Molecular mobility changes after high-temperature, short-time pasteurization: An extended time-domain nuclear magnetic resonance screening of ewe milk

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ABSTRACT

This study presents an extensive investigation on the effect of pasteurization on raw whole ewe milk. Milk samples have been analyzed, throughout lactation (from February to July), by time-domain nuclear magnetic resonance (TD-NMR), collecting the characteristic TD-NMR relaxation parameters, proton longitudinal and transverse relaxation times (^1H T_1 and T_2). Collected data aim at integrating previous NMR works, mainly focusing on dairy model systems (casein and whey proteins solutions and gels, reconstituted skim milk) and cheese, with specific reference to the effect of heat treatments. Whole ewe milk, from a single flock (Sarda sheep breed), was daily analyzed both as untreated (raw) and heat treated with a laboratory-scaled high-temperature, short-time treatment (72°C for 15 and 20 s). Moreover, molecular dynamics in milk were investigated by TD-NMR in different periods of lactation for the first time. As a consequence of high-temperature short-time treatment, ^1H T_1 and T_2 consistently shifted to lower values with respect to raw counterparts. Statistical analysis indicated a significant decrease of T_2 in treated samples, to an extent dependent on the heat treatment duration. A subset of dedicated experiments demonstrated that the observed T_2 shift is largely ascribable to protein molecular rearrangements and, to a lesser extent, to the interaction of fat globules with proteins or other nonfat components (or both). In light of the crucial importance of detecting the application of a heat treatment to milk, the results reported here suggest TD-NMR relaxation parameters were able to describe heat-induced changes in molecular dynamics and interactions of milk components in a water-rich environment. The use of TD-NMR can be considered a

potential suitable technique for quality control and assurance practices in the dairy industry. Upon statistical validation of methods, the application of TD-NMR in the dairy industry would take advantage of its low cost, reliability, and robustness.

Key words: sheep milk, time-domain nuclear magnetic resonance, heat treatment

INTRODUCTION

The application of heat treatments to milk is widely exploited currently, especially at an industrial level. Heat is applied to make milk suitable for human consumption and cheese/dairy production. Heat treatments primarily aim at destroying pathogenic flora and reduce undesirable gas and off-flavor-forming organisms in raw milk.

Heating conditions are usually optimized, with proper temperature and duration combinations, to ensure a minimized alteration of milk molecular properties. Nevertheless, depending on the applied heat treatment, several physico-chemical modifications are known to occur, inducing undesirable alterations in the functionality of specific milk components. Often, such molecular changes lead to an alteration of the cheesemaking properties of milk (Singh and Waungana, 2001). Heat treatments have been shown to cause a progressive monomerization and denaturation of whey proteins (deWit and Klarenbeek, 1984; Fox et al., 2015) and enzyme inactivation (Piga et al., 2013). Moreover, heat treatments promote interactions between serum proteins and membrane of fat globules (Corredig and Dalgleish, 1996), as well as between serum proteins and casein micelles (Fox et al., 2015). The abovementioned molecular rearrangements can result in sensible modifications of sensory and nutritional properties of treated milk and of the corresponding dairy products (Buchin et al., 1998; Mendia et al., 1999; Morales et al., 2000; Caboni et al., 2019).

Time-domain nuclear magnetic resonance (TD-NMR) is known for its ability to monitor complex

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molecular changes occurring in several food systems (van Duynhoven et al., 2010). Useful applications of TD-NMR for the dairy industry have been developed. Time-domain nuclear magnetic resonance has been exploited mostly on model systems and reconstituted skim milk so far. For example, the effect of heat treatment (60–80°C, Lambelet et al., 1989, 1992) and acidification (Mariette et al., 1993) on skim milk could be described in terms of changes in molecular mobility, with reference to the transverse relaxation time constant (T_2). Interestingly, curd syneresis can be monitored and described by NMR relaxation parameters, which turned out to be significantly affected by heat treatments (Tellier et al., 1993; Calvo and Balcones, 2000; Hansen et al., 2010; Curti et al., 2019). Monitoring the efficacy of heat treatments on milk is required for quality assurance and control in the dairy industry. The established official method for controlling milk pasteurization measures the residual activity of alkaline phosphatase (European Commission, 2006; ISO, 2013). However, alkaline phosphatase testing exhibits some criticalities and its reliability has been questioned (Scintu et al., 2000; Raynal-Ljutovac et al., 2007; Moatsou, 2010; Rankin et al., 2010). Preliminary investigations carried out in our laboratory showed the ability of magnetic resonance to detect heat-induced changes in milk, curd, and cheese (Mulas et al., 2013; Curti et al., 2019). The present work complements the abovementioned findings on a much larger data set by analyzing the effects of a laboratory-scale HTST pasteurization treatment on ewe milk, in different periods of lactation. The potential of TD-NMR in detecting heat-promoted molecular modifications, regardless of season and the associated compositional variability of milk, is described here. Conclusions are drawn by comparing raw milk samples and their HTST-treated counterparts, on a daily basis, from February to July and based on an additional subset of measurements aimed at understanding the influence of other factors such as pH, water evaporation, skimming, milk fat homogenization, and intrasample variability.

MATERIALS AND METHODS

Sheep Milk Supply

Fresh raw whole ewe milk was obtained from a single local flock (Sarda sheep breed; Agris Sardegna, Sassari, Italy) on a daily basis during 6 mo of lactation (February–July). Milk was transported to the laboratories in a plastic (polyethylene terephthalate) bottle, stored in refrigerator at 6°C, and analyzed within 12 h from milking.

Milk Sampling and Heat Treatment

Following gentle agitation, a batch of milk (≈ 50 mL) was extracted from the bottle and 3 aliquots were directly pipetted into 3 NMR glass tubes (10 mm diameter, 180 mm height, Bruker Biospin, Milan, Italy; Figure 1). The volume in each NMR tube was optimized to 680 μ L to ensure full immersion of the sample in the homogeneous magnetic field region of the spectrometer magnet. The NMR tubes were equilibrated at 25°C (Elma, Elmasonic S 30 H, Berlin, Germany) for 10 min before analysis.

