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Effect of calcium-sequestering salts and heat treatment on the rheological and textural properties of acid gels from blends of skimmed buffalo and bovine milk

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A B S T R A C T

The influence of adding 5 mM trisodium citrate (TSC) or disodium hydrogen phosphate (DSHP) and heat treatment (85 °C or 95 °C for 5 min) on the acid gelation properties of blends of skim buffalo and bovine milk (0:100, 25:75, 50:50, 75:25, 100:0) was investigated. Significant increases in gelation pH, final G′ values, firmness, and water-holding capacity of gels were observed with increasing proportion of buffalo skim milk and with higher heating temperature. Differences in gel firmness were linked to gel microstructure, where milk blends containing higher proportion of buffalo skim milk formed gels with denser protein network clusters. The addition of TSC or DSHP reduced the gelation pH, final G′ values and gel firmness, but increased gel water-holding capacity. These results provide a better understanding of acid gelation of buffalo and bovine milk blends which will subsequently promote the potential of using milk mixtures in modulating the gel texture.

1. Introduction

Consumption of milk and milk products has always been a significant part of human diet for centuries. Bovine milk remains at the forefront of the widely consumed milk species although utilisation of milk from non-bovine origin, particularly buffalo milk, is gaining popularity due to its health properties that are arguably superior to those of bovine milk (Salzano et al., 2021). In Asian countries, particularly India and Pakistan, where both buffalo and bovine milk are produced, sales of liquid milk or dairy products (paneer, khoa, stirred yoghurt, chhana) from blends of bovine and buffalo milk are common practise (Ahmad, 2013). These dairy products are normally subjected to a variety of processes including heat and/or addition of acid to render the product safe and to obtain certain organoleptic properties. However, these processes modify milk properties, resulting either desirable or undesirable sensory and nutritional characteristics based on the extent of processing conditions and milk composition (Hutkins, 2006).

The impact of heat treatment on bovine milk and how this affects the resulting acid gel properties had been studied extensively (Anema, Lee, Lowe, & Klostermeyer, 2004; Laursen, Rovers, Ipsen, & Ahrné, 2022; Lucey, Tamehana, Singh, & Munro, 1998) and was recently reviewed by Lucey, Wilbanks, and Horne. (2022). Milk components, particularly calcium and proteins, and their interactions during processing are crucial factors affecting the final properties of acid gels (Lucey et al., 1998). Modulating gel properties through the addition of calcium sequestering salts (CSS) such as citrate and phosphate to milk had also been explored (Johnston & Murphy, 1992; Ozcan-Yilsay, Lee, Horne, & Lucey, 2007; Udabage, McKinnon, & Agustin, 2001). The protein and mineral composition differ between buffalo and bovine milk (Ahmad, 2013), therefore similar processing conditions would have different impacts on the resulting dairy products. For instance, the higher protein content of buffalo milk compared with bovine milk contributed to a higher storage modulus and a characteristic densely clustered protein network for their acid-induced gels (Roy, Ye, Moughan, & Singh, 2020). A denser protein network and harder gels were also observed by Laursen et al. (2022) with gels from buffalo milk heated at 90 °C prior citric acid acidification at 85 °C, compared with those from cow milk. These observations suggest

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that compositional variations in milk from different species warrant specific processing parameters to obtain a certain desired product quality.

There has been a paucity of information on how the characteristics of acid-induced gels from buffalo milk and mixtures with bovine milk are affected by heat treatment and the addition of CSS. Significant heat-induced physicochemical and structural changes of buffalo skim milk and mixtures with bovine skim milk have been observed at heat treatment ≥85 °C (Mejares, Huppertz, & Chandrapala, 2023b) and these heat-induced changes were markedly modified with the addition of CSS before heat treatment (Mejares, Chandrapala, & Huppertz, 2023a). Thus, in the present study, the impact of heat treatment (85 °C and 95 °C for 5 min) and addition of 5 mM trisodium citrate (TSC) or disodium hydrogen phosphate (DSHP) prior heat treatment on the rheological and textural properties of acid gels from buffalo:bovine milk blends (0:100, 25:75, 50:50, 75:25, and 100:0) were investigated. Identifying the extent of rheological changes in acid gels afforded by CSS addition and heat treatment will enable understanding of curd formation in buffalo milk and mixtures with bovine milk. Furthermore, relating these rheological changes to the texture profile of the resulting gels will subsequently promote the potential use of heat treated buffalo:bovine milk mixtures with and without the addition of CSS in modulating the gel texture.

2. Materials and methods

2.1. Materials

Raw bovine and buffalo milk were provided by Saputo Dairy Australia (Laverton North, VIC, Australia) and Floridia Cheese (Thomastown, VIC, Australia) respectively. Sodium azide was immediately added at a concentration of 0.02% (w/v) to minimise microbial growth. Analytical grade glucono-δ-lactone (GDL), TSC dihydrate, DSHP dodecahydrate, Nile Red, and Fast Green FCF were all obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of Nile Red (1 mg mL^{-1} in dimethyl sulfoxide) and Fast Green FCF (1 mg mL^{-1} in Milli-Q water) dyes were prepared and a working solution of 1:100 (v/v) in Milli-Q water was used for staining the gels.

2.2. Skim milk preparation and heat treatment

Raw milk was skimmed by centrifugation at 3500 × g for 20 min at 20 °C in a Sorvall LYNX 6000 centrifuge with F9-6 × 1000 LEX fixed angle rotor (ThermoFisher, Osterode, Germany). Skimmed buffalo and bovine milk were subsequently blended at ratios of 0:100, 25:75, 50:50, 75:25 and 100:0. Each of the blends was subsequently divided into 3 portions: (1) without the addition of CSS, (2) with added 5 mM TSC, and (3) with 5 mM added DSHP. The solutions were then mixed for 15 min before subjecting them to heat treatment at 85 °C or 95 °C for 5 min using the protocol previously described by Mejares et al. (2023a).

2.3. Milk composition

Protein content of raw skim milk was quantified following the Kjeldahl method [Method 991.20 (AOAC, 2002)] while total calcium was determined by complexometric method of Davies and White (1962) using disodium salt of ethylenediaminetetraacetic acid.

