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## Formulation and characterization of hybrid milk containing bovine and *Spirulina* proteins

Jayani Samarathunga,<sup>1</sup> Thi Phuong Linh Le,<sup>a</sup> Max Gabard,<sup>b</sup> Katrina Strazdins,<sup>b</sup> Jeroen Rens<sup>b</sup> and Benu Adhikari<sup>1</sup>

The incorporation of microalgal protein into dairy products presents a sustainable and innovative approach to address rising global protein demands. This study developed model hybrid milk formulations (HMFs) by combining *Spirulina* protein concentrate (SPC) and milk protein concentrate (MPC), in which SPC replaced 25%, 50%, 75%, and 100% of the total protein content (w/w). A formulation with 100% MPC was used as the control. The fat and mineral contents of the HMFs were standardized using ghee and simulated milk ultrafiltrate (SMUF) respectively. Confocal laser scanning microscopy revealed a uniform distribution of fat globules in all formulations, characterized by small initial globule sizes; however, their size increased significantly after 15 days of storage. Increasing SPC levels led to a significant shift toward a greenish-brown hue. HMFs containing 25–75% SPC exhibited significantly higher creaming than those with either 100% MPC or 100% SPC. Increasing SPC levels led to higher viscosity and reduced thermal stability, with gelation occurring at lower temperatures. This reduction in thermal stability was supported by lower protein denaturation temperatures observed for SPC compared to MPC. Corresponding structural analysis showed a progressive loss of ordered conformation, with decreased  $\beta$ -sheet and increased random coil content, which contributed to the altered gelation behaviour. Among the formulations, the 25% SPC (75% MPC) blend most closely resembled the 100% MPC in terms of color, creaming, viscosity, thermal stability, and structural integrity, making it the optimal HMF developed using SPC and MPC.

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### Sustainability spotlight

This study supports the transition toward a more sustainable food system by formulating hybrid milk using bovine and *Spirulina* proteins. The hybrid formulations offer greatly increased nutritional value. Thus, the content of this paper aligns with United Nations Sustainable Development Goals (SDGs) particularly, zero hunger (SDG 2) and good health and well-being (SDG 3). By partially substituting dairy protein with *Spirulina*, the research supports more responsible consumption and production patterns (SDG 12) and contributes to climate action by reducing the environmental footprint of dairy products (SDG 13). Furthermore, the work promotes innovation in food systems (SDG 9), demonstrating the potential of alternative proteins to drive a more resilient and sustainable future for the dairy-food industry.

## 1 Introduction

Bovine milk is a widely consumed, nutritionally balanced staple in diets across the globe. Yet, rising consumer demand and evolving preferences have led to the growing popularity of mixed milk formulations containing blends of milk proteins with other protein sources, as well as dairy alternatives.<sup>1</sup> Among alternative protein sources, *Spirulina platensis* is of great interest due to its high protein content (60–67% dry weight) and the sustainable ways it can be cultivated.<sup>2</sup> *Spirulina* protein has been shown to interact compatibly with dairy proteins, suggesting potential applications in model milk and gelled products. To incorporate *Spirulina* protein and dairy proteins into

a mixed food system, the protein from *Spirulina* must first be extracted from the biomass. Additionally, its techno-functional properties such as solubility, water and oil absorption capacities, and foaming and emulsifying properties require improvement.<sup>3</sup> The techno-functional properties of *Spirulina* protein can be influenced by interactions with other proteins, solvents, and solutes. These properties are also influenced by processing parameters; for example, hydrophobic, hydrophilic, and electrostatic interactions in milk and plant protein systems are impacted by ionic strength, pH, and temperature.<sup>4</sup> Chen *et al.*<sup>5</sup> showed that proteins extracted from *Chlorella pyrenoidosa* and *S. platensis* using a phosphate buffer (1 : 10 w/v, pH 7) and ultrasonication (300 W, 30 min) exhibited minimal foam capacity, stability, solubility, and emulsifying activity at pH 5. Understanding these interactions is important for developing innovative products with greater consumer acceptability.