One of the 3 samples was analyzed untreated (raw = **R**), whereas the remaining 2 samples were analyzed after a heat treatment protocol simulating the HTST procedure (heat treated = **HT**). Two different heat treatments were tested: 72°C for 15 s (**HT15**) and 72°C for 20 s (**HT20**), consisting of a 2-step time- and temperature-controlled heating process (digital stainless-steel thermoprobe, VWR Collection, Milano, Italy). To perform the HTST treatment, each sample-loaded NMR tube was immersed in a water bath at 85°C (first step), with continuous manual agitation, to heat milk from room temperature (23°C) to 72°C (required time = 5 s, based on preliminary experiments) and immediately transferred into a second water bath at 72°C (second step), for 10 (HT15, i.e., 5 + 10 s) or 15 (HT20, i.e., 5 + 15 s) s. Immediately after the heat treatment, HT samples were quench-cooled in an ice-water basin (required time = 10 s to cool down to about 15°C, based on previous trials) with continuous manual agitation. The complete experimental plan included at least 3 and up to 6 milk batches (i.e., analysis of R, HT15, and HT20 per batch) on a daily basis (Figure 1), resulting in $\sim 1,500$ analyses overall. Heat treatments and acquisition of replicates of R, HT15, and HT20 samples were randomized in the daily schedule.

Measurement of pH

Milk pH was measured with a temperature probe-equipped pH meter (pH 210, Hanna Instruments Italy s.r.l., Padova, Italy). The pH was checked as the milk was received in the laboratory (after equilibration to 25°C) and before each analysis on raw milk batches progressively extracted from the bottle throughout the day.

Milk Composition

Samples of raw whole milk were analyzed for fat, protein, casein, lactose content, and SCC using a Milkoscan FT+ (Foss, Hillerød, Denmark).

¹H TD-NMR

The ¹H T₁ longitudinal (inversion recovery sequence; recycle delay 7 s, log-spaced interpulse range 1–12,000 ms, 20 data points) and ¹H T₂ transverse (Carr-Purcell-Meiboom-Gill pulse sequence; recycle delay 7 s, interpulse spacing 0.0567 ms, 8,000 data points) relaxation curves were obtained with a low-field NMR spectrometer (20 MHz, the miniSpec, Bruker Biospin, Milan, Italy), operating at 25.0 ± 0.1°C (JULABO, F25-ED, Milan, Italy).

Fitting of Experimental ¹H TD-NMR Data

The relaxation curves were fitted with the CONTIN algorithm (Provencher, 1982; software provided by Bruker, Milan, Italy) to obtain quasicontinuous distributions of T₁ (400 points; range: 1–5,000 ms) and T₂ (400 points; range: 1–500 ms). The distributions were then fitted with a Beta equation (“BetaAmp,” Equation [1], where a₀ = amplitude, a₁ = center, a₂ = width, a₃ = shape 1, and a₄ = shape 2) that well represents the observed shape (Table Curve software, trial version, Systat, San Jose, CA):

$$(x) = \frac{a_0 \times \left(\frac{x - a_1 + \frac{a_2(a_3 - 1)}{a_3 + a_4 - 2}}{a_2} \right)^{a_3 - 1} \times \left(1 - \frac{x - a_1 + \frac{a_2(a_3 - 1)}{a_3 + a_4 - 2}}{a_2} \right)^{a_4 - 1}}{\left(\frac{a_3 - 1}{a_3 + a_4 - 2} \right)^{a_3 - 1} \times \left(\frac{a_4 - 1}{a_3 + a_4 - 2} \right)^{a_4 - 1}} \quad [1]$$

Characteristic T₁ and T₂ relaxation times (ms), representative of the maximum of each distribution, were extrapolated (a₁ parameter, Equation [1]). Characteristic T₁ and T₂ relaxation times were then analyzed in terms of probability density (PDF) and cumulative distribution functions (Statistic Toolbox and Curve Fitting Toolbox, Matlab R2017a version, The MathWorks Inc., Natick, MA) and the best-fitting model was selected according to maximum likelihood estimation. Ranking criterion to select the best-fitting distribution was established according to both log-likelihood (Berger and Wolpert, 1988) and Akaike information criterion (Akaike, 1998).

Figure 1. Schematic representation of sampling experimental procedure. NMR = nuclear magnetic resonance; R = raw; HT15 = heat treatment at 72°C for 15 s; HT20 = heat treatment at 72°C for 20 s.

Table 1. Gross mean composition (g/100 g of milk) and SCC of milk during lactation in 2018

Item	Mean	Minimum	Maximum
pH	6.66	6.61	6.70
Fat	4.93	4.46	5.31
Protein	5.19	4.95	5.33
Casein	3.94	3.73	4.08
Lactose	4.63	4.57	4.70
SCC $\times 1,000/\text{mL}$	1,127	618	1,649

Statistical Analysis

To highlight the effect of thermization on characteristic T_1 and T_2 constants, R and HT (15 and 20 s) were statistically compared on a daily basis (Prism v 5.03, GraphPad Software, San Diego, CA). The R, HT15, and HT20 were compared by means of ANOVA, and R was individually compared with HT15 and HT20 by a *t*-test ($P \leq 0.05$).

To better evaluate the heat-driven differences between characteristic T_1 and T_2 values of milk (i.e., to highlight the shift of T_1 and T_2 of HT15 and HT20 with respect to R samples), delta (Δ) scores were calculated by subtracting each daily R value to each daily HT value. Given the intrinsic variability of milk relaxometric properties on different days, deltas were only obtained from samples analyzed the same day, and considering all the possible combinations ($\Delta_{\text{HTn-Rn}} C_{\text{day}}$, where n = number of daily measurements and C indicates all possible combinations according to the notation of combinatorial mathematics). Delta will be identified in the following as $\Delta T_{1 \text{ HT-R}}$ for T_1 , and $\Delta T_{2 \text{ HT-R}}$ for T_2 . Delta values (ms) represent the entity of time constant variation (absolute value) upon heat treatment and indicate the direction of the shift (positive or negative value

sign). Statistical comparison of the binary outcome of the $\Delta T_{1 \text{ HT-R}}$ and $\Delta T_{2 \text{ HT-R}}$ proportions was carried out by chi-squared test, followed by Tukey's honestly significant difference multiple comparison (Matlab Statistical Toolbox, The Mathworks Inc.).