2.4. Characterisation of acid gelation properties of milk

The rheological properties were determined in a stress-controlled Discovery Hybrid rheometer (DHR-2 model, TA Instruments, USA) equipped with a plate–plate geometry as described by Luo, Liu, and Pang (2019), with slight modifications. Milk samples were equilibrated at 30 °C, after which 2.5% GDL was added. After GDL addition and mixing, a portion (1.2 mL) of skim milk sample was placed on the rheometer plate and the remaining aliquot of 18.8 mL was transferred to a plastic container and incubated in a water bath (30 °C) to monitor pH changes in parallel to the gelation experiment. pH was recorded at 1-min intervals with a calibrated pH meter (Mettler Toledo, Columbus, OH, USA). For monitoring of acid gelation, the 40 mm plate geometry attached to the rheometer was lowered onto the sample to give a gap of 800 μm between the parallel plates. A solvent trap and cover arrangement was placed over the sample to prevent evaporation. Changes in the storage modulus (G′) were monitored during acidification at 30 °C for 150 min, followed by cooling from 30 °C to 5 °C at a rate of 2 °C min^{-1}, and subsequent holding at 5 °C for 15 min. Shear strain of 0.5 % and 1 Hz frequency were applied throughout the rheological analysis. Data processing was performed using TRIOS software. Gelation time and gelation pH were defined as the first time point and pH, respectively, at which G′ ≥1 Pa (Meletharayil, Patel, Metzger, & Huppertz, 2016).

2.5. Preparation of acid milk gels

The acid gelation of milk was induced by addition of 2.5% GDL, according to the procedure of Chandrapala, Bui, Kentish, and Ashokkumar (2014). Appropriate amount of GDL was added to milk equilibrated at 30 °C and the mixture was stirred for 1 min to incubation at 30 °C for 150 min. Acidified samples were transferred to a chiller (5 ± 1 °C) and kept overnight before measurement.

2.6. Characterisation of acid milk gels

2.6.1. Texture analysis

Texture analysis measurements of acid milk gels were carried out through penetration test using the TA.XTplus texture analyser (Stable Micro Systems, Godalming, UK) fitted with a 1-kg load cell and a 20 mm diameter stainless steel cylinder probe (P/20) attachment. The probe was set to operate at a speed of 1 mm s^{-1} and allowed to penetrate the gel to a distance of 4 mm. Exponent software ver. 6.1.11 (Stable Micro Systems) was used to collect measurement data on gel firmness. Three independent measurements were performed at 5 °C.

2.6.2. Water-holding capacity

The water-holding capacity (WHC) of acid gels were determined, in triplicate, using a centrifugation technique according to Luo et al. (2019) with slight modifications. Milk gels were formed in 2 mL centrifuge tubes using steps described in section 2.5. The samples were then centrifuged at 1000 × g for 10 min at 5 °C (Sigma 1-14K, Osterode, Germany). The supernatant was carefully decanted and weighed. WHC was expressed as the weight of the pellet expressed as a percentage of the sample weight before centrifugation.

2.6.3. Microstructure

Gel microstructure was studied using an inverted confocal laser scanning microscope (Nikon Eclipse Ti A1-R, Tokyo, Japan) with a 100× oil immersion objective without digital magnification. Nile Red (600 μL) and Fast Green FCF (600 μL) dyes were mixed with skim milk (10 mL) pre-warmed to 30 °C and then vortexed before the required amount of GDL was added. The solution was again vortexed for 1 min and a few drops of this mixture was placed onto the microscope slide, covered with a glass cover slip, and sealed with nail polish to prevent evaporation. The slide was placed in a petri dish and incubated at 30 °C for 150 min then stored at 5 °C overnight before microstructural analysis. Wavelengths of 532 nm and 635 nm were used to excite Nile Red and Fast Green respectively. Multiple fields were viewed, and typical micrographs were presented.

2.7. Data and statistical analysis

The experiment was carried out in two separate milk batches. Statistical analysis of data for effects of CSS addition and pasteurisation on properties of milk gels was performed by two-way Analysis of Variance (ANOVA) using SPSS statistical software version 28.0 (IBM, New York, NY, USA). Mean differences were analysed using Tukey's probability pairwise comparison test at $P < 0.05$.

3. Results

3.1. Milk composition

Composition of bovine milk, buffalo milk and blends thereof in terms of protein content and calcium content is shown in Table 1. Protein content of bovine milk was 3.2% and that of buffalo milk 4.2%. Blends of bovine and buffalo milk shows intermediate values, proportional to the fraction of bovine and buffalo milk used in the blends. Likewise, calcium content also increased with increasing proportion of buffalo milk in the blend, from 32 mM in bovine milk to 41 mM in buffalo milk (Table 1). The increase in Ca content relative to the increase in protein content of ~9 mmol Ca per 10 g of casein is in line expected casein mineralisation and suggests that the increase in Ca is due to micellar Ca rather than soluble Ca.

Table 1
Protein and calcium composition of buffalo:bovine skim milk blend.[a]

Buffalo:bovine milk ratio	Protein (%)	Total calcium (mM)
0:100	3.20 ± 0.19[a]	31.7 ± 0.9[a]
25:75	3.50 ± 0.15[ab]	34.6 ± 1.6[ab]
50:50	3.73 ± 0.08[bc]	36.9 ± 2.5[ab]
75:25	3.99 ± 0.07[cd]	37.5 ± 2.5[ab]
100:0	4.22 ± 0.12[d]	40.8 ± 3.6[b]

[a] Values are means ± standard deviation; different superscript letters in a column indicate significant difference ($P < 0.05$).

3.2. pH changes during gelation

The decline in pH was rapid at the first 20 min and a slow change thereafter (Supplementary material Fig. S1) in accordance with other studies (Lucey et al., 1998; Roy et al., 2020). Irrespective of the heat treatment or the type of salt added, bovine skim milk showed the highest pH decline as acidification progressed while the decline was less with increasing proportion of buffalo skim milk (Table 2). Notably, pH changes during acidification were similar for 25:75 and 50:50 buffalo:bovine milk blends as well as buffalo skim milk and 75:25 buffalo:bovine milk blend when heated at either 85 °C or 95 °C (Supplementary material Fig. S1A). Similar trends were observed for CSS-added milk irrespective of heating temperature (Supplementary material Fig. S1B,C), except in DSHP-added milk heated at 85 °C where bovine skim milk and 25:75 buffalo:bovine milk blend showed similar pH changes during acidification (Supplementary material Fig. S1C).