<sup>a</sup>School of Science, RMIT University, Melbourne, VIC 3083, Australia. E-mail: S3971507@student.rmit.edu.au; benu.adhikari@rmit.edu.au

<sup>b</sup>Bega Group, Melbourne, VIC 3008, Australia



To date, the whole biomass of *S. platensis* has been used in the food industry as a nutraceutical,<sup>6</sup> a coloring agent (phycocyanin for blue and phycoerythrin for red),<sup>7</sup> and as a food supplement.<sup>6,8,9</sup> Benelhadj *et al.*<sup>9</sup> determined three major protein fractions in *Arthrospira platensis* (the most commonly studied species of *Spirulina*)—albumins (51.5%), globulins (2.4%), and prolamins (46.1%)—using the Osborne sequential extraction method. These proteins, which are derived from what is commonly known as *Spirulina*, are characterized by their amino acid composition. This gives *Spirulina* protein its mildly acidic nature and a net negative charge at neutral pH, due to higher acidic-to-basic amino acid ratios.<sup>10</sup> These protein fractions significantly influence the physicochemical and techno-functional properties in food applications. Since albumins are soluble in water, glutelins in alkaline solutions, globulins in saline, and prolamins in water/alcohol mixtures,<sup>9,11</sup> the *Spirulina* protein concentrate is expected to contain water-soluble, alkaline-soluble, and salt-soluble fractions.

The native conformation of *Spirulina* protein is altered by denaturation during food processing. In this study, we evaluated how the physicochemical and techno-functional properties are influenced by partial unfolding of *Spirulina* protein during microfluidization and its cross-linking with other components in hybrid milk formulations. Lozober *et al.*<sup>12</sup> demonstrated that high-pressure homogenization of *Spirulina* protein concentrate (5% w/v) at 50 MPa (pH 6.5, 25 °C) enhanced protein solubility, which in turn promoted gel formation at a low gelation point, resulting in a stiff gel. In industrial milk production (pasteurized, UHT, and sterilized milk), thermal treatments are applied to extend shelf life. Similarly, in *Spirulina* protein-containing hybrid milk production, understanding the thermal stability and heat-induced gelation of *Spirulina* protein is essential. Additionally, high pressure homogenization/microfluidization, a key process in producing *Spirulina* protein-containing model milk, may have an impact on gelation, potentially posing challenges. This study, therefore, focused on the thermal stability of *Spirulina* protein in hybrid milk formulations.

Partially replacing bovine milk with alternative milk, or milk proteins with alternative proteins in milk systems, remains challenging, as protein–protein interactions are weak due to their non-covalent nature.<sup>1</sup> This can lead to phase separation through co-aggregation, driven by incompatibilities in internal factors such as structure, molecular weight, and free thiol groups, as well as external factors like pH, temperature, and ionic strength. Studies have been conducted to evaluate the application of cereal-based proteins (pea, soy, quinoa, barley, and rice proteins) as milk protein alternatives.<sup>13,14</sup> However, research on partially or fully replacing bovine protein with microalgal protein in milk systems remains scarce. Published studies have so far utilized whole algal biomass powder to produce dairy-mimetic products. For example, cheese analogues have been made from *Chlorella vulgaris* biomass powder,<sup>15</sup> yogurts fortified with *Spirulina* powder,<sup>16</sup> cheese enriched with *Spirulina* powder,<sup>17</sup> and fermented milk incorporated with *Spirulina* biomass.<sup>18</sup>

So far, there is no study on the behavior of *Spirulina* protein in milk systems. Given its potential as a sustainably sourced

protein, it is essential to investigate how *Spirulina* protein interacts with milk fat, lactose, and minerals to mimic the properties of bovine milk proteins. This work aims to optimize hybrid milk formulations designed to mimic the composition of natural milk for research purposes containing *Spirulina* protein and compare them to those made with bovine protein with respect to their physicochemical properties, techno-functional characteristics, and microstructure. The findings of this study will provide food producers with a scientific basis to utilize *Spirulina* protein in food formulations as a sustainable alternative to milk proteins.

