Chemical interactions among caseins during rennet coagulation of milk

J. C. Amaro-Hernández,¹ G. I. Olivas,¹ C. H. Acosta-Muñoz,¹ N. Gutiérrez-Méndez,² C. Rios-Velasco,¹
and D. R. Sepulveda^{1*}

¹Centro de Investigación en Alimentación y Desarrollo A.C., Unidad Cuauhtémoc, Av. Río Conchos S/N, Parque Industrial, Ciudad Cuauhtémoc, Chihuahua, México, C.P. 31570

²Facultad de Ciencias Químicas, Universidad Autónoma de Chihuahua, Circuito Universitario 8, Campus UACH II, Chihuahua, Chihuahua, México, C.P. 31125

ABSTRACT

Rennet milk curds were prepared under 4 different temperature and acidity conditions. The development of different types of inter-protein chemical bonds (disulfide, hydrophobic, electrostatic, hydrogen, and calcium bridges) was monitored for 60 min after curd cutting. Hydrophobic inter-protein interactions originally present in casein micelles in milk were substituted by electrostatic, hydrogen, and calcium bonds throughout the curd curing period. Disulfide bonds were not disturbed by the experimental conditions employed in the study, remaining at a constant level in all studied treatments. Acidification of curds increased the availability of soluble ionic calcium, increasing the relative proportion of calcium bridges at the expense of electrostatic-hydrogen bonds. Although pH defined the nature of the interactions established among proteins in curd, temperature modified the rate at which such bonds were formed.

Key words: milk curd, inter-casein chemical bonds, curd caseins interactions

INTRODUCTION

Bovine milk gels under the action of rennet, forming a complex protein-based tridimensional arrangement (Sinaga et al., 2017; Lamichhane et al., 2018; Mehta, 2018). Paracasein micelles in curd interact mainly by exposing their hydrophobic cores and through electrostatic and calcium-mediated ionic bonds (Panteli et al., 2015; Ono et al., 2017). After the onset of coagulation, some additional rearrangements of the structure occur (Lucey, 2014). External factors such as pH and temperature play an important role in determining the establishment of the various types of chemical bonds among caseins during this period (Corredig and Salvatore, 2016; Mehta, 2018). The continuous paracasein matrix

physically entraps fat and whey (Geng et al., 2011), creating a structure of high dimensionality supported by casein strands that get thicker through time (Fagan et al., 2017). Depending on the nature and abundance of the formed chemical bonds, the tridimensional protein structure develops a specific capacity to retain whey or to lose it (Mehta, 2018). The driving force behind whey loss is the formation of strong inter-casein interactions that promote micro-syneresis (Walstra et al., 2005), expelling whey as the protein structure becomes denser (Amaro-Hernández et al., 2020).

Although the general mechanism behind curd setting and curing has been abundantly discussed in the literature, the specifics regarding the role played by different types of inter-casein chemical bonds on the development of the proteinaceous curd structure during gel curing remain mostly unknown. An analytical technique reported by Van Camp et al. (1997) and further developed by Keim and Hinrichs (2004) has been suggested as a feasible method to quantify the relative proportion of hydrophobic, electrostatic, hydrogen, calcium, and disulfide bonds in milk gels and other dairy products (Gonçalves and Cardarelli, 2019), providing a means to advance the knowledge in this field.

Based on this, the objective of the present study was to determine the relative proportion of hydrophobic, electrostatic or hydrogen, calcium, and disulfide bonds in curd during a curing period of 60 min under different conditions of pH and temperature.

MATERIALS AND METHODS

The relative proportions of hydrophobic, electrostatic, hydrogen, calcium and disulfide bonds among proteins were measured on curd prepared from lactic acid bacteria-inoculated, and uninoculated milk, tempered at 30 and 40°C, resulting in 4 different treatments. Curd samples were taken every 15 min starting at cutting time, which was considered as time 0. Whey content and pH of each sample were determined as well throughout the curing period.