3.3. Rheological properties of acid gels

3.3.1. Gelation time and gelation pH

In unheated milk, gelation time, defined as the time at which $G' > 1$, significantly decreased with increasing proportion of buffalo skim milk in the sample (Table 3). Heat treatment significantly reduced the gelation time, but comparable gelation times were measured in milk blends and buffalo skim milk irrespective of heating temperature (Table 3). In unheated milk, DSHP addition significantly increased the gelation time of bovine milk and buffalo milk but reduced the gelation time of blends thereof (Table 3). However, TSC addition caused a significantly higher gelation time than DSHP and the control (no CSS added), particularly in buffalo skim milk and milk blends. The interaction between CSS addition and heat treatment was also significant ($P < 0.05$). Heat treatment significantly reduced the gelation time of milk with added CSS where TSC-added samples had significantly higher gelation time ($P < 0.05$) than DSHP and control milk samples at heating temperature of 85 °C (Table 3). When heat treatment temperature was increased from 85 to 95 °C, comparable gelation times were noted in TSC and DSHP-added milk blends, and values were still significantly higher than the

Table 2
pH after 0, 50, 100 or 150 min of incubation at 30 °C after addition of 2.5% glucono-delta-lactone of unheated or heated (5 min at 85 or 95 °C) mixtures of buffalo and bovine skim milk without (control) or with 5 mM added trisodium citrate (TSC) or disodium hydrogen phosphate (DSHP).[a]

Milk ratio	Acidification time (min)	Control milk			Milk with 5 mM TSC			Milk with 5 mM DSHP		
		Unheated	85 °C	95 °C	Unheated	85 °C	95 °C	Unheated	85 °C	95 °C
0:100	0	6.76	6.76	6.75	6.88	6.88	6.96	6.80	6.84	6.93
	50	4.87	4.94	4.46	4.92	4.99	4.61	4.92	5.03	4.66
	100	4.36	4.47	3.94	4.46	4.56	4.15	4.47	4.58	4.15
	150	4.13	4.23	3.76	4.26	4.33	3.95	4.24	4.32	3.93
25:75	0	6.78	6.76	6.76	6.88	6.90	6.88	6.86	6.84	6.83
	50	5.00	5.04	5.02	5.04	5.10	5.11	5.09	4.97	5.15
	100	4.58	4.61	4.63	4.65	4.71	4.72	4.69	4.54	4.81
	150	4.33	4.36	4.37	4.43	4.48	4.47	4.42	4.29	4.57
50:50	0	6.78	6.74	6.74	6.90	6.87	6.88	6.79	6.84	6.83
	50	5.11	5.05	5.01	5.18	5.13	5.12	5.15	5.08	5.09
	100	4.75	4.66	4.61	4.83	4.75	4.73	4.84	4.75	4.74
	150	4.48	4.37	4.33	4.59	4.50	4.48	4.60	4.48	4.49
75:25	0	6.79	6.74	6.74	6.80	6.87	6.86	6.73	6.82	6.83
	50	5.16	5.17	5.18	5.17	5.28	5.22	5.19	5.22	5.24
	100	4.88	4.90	4.94	4.86	4.99	4.96	4.91	4.94	4.96
	150	4.65	4.70	4.76	4.62	4.81	4.77	4.73	4.75	4.78
100:0	0	6.79	6.75	6.74	6.85	6.72	6.71	6.84	6.80	6.75
	50	5.26	5.24	5.23	5.29	5.25	5.24	5.25	5.31	5.25
	100	5.01	5.00	4.99	5.03	5.01	5.00	4.98	5.07	5.01
	150	4.85	4.85	4.84	4.86	4.86	4.84	4.82	4.92	4.87

[a] Milk ratio refers to buffalo:bovine skim milk.

Table 3
Gelation time and gelation pH during acidification with 2.5% glucono-delta-lactone of unheated or heated (5 min at 85 or 95 °C) mixtures of buffalo and bovine skim milk without added calcium sequestering salt (CSS) or with 5 mM added trisodium citrate (TSC) or disodium hydrogen phosphate (DSHP).[a]

Milk ratio	CSS added	Gelation time (min)			Gelation pH		
		Unheated	85 °C	95 °C	Unheated	85 °C	95 °C
0:100	None	64.6 ± 3.1^{Aa}	37.2 ± 1.2^{Ba}	26.7 ± 2.6^{Ca}	4.68 ± 0.04^{Aa}	5.10 ± 0.02^{Ba}	4.96 ± 0.06^{Ca}
	TSC	68.3 ± 1.5^{Aab}	52.0 ± 1.4^{Bb}	26.3 ± 1.5^{Ca}	4.72 ± 0.02^{Aa}	4.97 ± 0.02^{Bb}	5.09 ± 0.04^{Cb}
	DSHP	72.1 ± 1.9^{Ab}	42.6 ± 3.1^{Bc}	27.3 ± 1.1^{Ca}	4.69 ± 0.02^{Aa}	5.11 ± 0.04^{Ba}	5.07 ± 0.02^{Bb}
25:75	None	63.3 ± 4.1^{Aa}	34.3 ± 1.6^{Ba}	30.0 ± 2.0^{Ca}	4.79 ± 0.04^{Aa}	5.22 ± 0.02^{Ba}	5.22 ± 0.02^{Ba}
	TSC	65.6 ± 2.6^{Aa}	47.2 ± 4.6^{Bb}	39.5 ± 3.6^{Cb}	4.93 ± 0.02^{Ab}	5.13 ± 0.05^{Bb}	5.25 ± 0.06^{Cab}
	DSHP	45.3 ± 4.0^{Ab}	37.3 ± 1.2^{Ba}	36.8 ± 2.1^{Bb}	5.13 ± 0.04^{Ac}	5.11 ± 0.01^{Ab}	5.29 ± 0.04^{Bb}
50:50	None	46.2 ± 1.9^{Aa}	33.5 ± 1.1^{Ba}	31.2 ± 1.1^{Ba}	5.14 ± 0.02^{Aa}	5.23 ± 0.02^{Ba}	5.21 ± 0.02^{Ba}
	TSC	57.3 ± 1.2^{Ab}	43.3 ± 6.4^{Bb}	38.7 ± 1.8^{Cb}	5.12 ± 0.02^{Aa}	5.19 ± 0.06^{Ba}	5.23 ± 0.02^{Ba}
	DSHP	43.6 ± 2.3^{Aa}	34.5 ± 1.3^{Ba}	35.4 ± 2.4^{Bb}	5.21 ± 0.02^{Ab}	5.24 ± 0.01^{Aa}	5.25 ± 0.03^{Aa}
75:25	None	43.4 ± 3.2^{Aa}	33.3 ± 1.2^{Ba}	30.8 ± 1.3^{Ba}	5.22 ± 0.03^{Aa}	5.32 ± 0.02^{Ba}	5.36 ± 0.02^{Bab}
	TSC	54.2 ± 1.5^{Ab}	41.4 ± 1.1^{Bb}	36.2 ± 1.8^{Cb}	5.13 ± 0.01^{Ab}	5.36 ± 0.01^{Bab}	5.34 ± 0.02^{Ba}
	DSHP	40.5 ± 1.6^{Aa}	34.5 ± 1.0^{Ba}	34.2 ± 1.1^{Bab}	5.28 ± 0.02^{Ac}	5.37 ± 0.01^{Bb}	5.39 ± 0.01^{Bb}
100:0	None	26.7 ± 2.9^{Aa}	33.1 ± 1.0^{Ba}	28.7 ± 3.1^{Aa}	5.47 ± 0.04^{Aa}	5.38 ± 0.01^{Ba}	5.42 ± 0.03^{Ba}
	TSC	52.3 ± 1.5^{Ab}	38.2 ± 2.3^{Bb}	26.3 ± 5.6^{Ca}	5.28 ± 0.02^{Ab}	5.38 ± 0.04^{Ba}	5.45 ± 0.04^{Ca}
	DSHP	39.4 ± 2.3^{Ac}	34.3 ± 2.3^{Bab}	27.2 ± 2.8^{Ca}	5.35 ± 0.02^{Ac}	5.44 ± 0.02^{Bb}	5.46 ± 0.04^{Ba}