RESULTS AND DISCUSSION

Milk Composition

Table 1 shows the composition of raw milk during the season. As expected, fat, protein, and lactose contents are consistent with the average regional values [personal communication of ARA (Breeders Association of Sardinia)].

^1H TD-NMR

Representative ^1H T_1 and T_2 distributions of R and HT, obtained by CONTIN software, are shown in Figure 2a and 2b, respectively. The ^1H T_1 and ^1H T_2 distributions showed a mono-exponential behavior, with the T_1 population relaxing in the range 700–1,700 ms (peaking at ~ 900 –1,200 ms) and T_2 in the range 50–150 ms (peaking at ~ 80 –110 ms). Such features indicate a fast diffusion regimen of water molecules sampling all possible environments in the NMR time scale, but also reflect the complexity of milk at a molecular level, where very different proton domains and mobilities coexist. Similar T_2 relaxation times were reported in previous studies on milk model systems, milk protein solutions, and bovine skim milk (Hills et al., 1990; Mariette et al., 1993; Le Dean et al., 2004) and were related to water protons in fast exchange with labile protons of soluble whey proteins and lactose.

Figure 2. Representative ^1H longitudinal relaxation time (T_1 ; A) and transverse relaxation time (T_2 ; B) distributions (obtained using CONTIN software, Provencher, 1982; software provided by Bruker, Milan, Italy) of raw (R; solid line) and heat treated (HT; dashed line).

Characteristic milk T_1 and T_2 for each day of analysis (calculated from all samples from all batches in the same day) are reported in Tables 2 and 3, respectively. A preliminary statistical analysis (ANOVA, $P \leq 0.05$) gave an overall analysis of milk characteristic T_1 and T_2 changes. Statistics indicated 55.6% and 75.5% of significant differences between R and HT samples ($P \leq 0.05$) for T_1 and T_2 , respectively, regardless of the duration of heat treatment. The comparison between R and HT15 (t -test, $P \leq 0.05$) indicated 58.2% and 70.3% of significant differences for T_1 and T_2 , respectively. Comparing R to HT20, 59.6% and 80.4% of significant differences were found for T_1 and T_2 , respectively.

From February to July a progressive decrease of both T_1 and T_2 was observed together with a clear monthly grouping for both time constants, indicating some kind of relation between relaxation behavior and the seasonality of milk composition throughout lactation (Supplemental Figure S1; <https://doi.org/10.3168/jds.2019-17617>). As shown by PDF corresponding to the beginning (February) and the end (June–July) of lactation, T_1 and T_2 significantly spread, and with the progress of the season, generated tails in the distributions. More restricted ranges of time constant values were observed during the period from March to May, when the most frequent values of T_1 and T_2 were in fact observed.

As a general consideration, it is worth noting that HT samples showed a reduced molecular mobility with respect to R (distributions shifted toward lower characteristic T_1 and T_2) in all collected data (i.e., T_1 and T_2 distributions reported in Figure 2a and 2b; characteristic T_1 and T_2 data reported in Tables 2 and 3; and PDF of characteristic T_1 and T_2 reported in Supplemental Figure S1).

To further characterize this observed shift, it was calculated in the form of delta (Δ , ms) of characteristic T_1 and T_2 of HT samples with respect to R ($\Delta T_{1\text{ HT-R}}$ and $\Delta T_{2\text{ HT-R}}$), on a daily basis. Negative $\Delta T_{1\text{ HT-R}}$ and $\Delta T_{2\text{ HT-R}}$ scores (i.e., R > HT) represented the majority of cases (HT15: $T_1 \approx 80\%$, $T_2 \approx 92\%$; HT20: $T_1 \approx 83\%$, $T_2 \approx 94\%$), with a minor amount of trend-against data (no shift corresponding to $\Delta T_{1(2)\text{ HT-R}} \sim 0$ or shift toward longer times corresponding to $\Delta T_{1(2)\text{ HT-R}} > 0$). First of all, these data suggest a direct correlation between changes in T_1 and T_2 and the duration of heat application (i.e., the amount of thermal energy transferred). Second, the larger percentage of negative $\Delta T_{2\text{ HT-R}}$ suggests a higher sensitivity of T_2 to heat-induced molecular mobility changes with respect to T_1 .

Statistical significance was only observed in $\Delta T_{2\text{ HT-R}}$, for both HT15 and HT20 (Table 4). The $\Delta T_{1\text{ HT-R}}$ HT20 was not significant but very close to the significance threshold of $P = 0.05$.

Negative values of $\Delta T_{1\text{ HT-R}}$ (only HT20) and $\Delta T_{2\text{ HT-R}}$ were further classified according to their magnitude (Table 5). The $\Delta T_{1\text{ HT-R}}$ were prevalently larger than 20 ms, whereas the most frequent $\Delta T_{2\text{ HT-R}}$ observed fell from 0.5 to 1.5 ms. Increasing the duration of heating (i.e., from 15 to 20 s) produced an increase of larger $\Delta T_{2\text{ HT-R}}$ and a reduction of smaller ones (Table 5).

Several questions may arise when considering further key factors, other than heat treatment, potentially affecting the observed relaxometric changes. We first considered the possibility of water evaporation taking place during heating, but specific trials confirmed that no water evaporation occurred (Supplemental Table S1; <https://doi.org/10.3168/jds.2019-17617>). In our view, 3 more factors could likely bias the results presented above: sampling variability, pH, and the presence of fat globules. In light of the abovementioned results, a series of additional specific tests were performed to figure out to which extent $\Delta T_{1(2)\text{ HT-R}}$ values could be affected by other factors. The role of such factors as potential bias of the observed relaxometric changes will be separately assessed and discussed in the following paragraphs.