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*Corresponding author: dsepulveda@ciad.mx

Curd Manufacturing and Conditioning

Four pasteurized bovine whole milk aliquots (2 L each) were conditioned at 2 different temperatures (2 at 30°C, and 2 at 40°C). One sample of each temperature was inoculated with 0.8 U of lactic acid bacteria (**LAB**; RTS 743, Chr. Hansen). A resting period of about half an hour allowed inoculated samples to reach a pH value of 6.2 after a little lag time, ensuring strong LAB activity. Uninoculated samples remained at the original pH of milk (about 6.7). Once milk samples were LAB and temperature preconditioned, milk coagulation was performed by addition of 100 µL (50 international milk clotting units) of a solution of Chy-Max M 1000 (Chr. Hansen). Each sample was left undisturbed for 30 min until curd formation was complete. Afterward, the set curd was cut into cubes of about 1 cm and cured immersed in whey, undisturbed, for a period of 60 min. Curd samples were taken every 15 min (0, 15, 30, 45, and 60 min) for pH, whey content, and analytical characterization of inter-protein chemical bonds.

Whey Content and pH Determinations

The moisture content of curds was determined for each sample by the standard method 926.08 (AOAC International, 2006a). Soluble solids content of whey was measured employing standard method 990.20 (AOAC International, 2006b), to determine the whey content of curds based on their moisture and soluble solids content. The pH was measured potentiometrically (Orion Star A211, Thermo Scientific).

Inter-Protein Chemical Bonds

The method used to characterize chemical bonds is based on selective solubilization of curd protein in different buffer solutions. The assay was performed according to the methodology described by Gonçalves and Cardarelli (2019) with some modifications. Two grams of curd sample was placed in 85-mL polycarbonate tubes together with 20 mL of 1 of the 4 buffer solutions, A, B, C, or D (Table 1). Each buffer solution dissolves a given protein fraction depending on its particular bond-destabilizing properties. Curds in buffer were homogenized (Ultra-Turrax T25) at room temperature for 5 min at 13,500 rpm. Samples were then shaken for 20 min in an orbital agitation shaker (Incubator Shaker Series I26) and centrifuged at $15,000 \times g$ for 23 min at 20°C (Allegra 64R centrifuge/rotor F0685). Afterward, samples were filtered through 0.2-µm paper, and the total nitrogen content of each sample was determined using the Kjeldahl method (AOAC International, 2006c). The nitrogen content of each buffer (by itself)

Table 1. Chemical composition of buffers employed for selective protein solubilization

Buffer	Composition ¹	pH	Destabilized bonds ²
A	Tris 12.12 g/L SDS 1 g/L	7.2	EB, HB, Hy
B	Tris 12.12 g/L SDS 1 g/L	7.2	EB, HB, Hy, SS
C	DTT 5 g/L NaCl 9 g/L	7.2	EB, HB
D	NaH ₂ PO ₄ ·H ₂ O 7.8 g/L EDTA 14.6 g/L	8.0	EB, HB, CaB

¹Millipore water for buffer preparation; HCl/NaOH for pH adjustment; DTT = dithiothreitol.

²EB = electrostatic interactions; HB = hydrogen bridges; Hy = hydrophobic interactions; SS = disulfide bridges; CaB = calcium bridges.

was also determined and subtracted from the nitrogen content found for each sample, to block out the buffer's contribution.