[a] Milk ratio refers to buffalo:bovine skim milk. Values are means ± standard deviation; within the same gelation characteristic, means of samples in a row with different uppercase superscript letters differ significantly (P < 0.05); within the same type of milk, different lowercase superscript letters in the same column indicate significant difference (P < 0.05).

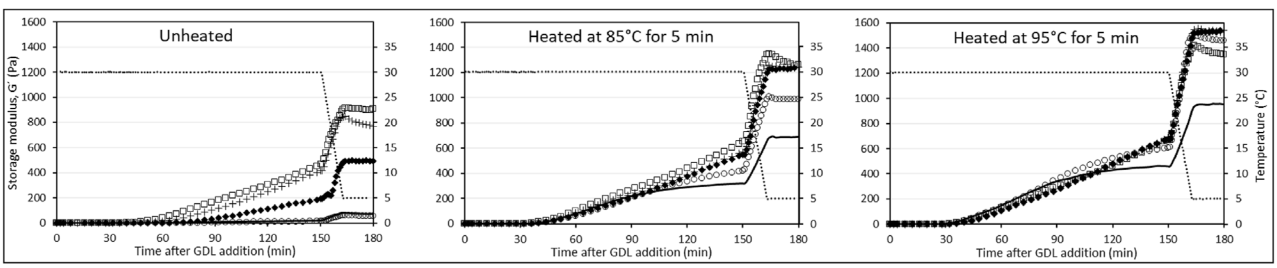

Fig. 1. Changes in the storage modulus (G′) of acid-induced gels from unheated and heated (5 min at 85 or 95 °C) buffalo:bovine skim milk at a ratio equivalent to 0:100 (—), 25:75 (○), 50:50 (◆), 75:25 (+), and 100:0 (□). The dotted line shows temperature as a function of time.

control. Notably, the addition of CSS did not induce significant change in the gelation time of buffalo milk or bovine milk heated at 95 °C (Table 3).

A significant increase ($P < 0.05$) in gelation pH with increasing proportion of buffalo skim milk in the blends was observed, irrespective of heat treatment and CSS addition (Table 3). CSS addition did not induce significant change in gelation pH of unheated bovine skim milk but significantly decreased the gelation pH of unheated buffalo skim milk. The gelation pH of all unheated milk blends were significantly increased ($P < 0.05$) with DSHP addition, whereas TSC addition effected a significant increase and decrease in gelation pH of 25:75 and 75:25 buffalo:bovine milk blend, respectively (Table 3). Heat treatment significantly increased the gelation pH of milk without added

Table 4
Effect of calcium sequestering salt (CSS) addition on the rheological and textural properties of acid gels made from unheated and heated mixture of buffalo and bovine skim milk.[a]

Milk ratio	CSS added	G′ at 150 min (Pa)			Firmness (N)			Water-holding capacity (%)		
		Unheated	85 °C	95 °C	Unheated	85 °C	95 °C	Unheated	85 °C	95 °C
0:100	None	23.0 ± 5.1^{Aa}	322.5 ± 4.8^{Ba}	454.7 ± 8.6^{Ca}	0.41 ± 0.09^{Aa}	0.48 ± 0.06^{Aa}	0.89 ± 0.06^{Ba}	73.3 ± 2.5^{Aa}	90.0 ± 2.0^{Ba}	91.7 ± 2.6^{Ba}
	TSC	16.7 ± 0.4^{Ab}	60.9 ± 1.4^{Bb}	269.1 ± 4.4^{Cb}	0.32 ± 0.02^{Aa}	0.31 ± 0.04^{Ab}	0.53 ± 0.03^{Bb}	79.4 ± 2.7^{Ab}	94.9 ± 1.4^{Bb}	95.7 ± 0.7^{Bb}
	DSHP	15.2 ± 1.2^{Ab}	31.8 ± 3.7^{Bc}	300.1 ± 5.1^{Cc}	0.33 ± 0.05^{Aa}	0.37 ± 0.06^{Ab}	0.60 ± 0.09^{Bb}	80.2 ± 2.9^{Ab}	92.3 ± 1.4^{Bc}	94.4 ± 1.5^{Cc}
25:75	None	14.4 ± 0.5^{Aa}	421.9 ± 7.5^{Ba}	607.7 ± 5.9^{Ca}	0.48 ± 0.10^{Aa}	0.90 ± 0.09^{Ba}	1.35 ± 0.05^{Ca}	75.5 ± 2.2^{Aa}	94.6 ± 1.9^{Ba}	95.6 ± 2.0^{Ba}
	TSC	12.9 ± 1.6^{Aa}	261.8 ± 6.6^{Bb}	292.6 ± 1.2^{Cb}	0.33 ± 0.03^{Ab}	0.58 ± 0.02^{Bb}	0.59 ± 0.04^{Bb}	86.0 ± 1.0^{Ab}	95.3 ± 1.0^{Ba}	96.3 ± 0.9^{Ba}
	DSHP	11.5 ± 0.5^{Aa}	129.3 ± 3.4^{Bc}	346.7 ± 4.1^{Cc}	0.34 ± 0.06^{Ab}	0.44 ± 0.10^{Ac}	0.60 ± 0.07^{Bb}	92.6 ± 1.0^{Ac}	96.0 ± 1.0^{Ba}	96.7 ± 0.7^{Ba}
50:50	None	192.5 ± 2.0^{Aa}	549.9 ± 3.4^{Ba}	680.3 ± 5.3^{Ca}	0.74 ± 0.13^{Aa}	1.10 ± 0.13^{Ba}	1.46 ± 0.07^{Ca}	95.2 ± 2.3^{Aa}	96.6 ± 1.6^{Aba}	97.6 ± 0.9^{Ba}
	TSC	54.8 ± 1.2^{Ab}	384.3 ± 3.4^{Bb}	545.0 ± 2.2^{Cb}	0.42 ± 0.03^{Ab}	0.75 ± 0.08^{Bb}	1.07 ± 0.06^{Cb}	97.2 ± 1.1^{Ab}	98.8 ± 0.8^{Ab}	98.7 ± 0.6^{Aa}
	DSHP	87.1 ± 7.2^{Ac}	433.3 ± 4.2^{Bc}	564.5 ± 3.6^{Cc}	0.45 ± 0.05^{Ab}	0.80 ± 0.10^{Bb}	1.33 ± 0.11^{Cc}	96.7 ± 1.0^{Aab}	97.0 ± 1.2^{Aab}	98.1 ± 0.5^{Aa}
75:25	None	413.3 ± 6.1^{Aa}	568.8 ± 4.0^{Ba}	689.5 ± 5.3^{Ca}	0.92 ± 0.10^{Aa}	1.22 ± 0.05^{Ba}	1.50 ± 0.16^{Ca}	97.0 ± 0.9^{Aa}	97.7 ± 1.2^{Aa}	98.4 ± 1.0^{Aa}
	TSC	142.3 ± 2.6^{Ab}	405.4 ± 2.8^{Bb}	542.4 ± 4.2^{Cb}	0.44 ± 0.07^{Ab}	0.77 ± 0.11^{Bb}	1.04 ± 0.03^{Cb}	98.4 ± 1.0^{Aa}	98.6 ± 0.8^{Aa}	98.5 ± 0.7^{Aa}
	DSHP	346.1 ± 3.3^{Ac}	545.5 ± 5.7^{Bc}	625.9 ± 4.1^{Cc}	0.71 ± 0.13^{Ac}	0.91 ± 0.10^{Bc}	1.37 ± 0.07^{Cc}	96.6 ± 1.1^{Aa}	97.6 ± 1.8^{Aa}	97.9 ± 1.4^{Aa}
100:0	None	467.8 ± 6.6^{Aa}	646.7 ± 7.0^{Ba}	675.8 ± 5.2^{Ca}	1.16 ± 0.10^{Aa}	1.25 ± 0.07^{Aba}	1.33 ± 0.13^{Ba}	97.1 ± 1.2^{Aa}	98.3 ± 1.0^{Aa}	98.5 ± 1.0^{Aa}
	TSC	325.0 ± 2.3^{Ab}	467.9 ± 2.7^{Bb}	1020.2 ± 9.8^{Cb}	0.59 ± 0.07^{Ab}	0.78 ± 0.12^{Bb}	2.32 ± 0.11^{Cb}	98.0 ± 0.8^{Aa}	98.6 ± 0.7^{Aa}	99.0 ± 0.5^{Aa}
	DSHP	504.7 ± 4.4^{Ac}	542.0 ± 5.6^{Bc}	532.4 ± 1.8^{Cc}	0.85 ± 0.11^{Ac}	0.92 ± 0.10^{Ac}	1.23 ± 0.07^{Ba}	96.2 ± 1.5^{Aa}	96.9 ± 1.9^{Aa}	97.7 ± 1.2^{Aa}

