	47.62	13.29	3.80	86.74	48.84	10.84	0.48	83.60	1.22	0.39	0.002

[1]RCT = rennet coagulation time (time to start of coagulation after rennet is added to milk); k_{20} = curd-firming time from start of coagulation until curd firmness of 20 mm is reached; a_{30} = curd firmness 30 min after addition of rennet to milk; a_{60} = curd firmness 60 min after addition of rennet to milk.

Milk Coagulation Properties Using Vegetal Rennet

When using the commercial vegetal rennet, 39 out of 1,302 milk samples (3.0%) did not start to coagulate within the 60 min of analysis. Moreover, 73 out of 1,302 samples (5.6%) did not achieve 20-mm curd firmness within the 60 min of analysis. Table 2 displays the descriptive statistics of MCP for bulk buffalo milk using animal and vegetal rennets. Considering the results obtained with vegetal rennet, the CV were 36% for RCT, 88% for k_{20}, 73% for a_{30}, and 22% for a_{60}.

Most studies of vegetal rennet have been performed using cow or sheep milk and rennets including species of the genus *Cynara* L. (Roseiro et al., 2003; Ben Amira et al., 2017a; Troch et al., 2017). Unlike that of animal rennet, coagulation activity of vegetal rennet is highly variable due to differences in the botanical composition of the rennet, the purity of the extract, the state of maturity of the plant, the part of the plant used (e.g., flowers vs. leaves), and the conditions of collection, season, and storage (Heimgarter et al., 1990; Ben Amira et al., 2017a; Troch et al., 2017). Vegetal rennets based on *Cynara cardunculus* are the most used in cheese production, due to the presence of the aspartic proteinases cardosin A and B (which are analogous to chymosin and pepsin, respectively, in terms of activity and specificity) that have the ability to hydrolyze the Phe105-Met106 bond of κ-casein (Veríssimo et al., 1995; Ben Amira et al., 2017a), which is necessary for rennet milk clotting.

Moreover, different techniques to assess MCP using vegetal rennet have been used and have provided different MCP traits than the ones determined in the present study. For example, Esteves et al. (2002; 2003a,b) used a dynamic rheometer, and Ben Amira et al. (2017b) used a rheometer with an oscillatory mode to evaluate the rennetability of vegetal rennet in cow milk. These 2 rheometers allow continuous measurement of the viscoelastic properties during the whole coagulation process by applying a strain (or stress) on the gelling sample. Thus, the parameters determined are the storage modulus (**G′**, expressed in Pa), which is the measure of gel strength; the gelation time (**GT**, expressed in min), which is the time needed for G′ to exceed a predefined pressure threshold; and the loss modulus (**G″**, expressed in Pa). When comparing the Formagraph with a rheometry method, an agreement has been observed between GT and RCT values (Ketto et al., 2015). Comparing our results with studies that used different plant extracts of *C. cardunculus* and *Cynara humilis* in cow milk, we obtained greater RCT than the GT reported by Esteves et al. (2002; 2003a,b) and Ben Amira et al. (2017b).

Vegetal Versus Animal Rennet on Milk Coagulation Properties

A greater CV for a_{30} was obtained with vegetal than with animal rennet. On the other hand, similar variability between animal and vegetal rennet has been observed for RCT, k_{20}, and a_{60}. On average, when using plant rennet, milk started coagulating 12 min later than with animal rennet ($P < 0.001$; Table 2; Figure 1). Ben Amira et al. (2017b) have also reported a longer time for *C. cardunculus* than chymosin rennet to start coagulating reconstituted skim cow milk (determined at G′ = 1 Pa; difference of 7 min in GT) at 30°C using a dose concentration of the enzyme of 0.5 mg/10 mL. However, in the same study, the authors indicated a longer time for chymosin than *C. cardunculus* in raw skim cow milk (difference of 4 min in GT). Esteves et al. (2003a), at 35°C of gelation temperature, reported a longer time for chymosin than for *C. cardunculus* or *C. humilis* to start coagulating reconstituted cow skim milk (determined at G′ = 0.5 Pa; almost 5 min of difference of GT).

Nevertheless, the same authors did not observe differences between the coagulation enzyme used when the assay was conducted at 32°C (Esteves et al., 2003a,b), even at different pH conditions (pH from 6.0 to 6.7; Esteves et al., 2003b). Moreover, in the present study, almost all the samples (99.52%; 1,255 out of 1,261) started coagulating before 30 min when using animal rennet, whereas only 70.42% (888 out of 1,261) of the samples started to coagulate within the first 30 min when using vegetal rennet (Figure 2). Manuelian et al. (2017) have reported a lower percentage of samples that coagulated within the first 30 min of the analysis (87%) using the same animal rennet in individual buffalo milk samples.