Role of Sampling-Induced Variability

As reported in Tables 2 and 3, we did observe a certain variability in characteristic T_1 and T_2 data when milk samples of the same day were compared. Such variability seemed to be amplified when the relaxometric properties of milk samples collected in different days were compared. Likely, sampling from the main tank at the farm site can be a first source of variability to be added to the sampling variability from the bottle to the 50-mL batches in the laboratory and from the batches to the NMR tubes (Figure 1). Therefore, to highlight the effect of heat on relaxometric properties of milk, deltas were calculated only by comparing R and HT samples analyzed the same day. Reasonably, this variability, originated from the sampling process, could have somewhat contributed to the relaxometric differences between R and HT samples in our study. Therefore, to separately address the effect of heating from that induced by sampling procedures, T_2 was measured before and after the heat treatment while keeping the analyzed milk sample in the same NMR tube. With respect to data reported above, the observed negative $\Delta T_{2\text{ HT-R}}$ increased in percentage from 92% to 98% in HT15 and from 94% to 100% in HT20, as expected. Moreover, the percentage of $\Delta T_{2\text{ HT-R}}$ classes 1.5–2 ms and >2 ms were also found to increase for both treatments (Table 5; Supplemental Table S2; <https://doi.org/10.3168/jds.2019-17617>). The observed shifts in relaxometric constants are in this case uniquely ascribed to the effect of the heat treatment, being independent from sampling

Table 2. Daily longitudinal relaxation time (T_1) values (ms, mean and SD) when at least 3 and up to 6 milk batches (raw, HT15, and HT20 per batch) were analyzed daily (n.m. = not measured)¹

Day	Raw		HT15		HT20	
1	1,132.3	11.3	1,134.2	3.8	1,125.3	5.1
2	1,157.5	8.6	1,132.4	10.5	1,131.8	8.4
3	1,102.3	4.4	1,099.7	7.1	1,101.0	1.6
4	1,133.8	6.6	1,141.5	12.9	1,143.3	12.9
5	1,113.4	6.8	1,119.2	6.0	1,111.5	5.6
6	1,127.2	27.9	1,093.8	5.8	1,080.3	6.8
7	1,127.3	5.7	n.m.	n.m.	1,122.2	3.6
8	1,117.7	6.6	1,104.4	8.4	1,105.9	3.0
9	1,111.2	14.4	1,107.8	5.2	1,105.6	6.3
10	1,137.7	11.2	1,138.5	7.7	1,135.2	4.5
11	1,111.8	5.8	1,113.1	10.8	1,112.4	4.5
12	1,126.8	5.2	1,116.4	6.8	1,117.4	6.3
13	1,145.4	16.3	1,127.4	10.7	1,128.9	15.9
14	1,119.1	7.2	1,111.1	5.8	1,113.7	2.7
15	1,137.6	3.1	1,127.2	7.9	1,137.3	11.3
16	1,147.9	6.2	1,149.1	4.5	1,144.5	3.8
17	1,125.0	5.0	1,110.9	7.6	1,113.5	4.0
18	1,173.4	4.9	1,154.1	4.7	1,157.3	7.8
19	1,126.8	4.6	1,120.9	2.8	1,116.3	5.2
20	1,081.2	7.5	1,072.3	7.2	1,076.6	7.4
21	1,136.0	16.3	1,133.0	14.7	1,129.1	11.8
22	1,155.6	15.5	1,150.4	14.6	1,154.7	14.1
23	1,169.6	8.0	1,165.1	4.4	1,161.5	7.7
24	1,159.2	5.2	1,156.2	3.9	1,159.0	9.8
25	1,143.1	6.4	1,136.0	3.4	1,129.6	10.2
26	1,177.6	12.0	1,176.2	4.8	1,180.9	10.1
27	1,152.5	2.7	1,132.1	2.5	1,129.6	7.2
28	1,099.9	3.3	1,086.4	4.0	1,084.5	4.6
29	1,158.7	3.4	1,153.9	5.2	1,157.9	0.9
30	1,146.4	7.7	1,114.9	4.1	1,113.9	9.5
31	1,147.0	4.9	1,146.6	4.8	1,141.2	8.9
32	1,106.2	6.6	1,102.8	5.5	1,094.2	10.0
33	1,102.6	8.0	1,082.9	6.2	1,086.7	5.4
34	1,162.6	1.7	1,157.9	9.0	1,159.8	4.2
35	1,088.1	4.6	1,075.0	11.4	1,090.1	47.6
36	1,152.2	7.9	1,144.8	3.8	1,147.3	6.4
37	1,114.2	7.9	1,091.0	5.1	1,088.0	10.5
38	1,198.4	2.9	1,152.0	4.1	1,150.8	4.2
39	1,152.2	3.6	1,145.7	6.2	1,145.9	8.9
40	1,068.7	9.5	1,070.2	12.9	1,067.0	9.6
41	1,102.8	9.3	1,097.9	7.3	1,091.4	8.1
42	1,087.0	21.0	1,076.5	12.4	1,070.9	4.0
43	1,173.0	4.4	1,148.2	4.8	1,151.7	0.3
44	1,173.8	9.8	1,158.1	8.6	1,157.8	9.1
45	1,169.6	3.6	1,162.6	3.6	1,163.5	7.8
46	1,136.1	7.9	1,133.4	13.4	1,132.6	12.1
47	1,133.5	5.1	1,107.4	4.3	1,101.7	4.5
48	1,095.9	6.8	1,095.1	6.8	1,094.9	9.2
49	1,132.4	10.1	1,108.7	9.6	1,110.8	5.1
50	1,155.5	6.3	1,153.8	6.5	1,152.0	8.6
51	1,141.3	5.5	1,115.2	11.0	1,108.4	8.1
52	1,054.7	12.8	1,046.4	6.0	1,042.6	5.1
53	1,074.8	9.6	1,079.6	6.9	1,082.7	28.0
54	1,101.5	11.2	1,099.5	28.7	1,084.3	19.7
55	1,084.0	6.7	1,078.6	7.3	1,073.3	13.6
56	1,114.3	5.7	1,104.8	7.7	1,107.0	9.3
57	1,133.8	6.2	1,129.6	7.1	1,126.3	9.2
58	1,082.9	7.0	1,071.3	7.2	1,059.0	9.1
59	1,098.7	8.9	1,075.7	11.4	1,078.4	12.2
60	1,137.2	5.7	1,120.5	3.9	1,119.2	4.7
61	1,066.4	5.2	1,049.9	7.8	1,045.0	9.7
62	1,059.2	14.3	1,051.7	1.8	1,052.2	6.3
63	1,070.4	8.6	1,050.8	11.6	1,056.7	12.5
64	1,099.8	8.5	1,085.9	21.1	1,082.2	4.2
65	1,083.4	2.5	1,065.1	1.2	1,059.0	6.7