[a] Milk ratio refers to buffalo:bovine skim milk. Heat treatment duration was 5 min. Values are means ± standard deviation; within the same gelation characteristic, means of samples in a row with different uppercase superscript letters differ significantly ($P < 0.05$); within the same type of milk, different lowercase superscript letters in the same column indicate significant difference ($P < 0.05$).

CSS, but comparable values were measured between samples heated at 85 °C and 95 °C (Table 3). Two-way ANOVA revealed that there was a significant interaction between the effects of CSS addition and heat treatment ($P < 0.05$) on the gelation pH of milk. When heated at 85 °C, TSC addition significantly decreased ($P < 0.05$) the gelation pH of milk with higher proportion of bovine skim milk whereas DSHP addition significantly increased the gelation pH of milk containing higher proportion of buffalo skim milk (Table 3). In contrast, the addition of either TSC or DSHP to milk with higher proportion of bovine skim induced a significant increase ($P < 0.05$) in gelation pH when milk was heated at 95 °C.

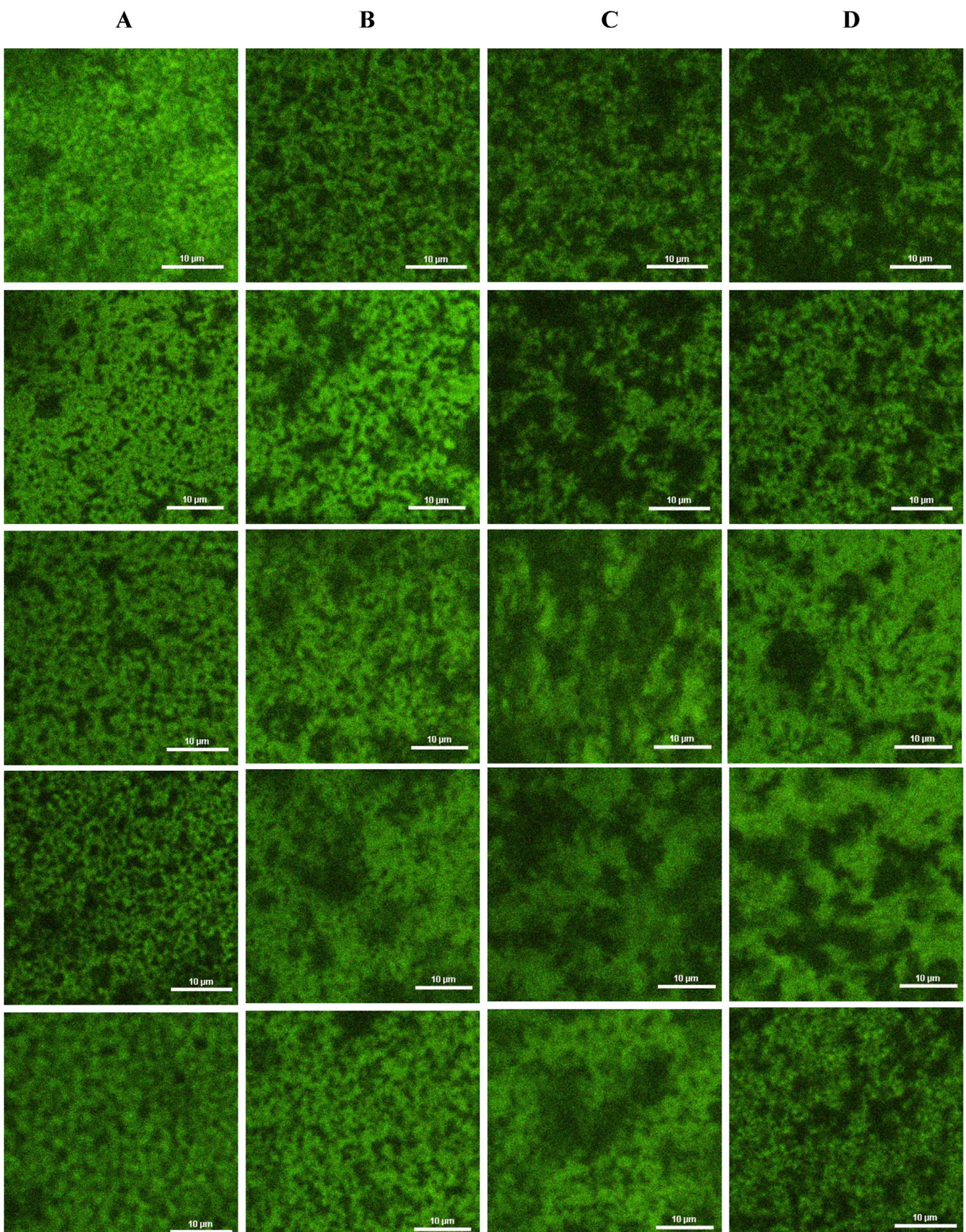

Fig. 2. Representative images of acid gels made from skim milk without heat treatment (column A) and heated at 95 °C for 5 min (columns B–D). Milk was without added calcium sequestering salts (column B), with 5 mM trisodium citrate added (column C), or with 5 mM disodium hydrogen phosphate added (column D) before heat treatment. Images from top to bottom correspond to buffalo:bovine milk ratio as 0:100, 25:75, 50:50, 75:25, and 100:0. Scale bar represents 10 μm.

3.3.2. Storage modulus (G′)

Irrespective of heat treatment and CSS addition, all milk samples showed similar trend in G′ values i.e., an initial slow increase followed by a steep increase as acidification progressed (Fig. 1; Supplementary material Fig. S1). After 150 min gelation and the temperature was reduced from 30 °C to 5 °C, all gels exhibited a sharp increase in G′ then formed a plateau or slightly decreased during annealing when the temperature was maintained at 5 °C for 15 min. Final G′ values significantly increased with increasing proportion of buffalo skim milk (Table 4). Although these G′ values significantly increased when milk was heated at 85 °C before acidification, milk blends particularly 50:50 and 75:25 buffalo:bovine milk blends, heated at 95 °C exhibited higher final G′ values than the buffalo or bovine milk (Table 4).

Similarly, the G′ values of gels from samples with added CSS increased with heat treatment and with increasing proportion of buffalo skim milk in the sample (Table 4). Two-way ANOVA revealed that there was a significant interaction between the effects of CSS addition and heat treatment ($P < 0.05$) on the G′ values of milk. Although a significant reduction in G′ values of acid gels was effected by CSS addition, samples with added DSHP generally had significantly higher ($P < 0.05$) G′ values than those with added TSC. A notable exception to this was TSC-added buffalo skim milk heated at 95 °C where G′ value was significantly higher than DSHP and control samples (Table 4). At the same temperature, DSHP-added 50:50 and 75:25 buffalo:bovine milk blends had significantly higher G′ values than DSHP-added pure milk.