## 2 Materials and methods

### 2.1. Materials

*Spirulina* biomass powder (BIOGLAN organic, New South Wales, Australia) was purchased from a local chemist warehouse outlet (Melbourne, Australia). Milk protein concentrate (MPC) was donated by Tatura milk industries Ltd, Victoria, Australia. According to the manufacturer, it contained 85.5% protein on dry basis of which 67.5% was casein and 16.2% whey protein. Ghee (clarified butter), labelled as containing 99.9% fat by the manufacturer, was purchased from the local supermarket (Maharajah's Choice, Melbourne, Australia). Food grade D-lactose monohydrate with purity  $\geq 98\%$  was purchased from Sigma-Aldrich Pty Ltd (New South Wales, Australia) and was used as the source of sugar for all *Spirulina* protein-based milk formulations. All other chemicals used were of analytical grade and were purchased from Sigma-Aldrich Pty Ltd Milli-Q water was used to prepare all of the milk mimetic formulations.

### 2.2. Protein extraction from *Spirulina* biomass

Preparation of *Spirulina* protein concentrate (SPC) was carried out according to Zhang *et al.*<sup>19</sup> with minor modification and the procedure is shown in the Fig. 1.

### 2.3. Determining proximate composition of freeze dried SPC powder

The crude protein content (Kjeldahl method; AOAC Method 991.20),<sup>20</sup> moisture (AOAC method 925.10),<sup>21</sup> fat (AOAC method 920.85)<sup>22</sup> and ash content (AOAC method 923.03)<sup>23</sup> of freeze dried SPC powder were determined. The mineral content (calcium-Ca, sodium-Na, magnesium-Mg, and potassium-K, iron-Fe, zinc-Zn and copper-Cu) of the freeze dried SPC powder, and MPC were analyzed using ICP-MS method (7700®, Agilent Technologies, Santa Clara, USA). This instrument was able to detect upper limit of 10 mg L<sup>-1</sup> (10 ppm) for Na, K, Mg, Ca and Fe and upper limit of 100 µg L<sup>-1</sup> (100 ppb) for Cu and Zn. In brief, a 2.0 g sample of powder was subjected to dry ashing in a muffle furnace (Thermo Scientific Thermolyne-Muffle Furnace, USA) at 550 °C for 16 hours. The resulting ash was dissolved in 2% HNO<sub>3</sub> to prepare the analyte solution, which was then filtered through a 0.45 µm membrane filter.



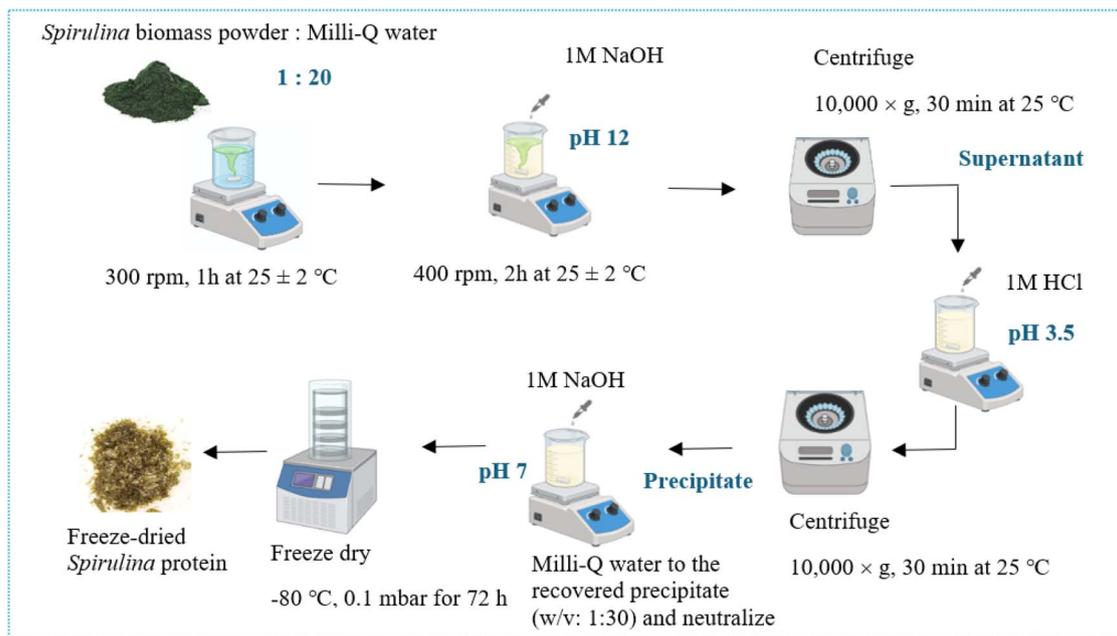


Fig. 1 Flow diagram of the procedure used to extract protein from *Spirulina* biomass powder.