Considering the different protein fractions solubilized by each buffer (Table 1), a set of equations was composed to determine the relative abundance of each type of bond from the assayed nitrogen contents:

$$P(SS) = \frac{Nbond,B}{Ns} - \frac{Nbond,A}{Ns}; \quad (1)$$

$$P(Hy) = \frac{Nbond,A}{Ns} - \frac{Nbond,C}{Ns}; \quad (2)$$

$$P(EB) + P(HB) + P(ub) = \frac{Nbond,C}{Ns}; \quad (3)$$

$$P(CaB) = \frac{Nbond,D}{Ns} - \frac{Nbond,C}{Ns}, \quad (4)$$

where $Nbond,A$, $Nbond,B$, $Nbond,C$, and $Nbond,D$ correspond to the nitrogen content measured in buffer solutions A, B, C, and D, respectively (g/g); Ns is the total nitrogen content of curd (g/g); and P is the proportion of protein (%) stabilized by a given specific interaction: electrostatic bonds (**EB**), hydrogen bridges (**HB**), hydrophobic interactions (**Hy**), disulfide bridges (**SS**), calcium bonds (**CaB**), and free protein (**ub**). Due to their similar nature, electrostatic and hydrogen bonds are quantified as a single group in the present study (Equation [3]).

Statistical Analysis

The experiment was independently repeated 3 times. For each response variable, ANOVA were conducted, considering a $2 \times 2 \times 5$ factorial experimental design,

with 2 inoculation levels (inoculated and uninoculated), 2 temperature levels (30 and 40°C), and 5 curing time levels (0, 15, 30, 45, and 60 min). Statistical analyses were performed using SAS software (SAS System for Windows 9.0; SAS Institute Inc.), considering a significance level of $P \leq 0.05$.

RESULTS AND DISCUSSION

The 4 studied treatment conditions produced different chemical and physical characteristics on the studied curd during the 60-min curing period. The pH of the studied samples developed as expected (Figure 1), showing a temperature-dependent decrease in inoculated samples caused by the metabolic activity of LAB. Uninoculated samples, by contrast, remained at a constant pH level around 6.7 for the duration of the experiment. Concomitantly, LAB-inoculated samples showed a steeper whey loss trend than uninoculated samples (Figure 2), being more pronounced at higher curing temperature (0.373 g of whey/min). Uninoculated samples cured at 40°C showed a whey loss trend similar to that of inoculated samples cured at 30°C (0.150 g of whey/min), with uninoculated samples cured at 30°C showing the least whey loss among the studied samples (0.076 g of whey/min). This behavior is consistent with previous reports indicating that curd syneresis increases as temperature and acidity increase (Van Vliet et al., 2004; Janhøj and Qvist, 2010).

Regarding the development of the different types of inter-protein chemical bonds during the curing period, it is relevant to mention that disulfide bridges remained at a constant average level of 1.58% for all treatments during the 60-min curing period, showing no statisti-

Figure 1. pH development of inoculated and uninoculated curds incubated at different temperatures. Empty markers correspond to uninoculated samples incubated at 30°C (□) and 40°C (Δ). Inoculated samples are represented by filled markers: 30°C (■) and 40°C (▲). Error bars represent SE.

Figure 2. Whey loss in inoculated and uninoculated curds incubated at different temperatures. Empty markers correspond to uninoculated samples incubated at 30°C (□) and 40°C (Δ). Inoculated samples are represented by filled markers: 30°C (■) and 40°C (▲). Error bars represent SE.

cally significant difference among them. (The whole model was not statistically significant, showing no effect of temperature, acidification, time, or any of their interactions.) This observation suggests that disulfide bonds are not disturbed by the experimental conditions employed in this study, which is consistent with previous reports by Gonçalves and Cardarelli (2019), who found 2% disulfide bridges in Mozzarella cheese during the stretching stage. Similarly, Keim et al. (2006) reported 0.5% disulfide bridges in rennet-induced milk gels. In both cases, the researchers mention that standard processing conditions are not able to change the proportion of disulfide bonds, probably due to their high intrinsic energy (Table 2). The small proportion of disulfide bridges found in curd, in any case, is due to the limited number of thiols in caseins, which is reduced to 2 in α_{S2} -casein and 2 in κ -casein (Broyard and Gaucheron, 2015; Fox et al., 2017). Reports mentioning higher proportions of disulfide bridges in curd typically include binding of cysteine-rich whey proteins (Table 3).