3.4. Firmness of acid gels

Acid gels from unheated bovine skim milk were significantly less firm ($P < 0.05$) than the gels from buffalo skim milk and gel firmness increased proportionally to the ratio of buffalo skim milk in the milk mixture (Table 4). While gel firmness significantly ($P < 0.05$) increased with heating of the milk prior to gelation, gels from buffalo:bovine milk blends heated at 95 °C had a significantly ($P < 0.05$) higher firmness than the gels made from pure buffalo or bovine milk. The addition of CSS significantly reduced gel firmness (Table 4). The interaction between CSS addition and heat treatment was also significant ($P < 0.05$). While TSC and DSHP effected comparable firmness in gels prepared from bovine skim milk and 25:75 buffalo:bovine milk blend, a significantly higher firmness ($P < 0.05$) was afforded by DSHP addition than TSC did in 50:50 and 75:25 buffalo:bovine milk blends as well as buffalo skim milk systems (Table 4). However, gels from TSC-added buffalo skim milk heated at 95 °C had significantly ($P < 0.05$) higher firmness than the control and DSHP-added counterpart (Table 4).

3.5. Water-holding capacity of acid gels

Acid gels prepared from unheated bovine skim milk and 25:75 buffalo:bovine milk blends had significantly lower ($P < 0.05$) WHC than the other samples (Table 3). Addition of TSC to bovine skim milk resulted a significant increase in WHC irrespective of heat treatment. In contrast, CSS addition and heat treatment did not significantly ($P > 0.05$) change the WHC of acid gels from 75:25 buffalo:bovine milk blend and buffalo skim milk. The WHC of 50:50 buffalo:bovine milk blend was significantly increased ($P < 0.05$) with the addition of CSS and heat treatment at 85 °C (Table 3) where the effect of TSC addition was comparable with that of DSHP.

3.6. Microstructure of acid gels

Irrespective of CSS addition, thin strands over dense aggregated protein clusters can be observed in the CLSM micrographs of gels prepared from unheated buffalo and bovine skim milk. These gels appeared to have a homogeneous structure with small pores (Fig. 2A; Supplementary material Fig. S3). In contrast, micrographs of gels from unheated milk blends showed the presence of thin strands attached on the sides of the aggregated protein clusters which visually conveyed the formation of larger globular and uneven pores than gels from individual milk (Fig. 2A; Supplementary material Fig. S3). Compared with gels from unheated milk, gels from heated milk can be seen to show denser clusters of aggregated protein particles with larger pore sizes that were covered with thin strands (Fig. 2B). The effect of heat treatment was clearly observed in micrographs of milk blends where the gel network appeared heterogeneous with larger interconnected clusters. Network of gels from heated CSS-added milk also appeared heterogeneous and dense but seemingly less interconnected since larger pores with irregularly spread small pores were more clearly visible (Fig. 2C, D).