Continued

Table 2 (Continued). Daily longitudinal relaxation time (T_1) values (ms, mean and SD) when at least 3 and up to 6 milk batches (raw, HT15, and HT20 per batch) were analyzed daily (n.m. = not measured)¹

Day	Raw		HT15		HT20	
66	1,086.3	5.4	1,069.9	3.9	1,074.7	7.2
67	1,079.6	15.1	1,052.2	7.8	1,028.6	67.9
68	1,105.0	7.6	1,093.5	10.5	1,091.4	6.1
69	1,052.7	6.3	1,039.4	4.6	1,034.6	9.4
70	1,114.3	9.6	1,096.5	5.6	1,089.3	8.1
71	1,075.0	13.0	1,061.7	3.1	1,060.5	7.0
72	1,112.8	5.3	1,084.6	3.6	1,085.3	8.1
73	1,055.8	10.6	1,046.1	10.4	1,042.9	11.5
74	1,079.2	3.3	1,064.3	4.1	1,060.8	7.8
75	1,145.5	11.8	1,121.9	5.6	1,118.5	9.0
76	1,098.1	7.7	1,091.3	7.9	1,085.6	6.5
77	1,099.8	4.9	1,083.4	8.8	1,086.3	3.9
78	1,134.4	8.4	1,109.6	2.9	1,106.4	9.7
79	1,113.3	2.2	1,091.8	9.6	1,091.7	3.3
80	1,133.6	14.9	1,098.1	7.1	1,091.4	3.5
81	1,076.0	7.3	1,056.8	11.5	1,066.4	8.6
82	1,115.3	10.6	1,097.6	4.7	1,097.5	6.8
83	1,106.1	6.4	1,091.8	4.3	1,090.4	5.5
84	1,070.7	9.0	1,056.5	4.6	1,055.5	8.8
85	1,109.3	2.7	1,080.3	3.5	1,070.9	7.0
86	1,016.9	9.9	1,001.9	6.7	993.1	6.2
87	1,035.8	8.7	1,015.9	9.6	1,014.2	2.9
88	1,039.3	15.4	992.3	5.7	989.4	7.1
89	930.9	13.5	921.4	13.8	920.2	12.8
90	1,022.1	3.4	1,006.3	4.3	1,010.9	4.3
91	1,002.3	5.1	990.7	3.8	990.1	9.3
92	1,066.9	12.9	1,042.8	4.4	1,036.0	7.7
93	1,082.3	10.9	1,022.8	3.6	1,023.4	3.6
94	968.3	5.6	957.6	4.4	954.5	4.9
95	1,026.3	4.2	1,003.2	2.5	1,000.9	0.9
96	958.7	4.1	957.4	2.0	956.4	0.9
97	998.1	2.2	979.1	4.7	974.2	0.8
98	1,043.6	8.1	1,003.4	9.0	1,006.8	13.3
99	976.1	3.1	962.8	7.5	959.5	4.7
100	984.2	6.1	968.4	6.6	963.3	3.9
101	973.8	4.7	959.4	5.8	959.8	1.6
102	986.2	13.2	964.5	3.3	963.4	4.1
103	980.9	2.0	968.5	3.5	971.8	3.8

¹HT15 = heat treatment at 72°C for 15 s; HT20 = heat treatment at 72°C for 20 s.

procedures. Based on these results, we can conclude that sampling-induced variability can partially mask the effect of heat treatment on TD-NMR relaxation parameters. Nonetheless, a daily analysis of real samples is still able to discriminate raw from HT milk.

Role of pH

Milk pH is paramount for colloidal equilibrium and well-known alterations in milk component stability occur as a consequence of a pH drop (e.g., solubilization of micellar calcium phosphate, dissociation of casein micelles; de la Fuente, 1998; Walstra et al., 2005). Several factors, including temperature, can influence milk pH. Heating affects salt distribution among the milk phases, with the formation of additional colloidal phosphate from the dissociation of calcium and phosphate, accompanied by H^+ ion release and pH decrease

(Walstra et al., 2005; Fox, 2015). The equilibrium is readily restored on cooling after heating to a moderate temperature (<95°C; van Dijk and Hersevoort, 1992) and the nonreversibility (or slow reversibility) of the reaction is proportional to the severity of treatment (Pouliot et al., 1989). The kinetics of pH equilibrium restoring process should be taken into account in the case of severe heating conditions (e.g., 24 h at 4°C after a heating treatment at 90°C for 10 min in bovine milk, according to Chandrapala et al., 2010). However, according to Walstra et al. (1999), irreversible reactions scarcely occur in milk submitted to a treatment milder than HTST pasteurization (e.g., 72°C, 15 s).