Statistical analyses for the rest of the studied types of bonds (hydrophobic, electrostatic+hydrogen, and calcium bridges) indicated statistically significant effects of temperature and LAB inoculation (acidification) in all of them, establishing the unequivocal dependence of bond formation with acidity and temperature. The effect of temperature in the case of formation of electrostatic+hydrogen bonds appeared to be interdependent on time, as a statistically significant interaction between temperature and time was found. In a similar way, hydrophobic bond formation showed a statistically significant interaction between temperature and acidification. Finally, calcium bond formation showed a

statistically significant effect of all studied factors and their interactions, although 80% of the model's sum of squares was taken by the acidification factor, indicating the great relevance of pH on calcium bond formation.

Inter-Protein Chemical Bonds in Uninoculated Curd at 30°C

The relative proportion of hydrophobic, electrostatic+hydrogen, and calcium bonds in uninoculated curd during the 60 min of curing at 30°C is shown in Figure 3. From a theoretical point of view, this treatment is the mildest studied condition. The absence of pH development ensures constant solubility, concentration, ionization, and general chemical reactivity characteristics, for all present chemical species throughout the studied period. The low temperature used in this treatment limits the rate of chemical reactions compared with the higher temperature used in other treatments.

Under these conditions, Figure 3 shows how some regions of paracasein micelles, structurally stabilized through hydrophobic interactions in their original configuration, are disassembled throughout the curing time, reducing the proportion of hydrophobic interactions from 58% to 33%.

Simultaneously, some new electrostatic+hydrogen and calcium bonds start to emerge in the newly formed proteinaceous structure, increasing from 21 to 38% and from 20 to 29%, respectively. Previous reports have established that casein micelle destabilization caused by external factors gives rise to the formation of new links (Choi et al., 2007; Dalglish, 2014). Casein amino acid residues such as Asp, Glu, and the HPO_4 ion of the phosphoserine complex, are capable of establish-

ing both electrostatic and calcium bonds, whereas Lys forms only electrostatic bonds (Gaucheron, 2011; Koutina et al., 2015; Mehta, 2018). Amino acid residues of Asn, Thr, Ser, Gln, Met, Tyr, and Trp are capable of establishing hydrogen bridges, whereas His and Arg are capable of forming both electrostatic and hydrogen interactions (Keim, 2005).

Electrostatic and hydrogen bonds developed more quickly than calcium bonds under the studied conditions, probably because soluble ionic calcium is not readily available at this pH level (pH 6.7). The moderate development of new electrostatic+hydrogen and calcium inter-protein bonds resulted in a moderate increase in protein-protein interactions, translating into a limited whey loss (4.8% loss), as shown in Figure 2, for this treatment.

The hydrophobically stabilized region in the original micelle structure was only possible thanks to the colloidal stability conferred upon micelles by the glycomacropeptide in κ -caseins (Fox and Guinee, 2013). Once absent, the old structure must turn into a new more thermodynamically stable one, which, to be formed, requires the dissolution of old bonds and the establishment of new ones (Huppertz, 2013). This bond reorganization process takes place following a hierarchical order, $\text{CaB} > \text{EB} > \text{HB} > \text{Hy}$, in which stronger bonds tend to replace weaker bonds as the disaggregation of the structure exposes new reactive sites (Table 2).

Some punctual values similar to those obtained in the present study have been reported in the literature (Table 3). Lefebvre-Cases et al. (1998) reported HB values of 30% in rennet skim milk gels of constant pH at 30°C. Keim et al. (2006) reported values of 23% for EB+HB in rennet gels under similar conditions. Benyahia-Krid et al. (2016) reported values of 42 to 46% for