The k_{20} was reached 1.9 min earlier using animal compared with vegetal rennet (Table 2) due to a faster velocity of aggregation from RCT to k_{20}, which was 4.09 and 2.94 mm/min for animal and vegetal rennet, respectively (Figure 1). Moreover, considering the beginning of the assay, when the enzyme was added to the milk sample, the time difference between both rennets to achieve 20-mm coagulum was even greater (12 min faster with the animal rennet; Figure 1). Because of the delay in starting the aggregation process when using vegetal rennet, a_{30} was almost twice as firm in animal than in vegetal rennet (Table 2; Figure 1). However, a_{60} differed by only 1.21 mm between both rennets (Table 2; Figure 1). Measurements between 30 and 60 min after enzyme addition would be interesting, to better determine the time at which the enzymes produce the same coagulum thickness. In addition, considering the prolonged coagulation time of the vegetal rennet used

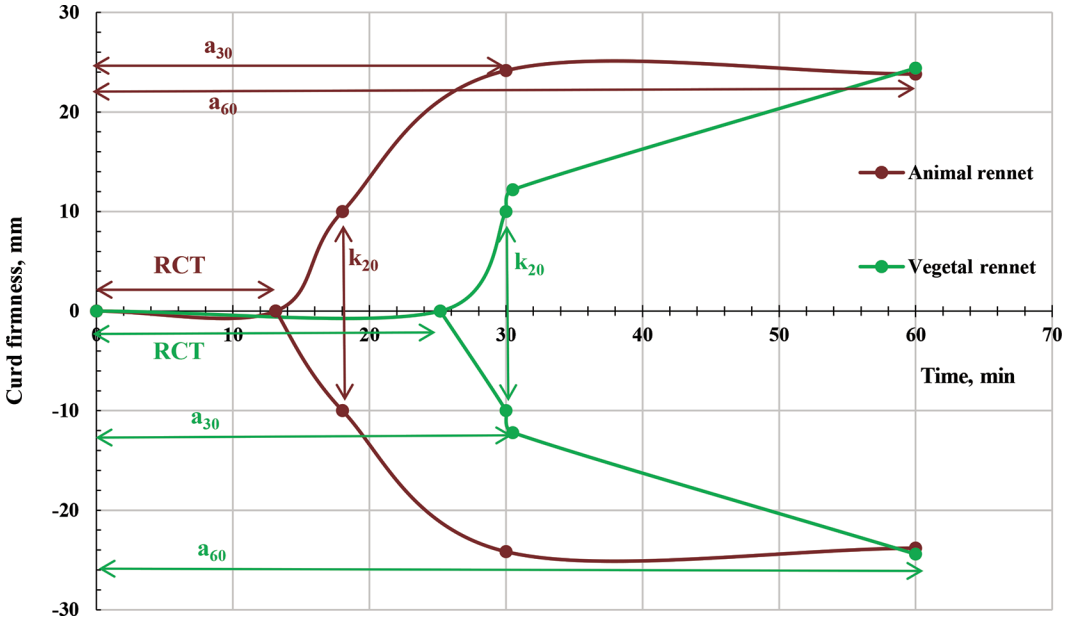

Figure 1. Diagram of coagulation and curd firmness of Mediterranean buffalo bulk milk samples as a function of time and rennet used (animal or vegetal). The same bulk milk sample was tested using animal and vegetal rennet. Dots represent the average values for RCT (rennet coagulation time, time to start of coagulation after addition of rennet to milk; n = 1,261); k_{20} (curd-firming time from start of coagulation until curd firmness of 20 mm is achieved; n = 1,226); a_{30} (curd firmness 30 min after addition of rennet to milk; n = 962); and a_{60} (curd firmness 60 min after addition of rennet to milk; n = 1,242).