It has been demonstrated that a variation of pH can result in changes in NMR transverse relaxation (T_2) in reconstituted skim milk and caseinate-whey mixtures (Mariette et al., 1993). In particular, the transverse relaxation rate R_2 (where $R_2 = 1/T_2$) decreases with

Table 3. Daily transverse relaxation time (T_2) values (ms, mean, and SD); at least 3 and up to 6 milk batches (raw, HT15, and HT20 per batch) were analyzed daily¹

Day	Raw milk		HT15		HT20		Day	Raw milk		HT15		HT20	
	Mean	SD	Mean	SD	Mean	SD		Mean	SD	Mean	SD	Mean	SD
Day 1	109.3	1.5	108.7	1.0	108.3	1.1	Day 52	96.7	0.3	96.3	0.8	97.2	3.1
Day 2	110.8	0.8	109.8	0.8	109.8	0.9	Day 53	99.8	1.7	101.1	4.9	98.7	2.0
Day 3	105.1	0.5	104.2	0.2	104.3	0.2	Day 54	103.3	0.1	102.2	0.2	102.2	0.2
Day 4	115.9	0.9	115.7	1.5	116.1	1.5	Day 55	104.5	0.8	103.7	0.3	103.6	0.3
Day 5	110.3	0.8	111.0	0.8	110.0	0.8	Day 56	105.6	0.8	104.8	0.8	105.4	1.7
Day 6	105.1	1.1	103.4	0.3	103.4	0.3	Day 57	96.6	0.1	95.5	0.2	95.3	0.2
Day 7	108.4	1.1	n.m.	n.m.	107.0	0.2	Day 58	100.4	0.1	99.1	0.1	99.0	0.1
Day 8	106.2	0.1	104.5	0.6	104.9	0.2	Day 59	105.7	0.2	105.0	0.4	105.0	0.3
Day 9	108.7	0.3	108.0	0.3	107.9	0.3	Day 60	96.9	0.3	95.7	0.4	95.8	0.4
Day 10	111.0	0.3	110.0	0.7	110.0	0.7	Day 61	101.1	0.2	100.1	0.2	100.0	0.2
Day 11	109.1	0.2	108.3	0.1	108.0	0.3	Day 62	99.2	0.1	98.7	0.8	98.9	1.0
Day 12	111.0	0.1	110.1	0.2	110.0	0.3	Day 63	100.1	0.5	99.8	1.9	98.5	0.2
Day 13	109.7	0.2	109.1	0.7	108.8	0.1	Day 64	103.1	0.3	102.1	0.1	101.8	0.2
Day 14	105.1	0.0	104.4	0.2	104.4	0.1	Day 65	100.4	0.1	99.7	0.3	99.5	0.2
Day 15	111.0	0.3	110.0	0.7	110.0	0.7	Day 66	99.3	0.7	98.2	1.0	97.6	1.1
Day 16	109.3	0.3	109.2	0.6	108.5	0.1	Day 67	100.2	0.2	99.3	0.1	99.3	0.1
Day 17	103.3	0.3	102.3	0.3	102.3	0.9	Day 68	93.7	0.2	92.9	0.3	92.7	0.4
Day 18	116.2	0.3	115.2	0.2	115.1	0.2	Day 69	100.5	0.2	99.6	0.2	99.6	0.2
Day 19	111.4	0.2	110.7	0.2	110.7	0.2	Day 70	95.5	0.3	94.6	0.2	94.7	0.3
Day 20	102.3	0.3	101.4	0.3	101.4	0.3	Day 71	98.7	0.6	97.4	0.3	98.1	1.5
Day 21	115.5	0.4	114.7	0.2	114.4	0.2	Day 72	94.9	0.2	94.3	0.8	93.8	0.3
Day 22	109.5	0.2	108.8	0.7	109.1	1.2	Day 73	99.2	0.1	98.4	0.2	98.0	0.3
Day 23	112.1	0.4	111.4	0.2	111.4	0.2	Day 74	106.2	0.4	105.2	0.2	105.2	0.3
Day 24	109.3	0.2	108.7	0.3	109.0	1.0	Day 75	103.3	0.4	102.3	0.4	101.9	0.2
Day 25	106.0	0.1	105.5	0.3	105.2	0.1	Day 76	105.0	0.2	104.1	0.8	103.7	0.1
Day 26	114.4	1.3	113.4	0.3	113.4	0.3	Day 77	107.1	1.6	105.4	0.4	105.3	0.4
Day 27	110.3	0.2	109.3	0.1	109.3	0.1	Day 78	105.9	0.3	105.1	0.5	104.9	0.4
Day 28	106.8	0.6	106.0	0.3	105.8	0.2	Day 79	103.9	0.5	103.1	0.7	102.8	0.5
Day 29	105.7	1.3	103.8	0.6	103.9	0.1	Day 80	93.9	0.3	93.0	0.5	93.0	0.4
Day 30	109.3	0.5	108.8	0.3	108.4	0.2	Day 81	104.4	0.3	103.4	0.1	103.4	0.2
Day 31	104.7	0.3	103.5	0.2	103.4	0.3	Day 82	102.5	0.2	101.2	0.2	101.3	0.2
Day 32	104.1	0.1	103.3	0.2	103.1	0.6	Day 83	99.7	0.1	99.0	0.6	98.2	0.3
Day 33	113.5	0.1	112.9	0.2	112.8	0.2	Day 84	104.0	0.1	102.9	0.1	102.8	0.2
Day 34	102.2	0.6	101.3	0.4	101.3	0.2	Day 85	87.9	0.3	87.0	0.5	87.0	0.6
Day 35	110.4	0.2	109.7	0.2	109.6	0.1	Day 86	92.0	0.2	90.4	0.2	90.4	0.3
Day 36	102.6	0.2	101.7	0.2	101.5	0.2	Day 87	86.4	0.3	84.9	0.6	84.9	0.7
Day 37	111.0	0.1	109.9	0.1	109.7	0.1	Day 88	85.4	0.2	84.6	0.3	84.5	0.4
Day 38	109.7	0.2	108.9	0.1	108.6	0.2	Day 89	90.9	0.7	90.0	0.9	90.0	0.4
Day 39	99.1	0.1	98.1	0.2	98.1	0.3	Day 90	87.0	0.4	86.5	0.5	86.0	0.9
Day 40	106.0	1.1	105.0	0.2	104.8	0.1	Day 91	94.3	0.3	93.1	0.3	92.5	0.5
Day 41	105.7	2.7	103.3	0.6	103.3	0.6	Day 92	92.1	0.4	90.6	0.8	90.7	0.5
Day 42	109.8	0.7	108.6	0.1	108.3	0.1	Day 93	81.0	0.2	80.3	0.5	79.9	0.2
Day 43	110.6	0.2	109.6	0.3	110.2	1.1	Day 94	90.0	0.2	89.4	0.2	89.1	0.1
Day 44	110.3	0.2	109.7	0.2	109.6	0.1	Day 95	89.8	0.7	88.7	0.2	88.7	0.3
Day 45	107.8	0.8	107.5	1.2	106.7	0.7	Day 96	91.1	1.1	89.1	0.6	89.4	0.5
Day 46	104.7	0.1	103.7	0.1	103.6	0.3	Day 97	79.0	0.7	77.5	0.6	77.3	0.3
Day 47	102.7	0.4	102.0	0.1	102.0	0.4	Day 98	82.5	0.7	80.4	0.6	80.7	1.5
Day 48	104.2	0.1	103.2	0.1	103.0	0.2	Day 99	79.0	0.4	77.5	0.3	76.9	1.0
Day 49	106.0	0.1	105.3	0.2	105.2	0.1	Day 100	83.2	0.6	81.5	0.2	81.2	0.4
Day 50	108.0	1.0	106.9	1.1	106.2	0.5	Day 101	82.0	0.5	80.2	0.4	80.5	0.4
Day 51	94.6	0.2	93.9	0.4	93.7	0.4							