Table 2. Properties of various interactions stabilizing inter-casein structures

Type of interaction	Energy (kJ/mol)	Functional groups involved	Reference
Peptide bond	300–400	NH–CO	Messens et al. (1997) Keim (2005)
Disulfide bridges (SS)	200	Cystine S–S	Messens et al. (1997) Keim (2005)
Calcium bridges (CaB)	90–97	H donor Tyr, Thr, Ser residues Residue HPO_4^- Asp and Glu Deprotonated COO^-	Keim (2005) Gaucheron (2011) Permyakov and Kretsinger (2011)
Electrostatic interactions (EB)	42–84	Carboxyl COO^- (Asp, Glu) Amino NH_4^+ (His, Arg, Lys)	Messens et al. (1997) Keim (2005)
Hydrogen bridges (HB)	8–40	H atom of OH, NH (donor), or CO (acceptor) groups	Messens et al. (1997) Keim (2005)
Hydrophobic interactions (Hy)	4–12	Residues with aliphatic (Leu, Ile, Val) or aromatic (Phe, Tyr) side chains	Messens et al. (1997) Keim (2005)

Table 3. Reported values of chemical bonds present in different milk gel types

Gel type ¹	Type of bond ²	Bonds destabilized ¹ (%)	Dissociating agent ³	Reference
Whey protein concentrate gels at pH 6.0	HB+CaB Hy SS	63 8 24	(1) Demineralized water (2) 1 + Tris + Gly + Na ₂ EDTA (3) 2 + Urea + SDS (4) 3 + DTT	Van Camp et al. (1997)
Skim milk gels: acidified/rennet	HB Hy CaB	30/30 ~12/60 27/76	Urea (1 M) SDS (1 g/L) EDTA (2 mM)	Lefebvre-Cases et al. (1998)
Whey protein isolate gels, different pressure application: build up/holding/release time	Non-covalent SS	98/18/8 ~0/63/71	Tris + SDS Tris + SDS + DTT	Fertsch et al. (2003)
Whey milk gels: nontreated/induced by high pressure (600 MPa)	EB+HB Hy SS	95/12 5/5 <5/80	NaCl + NaH ₂ PO ₄ Tris + SDS Tris + SDS + DTT	Keim and Hinrichs (2004)
Rennet-induced gel/heat-induced gel/pressure-induced gel (reconstituted milk)	EB+HB Hy SS	23/27/51 ~0/~0/4 0.5/21/9	NaCl + NaH ₂ PO ₄ Tris + SDS Tris + SDS + DTT	Keim et al. (2006)
Cheddar cheese: full fat/half fat (heating to 180°C for 25 min)	CaB HB Hy SS	74/67/40 76/82 57/58 42/41	EDTA + Na ₃ citrate Urea SDS Mercaptoethanol	Kim et al. (2011)
Rennet milk gels: raw milk/homogenized-pasteurized milk/ultra-high pressure homogenization milk (3.6% fat content)	CaB HB Hy	41/42 66/73/49 99/100/83	EDTA Urea SDS	Zamora et al. (2012)
Rennet milk gels: rennet/chicken pepsin (skim milk powder)	CaB HB Hy	16/20/20 46/42 67/65	EDTA Urea SDS	Benyahia-Krid et al. (2016)
Mozzarella cheese: during/after stretching	EB+HB Hy SS CaB	32/28 ~0/35 24/10 2/<2 ~0/42	EDTA NaCl + NaH ₂ PO ₄ Tris + SDS Tris + SDS + DTT EDTA + Na ₃ citrate	Gonçalves and Cardarelli (2019)

¹Values in the “Bonds destabilized” column separated by a slash refer to the corresponding parameters separated by slashes in the “Gel type” column.²EB = electrostatic interactions; HB = hydrogen bridges; Hy = hydrophobic interactions; SS = disulfide bridges; CaB = calcium bridges.³DTT = dithiothreitol.

Figure 3. Inter-protein chemical bonds in uninoculated curd samples at 30°C are represented by different markers: hydrophobic interactions (●), electrostatic bonds and hydrogen bridges (■), and calcium bonds (▲). Error bars represent SE.

HB, and 28 to 32% for CaB in skim milk powder gels prepared with different types of coagulant agents. Finally, Zamora et al. (2012) reported CaB values around 20% in curd made of milk subjected to high pressure.