4. Discussion

4.1. Properties of acid gels from unheated milk as affected by milk ratio

The reduction in milk pH during acidification proceeded at various rates depending on the buffalo:bovine milk ratio. Increasing the concentration of bovine skim milk effected an increased rate in pH reduction (Table 2) that is likely due to the lower buffering capacity of bovine milk than buffalo milk (Ahmad et al., 2008). Reduction in milk pH results in an increased dissolution of colloidal calcium phosphate (CCP) leading to release of micellar calcium and phosphorus from the micelles along with a progressive decrease in the net charge of the caseins (Li & Zhao, 2019; Lucey, 2016; Sinaga, Bansal, & Bhandari, 2017). This also reduced the electrostatic repulsion and steric stabilisation of casein micelles leading to aggregation and subsequently allowing gelation to proceed (Lucey, Wilbanks, & Horne, 2022). In this study, it was observed that gelation pH significantly differed with milk ratio. Buffalo skim milk had the highest gelation pH, which decreased as the concentration of bovine skim milk increased (Table 3). This is probably related to the higher casein concentration, particularly κ-casein, in buffalo milk (Ahmad, 2013) as well as potentially the higher calcium concentration in buffalo milk than bovine milk (Table 1). About 80% of the total calcium is in the micellar phase of buffalo milk (Ahmad et al., 2008) therefore as milk pH is reduced, the casein micelles of buffalo skim milk would mostly be affected leading to partial rearrangement of the inner structure of casein micelles, reduced electrostatic repulsion and steric stabilisation of κ-casein hence earlier onset of aggregation and gelation. Roy et al. (2020) also reported that unheated buffalo milk started to gel at a significantly higher pH (5.7) than bovine milk (pH 4.7). Mixing buffalo skim milk with bovine skim milk significantly ($P < 0.05$) reduced the gelation pH compared with pure buffalo milk, probably due to the contribution of bovine skim milk caseins that started to gel at pH 4.7 (Table 3) which is close to the isoelectric point of casein (pH 4.6).

The pH-induced casein aggregation results in changes in rheological and textural properties of acid gels. After an initial gel formation, casein micelles continuously fuse due to rearrangement of both inter- and intramolecular forces (Roefs, De Groot-Mostert, & Van Vliet, 1990) as pH continuously drops. This is evident from the continuous increase in G′ with time, irrespective of milk ratio (Fig. 1). When gels are cooled, casein particles swell leading to an increase in the contact area between particles (Lucey, 2016). Similarly, there is a drastic increase in the number and/or strength of the elastically effective bonds between the protein molecules of different casein particles (Roefs et al., 1990) which results in an increase in the G′ value. This would explain the observed steep increase in G′ during cooling of acid gels irrespective of milk ratio (Fig. 1). The final G′ values were observed to be higher with increasing proportion of buffalo skim milk (Table 4). This is parallel to an increase in gel firmness (Table 4), which is likely due to the differences in total solids, particularly casein concentration between buffalo and bovine milk. Buffalo milk has a significantly higher protein content than bovine milk (Table 1), which means that buffalo skim milk has higher casein concentration hence higher structural interconnectivity and contact points at a given unit area. In addition, bovine milk caseins are more hydrated than buffalo milk caseins (Ahmad et al., 2008) and this higher amount of water associated with caseins was reported to be associated with softer gel texture (Laursen et al., 2022). Additionally, a denser network with larger pores can be observed in the confocal images of gels from unheated buffalo skim milk and mixtures (Fig. 2A). This interconnected protein network could possibly have entrapped the serum phase which in turn will act as fillers in a protein gel matrix hence reduced separation of fluid during gel centrifugation resulting in higher WHC.

4.2. Influence of calcium sequestering salts on the properties of acid gels from unheated milk

Irrespective of the type of salt added, the rate of GDL-induced acidification during gel formation was observed to be increasing as the concentration of bovine skim milk increased (Supplementary material Fig. S1,C) similar to the control sample (no CSS addition, Supplementary material Fig. S1A). Likewise, the addition of CSS had a significant effect on the gelation time and pH of milk, but this was dependent on the buffalo:bovine milk ratio and the type of CSS added. TSC and DSHP have similar calcium-binding ability (Mejares et al., 2023a) and their preheat addition in skim milk resulted to a significant increase in gelation time. This indicates that CSS-induced casein dissociation as a result of calcium chelation inhibits protein aggregation. Citrate chelates casein-bound calcium and the loss of calcium phosphate crosslinks in the casein micelles exposes charged phosphoserine residues and increases charge repulsion between caseins, which inhibits casein aggregation (Deshwal, Gómez-Mascaraque, Fenelon, & Huppertz, 2023) and thus gel formation. Formation of the soluble calcium−citrate complex reduces the interaction of calcium with caseins, hence inhibiting aggregation (Tsioulpas, Koliandris, Grandison, & Lewis, 2010). This would likely explain the observed significant increase in gelation time of TSC-added milk. It appears though that irrespective of the level of calcium in milk, the onset of gelation for buffalo skim milk is due to the early collapse of casein micelle stabilising layer and for bovine skim milk, gelation only starts when isoelectric point of casein is approached. For milk blends, the onset of gelation is dictated by the dominant milk species.

Addition of CSS significantly reduced ($P < 0.05$) the G′ value as well the firmness of gels (Table 4). This is presumably a result of reduction in the number and/or strength of bonds between casein particles due to calcium sequestration. CSS-induced dissolution of CCP results to release of caseins and this reduction in the number of CCP crosslinks leads to an increase in electrostatic repulsion between caseins (Lucey, 2016). This is particularly evident in calcium sequestration by citrate. Calcium-citrate complexes cannot associate with dispersed caseins but calcium−phosphate complexes are capable of such association (Mizuno & Lucey, 2007), which could explain significantly lower G′ values of gels from TSC-added milk than their DSHP-added counterpart (Table 4). Additionally, the extent of TSC-induced reduction in G′ value of acid gels increased as the concentration of buffalo skim milk increases. This is mainly due to the presence of high amounts of micellar calcium in buffalo milk which then get affected by TSC to a greater extent. However, the higher total casein concentration in buffalo skim milk dominantly influenced gel formation as confirmed by a progressive increase in G′ value with increasing proportion of buffalo skim milk (Table 4).

Similarly, gel firmness was significantly reduced ($P < 0.05$) with TSC addition (Table 4). The addition of CSS, however, did not have a significant effect on the WHC of 75:25 buffalo:bovine milk blend and buffalo skim milk but significantly increased the WHC of bovine skim milk as well as 25:75 and 50:50 buffalo:bovine milk blends despite marked reduction in gel firmness. It is possible that the interconnected networks of smaller casein particles previously released due to dissolution of CCP could have efficiently trapped the aqueous phase within the protein gel matrix. This is further evident from presence of uneven pores within aggregated protein clusters as observed in the micrographs of CSS-added milk (Supplementary material Fig. S3).