¹HT15 = heat treatment at 72°C for 15 s; HT20 = heat treatment at 72°C for 20 s; n.m. = not measured.

decreasing pH values, reaching a minimum at pH = 5.3. Such effect has been ascribed to both a change in the hydration status of micelles, due to the alteration in composition and structure of casein particles upon acidification, and to the dissolution of micellar calcium and phosphorus (Mariette et al., 1993).

According to the abovementioned dependence of milk T_2 on pH, an increase in T_2 should be expected upon heat treatment, due to the inverse relation between pH and T_2 . However, changes of pH following heat treatment, in agreement with previous findings, were negligible or very subtle (Supplemental Table S3;

Table 4. Multiple comparisons (chi-square with associated Tukey's honestly significant difference test) among the binary outcomes of delta longitudinal relaxation time (T_1) and transverse relaxation time (T_2) values¹

Item	Differences	SE	P-value	Decision
$\Delta T_{1 \text{ HT15-R}}$	0.2396	4.603	$P = 0.90742$	NS
$\Delta T_{1 \text{ HT20-R}}$	1.9932	3.128	$P = 0.05574$	NS
$\Delta T_{2 \text{ HT15-R}}$	10.597	2.18	$P = 0.00972$	**
$\Delta T_{2 \text{ HT20-R}}$	12.3507	1.115	$P = 2.1034 \times 10^{-5}$	***

¹Listed parameters include cumulative difference values after arcsin transformation and SE, with a given significance level of $P < 0.05$ to assess the significant threshold of the multiple comparisons.

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

<https://doi.org/10.3168/jds.2019-17617>) and often fall within the experimental error of the pH meter (0.01 pH points).

We decided to accurately estimate the dependence of NMR relaxation on pH in whole ewe milk by means of an NMR relaxation-based titration (Supplemental File S1; <https://doi.org/10.3168/jds.2019-17617>). Results are in line with those previously reported in literature for skim milk and other dairy protein solutions (Mariette et al., 1993). According to these results, considering a heat-driven decrease of pH (i.e., $\text{pH}_{\text{HT}} < \text{pH}_{\text{R}}$), a corresponding T_2 increase would be opposite to its decrease reported in the present data. The lack of an observation of T_2 increase upon heating allowed the exclusion of any role of pH on the relaxometric constants shift. In any case, very subtle variations of milk pH could contribute to the observed sample variability of relaxation time constants by partially masking the heat-induced T_2 shift.

Role of Fat Globules

The presented relaxometric responses of whole milk to a heat treatment are similar to those previously obtained in fat free milk-like models and reconstituted skim milk of different origin (Lambelet et al., 1992; Mariette et al., 1993). To accomplish a more accurate description of the behavior of whole milk, the role played by fat in the relaxometric profiles upon heat treatment was specifically evaluated by dedicated experiments.

Table 5. Classification of delta ($\Delta T_{1 \text{ HT-R}}$ and $\Delta T_{2 \text{ HT-R}}$), expressed as percentage of the subset of negative delta¹

$\Delta T_{1 \text{ HT-R}}$	HT15	HT20	$\Delta T_{2 \text{ HT-R}}$	HT15	HT20
1–5 ms	10.7	10.1	0.1–0.5 ms	12.7	8.1
5–10 ms	17.4	17.9	0.5–1 ms	41.9	36.2
10–15 ms	18.4	15.5	1–1.5 ms	30.2	35.7
15–20 ms	16.0	15.7	1.5–2 ms	9.1	11.7
>20 ms	37.4	40.7	>2 ms	6.2	8.4

¹ T_1 = longitudinal relaxation time; T_2 = transverse relaxation time; HT-R = heat treated raw; HT15 = heat treatment at 72°C for 15 s; HT20 = heat treatment at 72°C for 20 s.

Role of Fat Globule Inhomogeneity. Official methods for characterizing milk physico-chemical properties require the homogenization of milk fat fraction (925.21, Association of Official Analytical Chemists, 1990) before further measurements. In fact, a certain degree of inhomogeneity can be observed in the fat phase (Marth, 1978), especially when milk is kept at refrigerated temperatures after milking. In this study, milk was intentionally not homogenized before NMR analysis (as suggested by official methods), to avoid any additional heat transfer beyond pasteurization. However, specific trials were conducted following the official method to verify if the observed variability could be ascribable to the inhomogeneity of fat. The relaxometric differences between raw and heat treated were still present in the homogenized samples (Supplemental Figure S2; <https://doi.org/10.3168/jds.2019-17617>), suggesting that they were not ascribable to inhomogeneities in the milk fat globule phase.