Inter-Protein Chemical Bonds in Uninoculated Curd at 40°C

Figure 4 shows the relative proportion of hydrophobic, electrostatic+hydrogen, and calcium bonds found in uninoculated curd cured at 40°C for 60 min. This treatment, similar to the one previously described, did not induce curd pH changes during the studied period, ensuring constant chemical reactivity properties of all the present chemical species. The higher temperature employed in this treatment, however, increased the kinetic energy of the system, roughly doubling the reaction rate of all chemical changes, as established by the Arrhenius law. In agreement with this, the relative proportion of all studied chemical bonds at 40°C after 30 min coincided almost exactly with the values found at 30°C after 60 min, showing an average difference of only 0.64%.

Curd tempering at 40°C induced faster changes from the beginning of the curing process. By the first measurement (time 0), a large percentage of the hydrophobic interactions had already been severed, quickly reaching an asymptotic value around 34%, which is nearly the same level of hydrophobic bond content found after 60 min at 30°C (Figure 3). The reduction of hydrophobic bonds was obviously caused by formation of new electrostatic+hydrogen bonds, which accounted for a much larger proportion (44%) at time 0 in curd at 40°C. Calcium bridges, on the other hand, again showed a low initial proportion (22%), similar to what was observed at 30°C, confirming the unavailability of soluble ionic

calcium at high pH (Lamichhane et al., 2018; Horne, 2020). Calcium bridges increased up to 33% after 60 min at the expense of electrostatic+hydrogen bonds, which gradually yielded reaction sites to calcium. Whey loss increased under these conditions, roughly doubling whey loss at 30°C after 60 min.

Inter-Protein Chemical Bonds in Inoculated Curd at 30°C

The relative proportions of chemical bonds in LAB-inoculated curd samples cured at 30°C are shown in Figure 5. The low temperature employed in this treatment induced low chemical reaction rates, similar to what was observed in uninoculated curds at 30°C. Nevertheless, the pH decrease resulting from moderate LAB metabolic activity in this treatment modified the reactivity of some chemical species and increased the availability of soluble ionic calcium, as previously reported (Bijl et al., 2013; McMahon and Oommen, 2013). Soluble ionic calcium may interact with non-protonated Glu and Asp, whose abundance depends on pH, becoming scarcer as pH lowers. Proteins are also capable of reacting with calcium through the amino acids Ser, Thr, and Tyr via intermediation of a phosphate group forming calcium-reactive phosphoserine, phosphothreonine, and phosphotyrosine (Keim, 2005; Horne, 2016). The relative abundance of these amino acids in individual caseins determines their potential for interaction through calcium bridges or other electrostatic interactions (Cooke and McSweeney, 2017; Sinaga et al., 2017; Kern et al., 2018).

The effect of the increase in soluble ionic calcium is immediately apparent, as the relative proportion of calcium bridges between proteins in this treatment is notably larger than in the rest of the studied treat-

Figure 4. Inter-protein chemical bonds in uninoculated curd samples at 40°C are represented by different markers: hydrophobic interactions (●), electrostatic bonds and hydrogen bridges (■), and calcium bonds (▲). Error bars represent SE.

ments (68%). The observed values are similar to those reported by Keim et al. (2006) under similar conditions. The gradual decrease in hydrophobic bonds observed in uninoculated curds at 30°C in this case occurs abruptly even before the first measurement is conducted (time 0), probably forced by the sturdy structure developed by strong calcium bridges, reducing it below the levels previously observed (10%). Although similar to what was observed in uninoculated curds at 40°C, in this case hydrophobic interactions are mainly extinguished by calcium bridges and, to a lesser extent, by generic electrostatic+hydrogen bonds. Curd structure remains mainly calcium bridge-stabilized until the end of the 60-min curing period, although a slight recovery of hydrophobic interactions occurs at the expense of electrostatic+hydrogen bonds (32% reduction) and, to a lesser extent, of calcium bridges (13% reduction). This increase of hydrophobic bonds during the curing period may be related to the decrease of electrostatic repulsion experienced among caseins as pH lowers, reducing their net charge (Gonçalves and Cardarelli, 2019). Curd whey content remained relatively high throughout the curing period, similar to what was observed in uninoculated curd cured at 40°C. The limited pH reduction marginally modified the water-holding capacity of curds (Lin et al., 2018; Mehta, 2018).