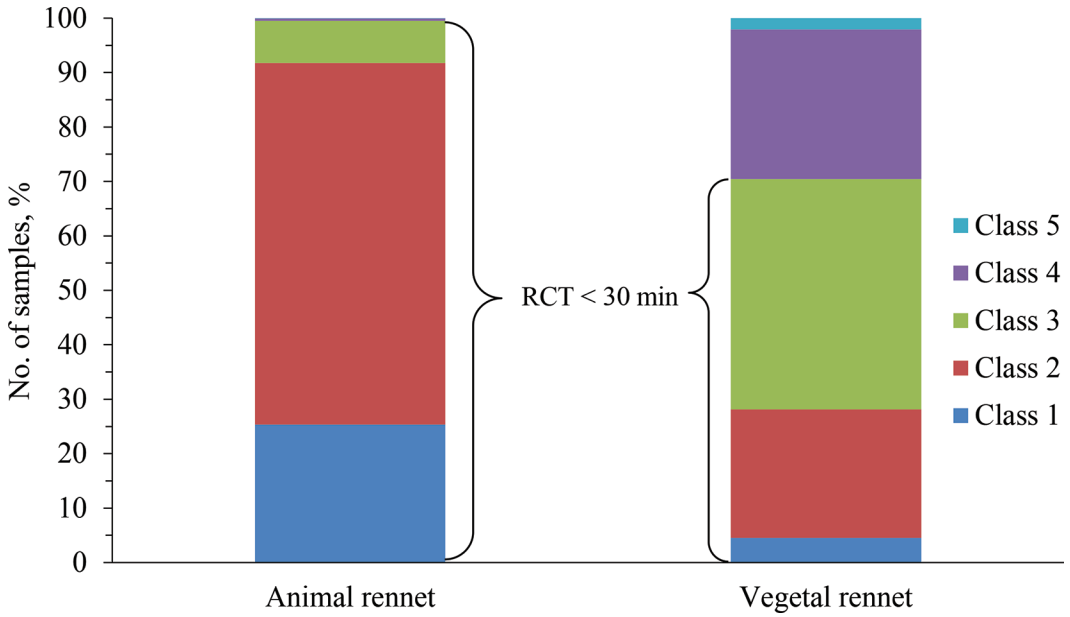

Figure 2. Proportion of Mediterranean buffalo bulk milk samples classified according to their rennet coagulation time (RCT, time to start of coagulation after addition of rennet to milk) using animal or vegetal rennet. Class 1: RCT < 10 min; class 2: 10 min ≤ RCT < 20 min; class 3: 20 min ≤ RCT < 30 min; class 4: 30 min ≤ RCT < 45 min; and class 5: 45 min ≤ RCT < 60 min.

and the subsequent whey drainage step, the quantity of vegetal rennet added might be adjusted to better fit the coagulation behavior. Nevertheless, to produce Mozzarella di latte di Bufala, after forming and cutting the curd, fermentation continues under the whey for about 3h, and then the cheese is put on steel racks to drain for a few more hours. Thus, a delay of about 20 to 30 min when using the vegetal rennet could be considered marginal in proportion to the complete process (6 to 7 h).

The slight difference observed for a_{60} between animal and vegetal rennet in the present study was consistent with previous studies that assessed G′ using chymosin or vegetal extracts from the genus *Cynara* L. (Esteves et al., 2003a; Ben Amira et al., 2017b). The G′ performed similarly to curd firmness determined with Formagraph: at higher coagulum thickness, greater pressure must be applied (Ketto et al., 2015). Ben Amira et al. (2017b) reported a similar G′ between chymosin and vegetal extracts at the end of the assay (60 min after addition of the coagulant) in raw skimmed cow milk at 30°C; similar results were reported by Esteves et al. (2003a) at the end of the assay (3 h after addition of the coagulant) when comparing chymosin and *C. cardunculus* rennets at 32°C and 35°C of gelation temperature in reconstituted cow skim milk. On the other hand, Esteves et al. (2003b) reported a greater G′ at the end of the assay (6 h after the addition of the coagulant) when using chymosin than when using plant extracts from the genus *Cynara* L.; however, that difference was lower at pH 6.7, being 12 Pa instead of 20 Pa.

CONCLUSIONS

The present study compared the clotting activity of commercial animal and vegetable rennets (*C. cardunculus*) on buffalo bulk milk. Milk samples with vegetal rennet took more time to begin coagulation than when using animal rennet. However, at the end of the assay (60 min), both rennets presented similar coagulum thickness, suggesting similar viscoelastic properties of the final product. Therefore, from an analytical point of view, vegetal rennet could be used to produce Mozzarella and other cheeses from buffalo milk, allowing more time for the enzyme to actuate. Further studies are needed to assess the sensory properties and texture characteristics of the product, to ensure consumer acceptability. In addition, the cost effectiveness of the use of vegetal rennet by the dairy industry should be evaluated, as it might be necessary to use greater amounts of vegetal rennet to achieve a_{30} comparable to that of cheeses made with animal rennet.

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