Role of Milk Skimming. Skim milk always exhibited higher T_2 values than whole milk (Supplemental Figure S3; <https://doi.org/10.3168/jds.2019-17617>). Such result can be preliminarily explained with the dilution effect due to skimming. Furthermore, NMR relaxation is likely influenced by the effect of fat globules that either slow down the overall molecular mobility in milk, making it a more viscous solution (Bakshi and Smith, 1984) than skim milk, and promote chemical exchange between water protons and their faster relaxing protons. Additionally, it could be reasonably argued that proton relaxation is enhanced by water diffusion through magnetic gradients generated by the presence of fat globules (Hills et al., 1990).

To more deeply understand how fat actually modulates the NMR relaxation of whole milk upon heating, $\Delta T_{2 \text{ HT-R}}$ of whole and skim milk were derived. In fact, if the presence of fat were a significant driving factor of the $\Delta T_{2 \text{ HT-R}}$ increase of whole milk, then the expected $\Delta T_{2 \text{ HT-R}}$ of skim milk would be smaller. According to experimental data, however, $\Delta T_{2 \text{ HT-R}}$ of skim milk were similar to those of whole milk (0.5–2 ms; Supplemental Table S4; <https://doi.org/10.3168/jds.2019-17617>), suggesting that relaxometric changes are most largely

influenced by molecular changes in the protein fraction or in other soluble molecular components rather than by fat.

We further investigated this behavior by comparing T_2 of milk which was heat treated and *then* skimmed (treated skim) to milk that was *first* skimmed and then heat treated (skim treated; Supplemental Table S5; <https://doi.org/10.3168/jds.2019-17617>). Treated skim samples had significantly higher T_2 than skim treated counterparts (*t*-test, $P < 0.001$). These data confirm previous evidence of strong interactions between fat globules and other components, due to milk heating (Corredig and Dalgleish, 1996), and corroborates the hypothesis that the heat treatment itself changes the relaxation properties of milk by involving not only polar (proteins, lactose, and so on) but also fat components. It is reasonable to assume that molecular interactions are established between β -LG and denaturated milk fat globule membrane (MFGM) proteins during heating. Moreover, fat could cause a partial “retention” of whey proteins and other species during the skimming process, resulting in a reduced availability of relaxation sinks (i.e., relaxation-promoting agents) in the corresponding treated skim sample (Corredig and Dalgleish, 1996; Ye et al., 2004).

Can TD-NMR Relaxometric Profiles Be Related to Heat-Induced Molecular Changes in Milk?

Previous studies investigated the modifications of milk components when a heat treatment is applied. Whey proteins exhibit reversible modifications at temperatures $<60^\circ\text{C}$ (Fox et al., 2015), with partial unfolding that can evolve to irreversible aggregation (via disulfide bonds formation between denatured β -LG) at more severe heating conditions (e.g., temperature $>70^\circ\text{C}$; deWit and Klarenbeek, 1984; Wijayanti et al., 2014; Fox et al., 2015). β -Lactoglobulins can also interact with κ - and α_{S2} -caseins, resulting in the “coating” of casein micelles by whey proteins. The aforementioned phenomena were described in bovine milk (Vasbinder and De Kruif, 2003), but since ruminant whey protein polymorphism consists of few genetic variants, without significant amino acid substitutions, it is reasonable to infer that a similar protein unfolding mechanism may also characterize whey proteins denaturation in sheep milk (Juarez and Ramos, 2003; Hernández-Ledesma et al., 2008). This is especially convincing if the lower thermostability of ovine β -LG is considered (El-Zahar et al., 2004; Balthazar et al., 2017). In the same way, proteins of MFGM are affected by heat (temperature $>70^\circ\text{C}$) and can interact with whey proteins, via disulfide interac-

tions, leading to the formation of aggregates (Corredig and Dalgleish, 1996; Ye et al., 2004; Huppertz and Kelly, 2006).

Based on our data, a change in ewe milk molecular dynamics (decrease in T_1 and T_2), following HTST treatment, is consistently observable. Previous NMR-relaxometry reports considered more severe heating conditions in milk model systems (from 20°C to 90°C for an extended time, 30 min; Lambelet et al., 1992) and associated an increase in the transverse relaxation rate R_2 ($R_2 = 1/T_2$) to a rigidity gain in whey protein backbone protons, due to protein denaturation. The time-temperature conditions of the heat treatment applied in this experimentation were likely not sufficient to allow the same degree of protein denaturation of that measured in previous trials. However, the consistent shift toward shorter times upon industrial-like heat treatment shown here indicates a coherent relaxometric change ascribable to dynamic perturbations in protein molecular arrangement. With proteins in a pre-denaturational stage, the observed faster relaxation showed by HT samples could be related to a variation in their hydration status that promotes proton relaxation (Mariette et al., 1993; Hinrichs et al., 2004). Other possible relaxation-promoting factors are the establishment of a higher degree of disorder (random coil structure, molten globule state; Bhattacharjee et al., 2005) and aggregation caused by disulfide-links and noncovalent interactions involving whey proteins (Hoffmann and van Mil, 1997; Halle, 2004). A contribution to the faster relaxation could be attributed to an interaction of MFGM with whey proteins, as previously discussed.

CONCLUSIONS

In this work we provided an extensive collection of relaxometric data on whole ewe milk related to different periods of lactation. We considered important the choice to investigate a “real” milk system to evaluate the potentiality of TD-NMR applied to complex systems that were scarcely considered in previous literature. The observed data demonstrate TD-NMR is able to detect heat-induced changes in whole ewe milk molecular dynamics, which are uniquely ascribable to the application of a heat treatment. In fact, the analyzed contribution of other factors beyond heat, such as pH and fat, to the observed modifications of NMR relaxation parameters was not significant. The presented results suggest the possibility of further exploiting TD-NMR as a technique for dairy industry applications and paving the way for novel applications of TD-NMR in quality control and assessment.

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