4.3. Effect of heat treatment of milk on acid gelation properties

Whey proteins denature during heat treatment, leading to their association with themselves or with casein micelles, which subsequently affects the gelation properties of acid gels (Lucey et al., 2022). As shown in Table 3, gelation pH of milk significantly increased with heat treatment, irrespective of milk ratio. This is in line to a significant reduction in gelation time after heat treatment observed in other studies (Anema et al., 2004; Lucey et al., 1998; Lucey et al., 2022). This significant increase in gelation pH (Table 3) is mainly attributed to increased aggregation of denatured whey proteins as the pH of milk during acidification approaches the isoelectric point of the whey proteins which is about pH 5.3 for β-lactoglobulin (Anema et al., 2004). Similar to unheated milk, the gelation pH of heat-treated milk increased with increasing proportion of buffalo skim milk (Table 3) which further substantiate the influence of casein concentration in regulating the onset of gelation (Roy et al., 2020).

The gelation pH of milk heated at either 85 °C or 95 °C did not significantly differ ($P > 0.05$), but heating temperature had a pronounced influence on G′ and firmness of the formed gels (Table 4). This is possibly related to the degree of whey protein denaturation which was reported to increase as heating temperature increased regardless of milk ratio (Mejares et al., 2023b). Additionally, heating induces alteration in the salt balance of milk leading to dissociation of some caseins from the micelles during heating (Anema & Klostermeyer, 1997) and this mostly affected buffalo skim milk (Mejares et al., 2023b). This could lead to increased association of casein micelles with denatured whey proteins which produce acid gels with high storage moduli values (Lucey et al., 1998) and thus more firm gel. However, it appears that the formation of different proportions of micelle-bound and soluble protein complexes exhibit distinct effects in the acid gels from milk blends or individual milk in agreement with Anema et al. (2004). In acid gels from 50:50 and 75:25 buffalo:bovine milk blends heated at 95 °C, a significantly higher ($P < 0.05$) final G′ values and gel firmness (Table 4) than their individual milk counterpart were measured. It was previously reported that heat treatment induced higher reduction in the level of non-sedimentable whey proteins in milk mixtures of buffalo and bovine skim milk than in individual milk system (Mejares et al., 2023b). This implies that denatured whey proteins in mixed milk readily associated with casein micelles forming large aggregates that mainly contributed to the observed high G′ values and thus a firmer gel (Table 4).

In individual milk, it appears that the heat-induced significant increases in final G′ values and textural properties were primarily related to the heat-induced changes in the levels of soluble proteins.

As shown in Table 4, acid gels from buffalo skim milk have significantly higher final G′ values and firmer than gels from bovine skim milk irrespective of heating temperature used. Bovine whey proteins are more heat labile than their buffalo counterparts (Mane & Chatli, 2015) which could be related to the variation of the genetic variant of β-lactoglobulin. Buffalo milk β-lactoglobulin was reported to be homologous to bovine β-lactoglobulin B variant, but bovine milk whey also contains β-lactoglobulin A that is absent in buffalo milk (Ahmad, 2013). β-Lactoglobulin A variant has been reported to have a lower heat stability than the B variant (Keppler, Sönnichsen, Lorenzen, & Schwarz, 2014). Heat treatment resulted in higher levels of soluble caseins and whey proteins in buffalo skim milk than bovine skim milk (Mejares et al., 2023b). This suggests that heating induced whey proteins denaturation and their subsequent association with caseins but most of the formed aggregates remained in the serum phase which significantly contributed to gel structure.

This heat-induced denaturation of whey proteins and their subsequent attachment to casein micelles result to an increased connection and compact protein network which subsequently immobilises more water inside the gel structure (Mahomud, Katsuno, Zhang, & Nishizu, 2017), which resulted a higher WHC (Table 4). It appeared that this increase in WHC is a result of the combined effect of both micelle-bound and soluble protein complexes as shown by a significant increase in WHC of gels from heated bovine skim milk and 25:75 buffalo:bovine milk blend. This interconnectivity of the protein network was observed by confocal micrographs of gels made from heated milk (Fig. 2B), which further supports the linear relationship between final G′ values, gel firmness, and WHC. This correlation is expected because water is entrapped more effectively in a firm gel than in softer gels (Kneifel & Seiler, 1993).

4.4. Acid gel properties as affected by CSS addition and heat treatment

The addition of CSS before heat treatment significantly influenced the gelation pH but this effect was determined by milk ratio and the type of CSS (Table 3). TSC and DSHP significantly increased ($P < 0.05$) the gelation pH of bovine skim milk and 25:75 buffalo:bovine milk blend heated at 95 °C (Table 3), which is due to CSS-induced casein dissociation. It was previously reported that CSS addition increased non-sedimentable caseins and whey proteins in heated milk (Mejares et al., 2023a) which are likely to condensate on casein micelles thus accelerating gelation (Asaduzzaman, Mahomud, & Haque, 2021). This also resulted to a higher gelation pH of 50:50 and 75:25 buffalo:bovine milk blends as well as buffalo skim milk systems but such change was not significant.

The final G′ values and gel firmness were significantly reduced ($P < 0.05$) with the addition of CSS (Table 4) which is mainly due to reduction in the CCP crosslinks as discussed previously. The notable exception to this is TSC-added buffalo skim milk heated at 95 °C where the final G′ value and gel firmness (Table 4) was almost twice that of other milk gels parallel to a high WHC (99%). Due to its high micellar calcium and phosphate concentration, TSC-induced casein dispersion as a result of CCP removal was the highest in buffalo skim milk (Mejares et al., 2023a). Additionally, casein dissociation as a result of the heat-induced alteration in the salt balance of milk was highest in buffalo milk (Mejares et al., 2023b). This could potentially lead to increased molecular flexibility (Ozcan-Yilsay et al., 2007) and thus increased the interaction with denatured whey proteins resulting to formation of more aggregates thereby improving gel stiffness. This is further evident in the microstructure of gel prepared from heated TSC-added buffalo skim milk (Fig. 2C) where presence of thin strands and dense clusters of aggregated protein particles can be observed. It is also possible that micelle-bound and soluble protein complexes equally contributed to gel structure.

5. Conclusion

Variation in rheological and textural properties of acid gels produced from blends of buffalo skim milk and bovine skim milk are mainly attributed to the differences in protein and mineral composition between these milk species. Higher protein content and more mineralised casein micelles of buffalo skim milk mainly contributed to the early onset of gelation and formation of more firm gels with greater WHC than gels prepared from bovine skim milk or milk mixture containing higher proportion of bovine skim milk. The addition of CSS greatly increased the onset of gelation, and the resulting gels were less firm but with higher WHC. Irrespective of the type of salt added, heat-induced changes on the rheological and textural properties of acid gels from milk blends were primarily influenced by the presence of micellar whey protein-casein complex whereas those of acid gels from individual milk were dominantly affected by protein aggregates remaining in the serum phase. The presumably equal contribution of micelle-bound and soluble protein complexes to gel structure is manifested in TSC-added buffalo skim milk heated at 95 °C.

Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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