Inter-Protein Chemical Bonds in Inoculated Curd at 40°C

Finally, assessment of the relative proportions of chemical bonds found in LAB-inoculated curd cooked at 40°C for 60 min is shown in Figure 6. In this treatment, hydrophobic bonds maintained relative abundance levels around 22% throughout the studied 60-min period. This value is roughly double what was observed at the beginning of the curing period at 30°C, as higher temperatures increase the dominance of hydrophobic interactions, reducing the contribution of electrostatic interactions (Giroux et al., 2014; Lamichhane et al., 2018). Generic electrostatic+hydrogen bonds developed more quickly at 40°C than at 30°C, as predicted by the Arrhenius law, limiting the proportion of calcium bonds formed in the initial stages of the curing period. Nevertheless, calcium bonds quickly recovered their preponderance. As the curing period proceeded, calcium bonds gradually substituted electrostatic bonds, accumulating over time. The sum of calcium and electrostatic+hydrogen bonds remained practically constant throughout curing time. The relative proportion of calcium bonds found in inoculated curd cured at 40°C for 60 min seems to coincide with the value which calcium bonds in inoculated curd cured at 30°C approached after 60 min (52%).

Figure 5. Inter-protein chemical bonds in inoculated curd samples at 30°C are represented by different markers: hydrophobic interactions (●), electrostatic bonds and hydrogen bridges (■), and calcium bonds (▲). Error bars represent SE.

The water-binding capacity of curd was drastically reduced at 40°C, as pH in this treatment is much lower than at 30°C (Giroux et al., 2014; Lamichhane et al., 2018). Therefore, although the proportion of hydrophobic bonds to combined calcium, electrostatic, and hydrogen bonds at 60 min is roughly 20:80 in both 30 and 40°C treatments, their whey content differs greatly (Figure 2).

CONCLUSIONS

Although visually all studied conditions coagulated milk in a similar manner, different temperature and acidity conditions strongly affected the relative proportions of inter-protein chemical bonds found in curd. Disulfide bridges were not affected by the experimental conditions, remaining at a constant level of 1.58% in all treatments. Electrostatic interactions (including

Figure 6. Inter-protein chemical bonds in inoculated curd samples at 40°C are represented by different markers: hydrophobic interactions (●), electrostatic bonds and hydrogen bridges (■), and calcium bonds (▲). Error bars represent SE.

calcium and hydrogen bridges) quickly substituted the hydrophobic interactions that stabilized the original structure of casein micelles in milk before the action of chymosin. Treatment temperature affected reaction rate, as established by the Arrhenius law. pH-dependent soluble ionic calcium availability defined the balance between electrostatic+hydrogen bonds and calcium bridges. In general, stronger bonds tended to replace weaker bonds as the disaggregation of the structure exposed new reactive sites, following the hierarchical order $\text{CaB} > \text{EB} > \text{HB} > \text{Hy}$. The different combinations of pH and temperature during curd curing promoted the establishment of different proteinaceous structures. Although pH defined the nature of the interactions among proteins in curd, temperature modified the rate at which such bonds were formed. Determining how the differently stabilized curd structures translate into the microstructure and physical properties of finished cheese and other intermediate products remains a relevant topic.

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ORCIDS

- G. I. Olivas <https://orcid.org/0000-0001-8242-8614>
 N. Gutiérrez-Méndez <https://orcid.org/0000-0002-0791-3211>
 D. R. Sepulveda <https://orcid.org/0000-0002-0400-8873>