#### 2.4. Formulation of milk

Milk was formulated to mimic the composition of cow's milk (fat, protein, sugar and minerals). Typically, cow's milk contains 3.5% fat, 3.3% protein, and 5% lactose.<sup>24</sup> The pure ghee was used as the source milk fat after melting it at 40 °C. The mineral composition of milk was replicated using simulated milk ultrafiltrate (SMUF), prepared according to the protocol of Jenness<sup>25</sup> with minor modifications as described in de Groot *et al.*<sup>26</sup> Formulation of the *Spirulina*-based milk was carried out by replacing milk proteins (casein : whey; 80 : 20) using SPC at 0%, 25%, 50%, 75% and 100% (w/w). All the ingredients (Table 1) were homogenized under 62 MPa in 3 passes using a microfluidizer (Microfluidics®-M110L-UHPH, Germany). Experiments were carried out at 25 °C and stored at 4 ± 1 °C for further analysis.

The SPC and MPC contents (33.3 g protein/1000 mL) required for each milk formulation system was calculated based on the protein content in them. Prior to homogenization, the protein component was dissolved in 500 mL SMUF and mixed by agitating at 600 rpm for 2 h at 35 ± 2 °C. The pH of the

protein solutions was adjusted to 6.70 using 1 M NaOH/1 M HCl to match the pH of bovine milk. These protein solutions were stored at 4 °C overnight for complete hydration. Then, course emulsions were prepared by mixing these protein solutions (30 °C) with melted ghee using Ultra Turrax high shear homogenizer (IKA T25 digital® Ultra-Turrax, Malaysia) at 15 000 rpm for 30 s. Finally, model milk samples were produced by passing the course emulsions through microfluidizer three times. Temperature during the microfluidization process was maintained within 20 ± 5 °C using an ice bath. The milk samples produced in this way were stored at 4 ± 1 °C for further analysis.

#### 2.5. Physicochemical properties of milk formulations

**2.5.1. Viscosity of the milk formulations.** Viscosity of the model milk samples was measured using a rheometer (Discovery HR3 Hybrid Rheometer, TA instruments, Australia) operated by TRIOS software. Measurements were taken using a 60 mm measuring plate with a 2° angle cone head at 25 °C. The viscosity *versus* shear rate data was recorded in 1–100 s<sup>-1</sup> range.

Table 1 Proportions of different ingredients in the milk formulations<sup>a</sup>

Sample	Description	Protein	Fat (melted ghee)	Lactose	SMUF (minerals)
100% MPC	100% Milk protein (80 : 20; casein : whey)	3.3% (w/v)	3.5% (v/v)	5% (w/v)	500 mL
25% SPC	25% <i>Spirulina</i> protein + 75% dairy protein	3.3% (w/v)	3.5% (v/v)	5% (w/v)	500 mL
50% SPC	50% <i>Spirulina</i> protein + 50% dairy protein	3.3% (w/v)	3.5% (v/v)	5% (w/v)	500 mL
75% SPC	75% <i>Spirulina</i> protein + 25% dairy protein	3.3% (w/v)	3.5% (v/v)	5% (w/v)	500 mL
100% SPC	100% <i>Spirulina</i> protein	3.3% (w/v)	3.5% (v/v)	5% (w/v)	500 mL

<sup>a</sup> 100% MPC – formulation with 100% MPC; 25% SPC – formulation having 25% SPC; 50% SPC – formulation having 50% SPC; 75% SPC – formulation having 75% SPC and 100% SPC – formulation having 100% SPC.



**2.5.2. Measurement of color parameters.** Color parameters of the milk samples were measured within 24 hours after their preparation using the chromameter (Chroma Meter CR-400, Konica Minolta, Australia). Color space measurements  $L^*$  (Lightness),  $a^*$  (red-green range) and  $b^*$  (blue-yellow range) were measured. Whiteness index (WI) of the formulations was calculated using the eqn (1).<sup>27</sup>

$$WI = \left( 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}} \right) \quad (1)$$

**2.5.3. pH during storage period.** pH of the model milk formulations was measured using laboratory pH meter day 0, 5, 10, and 15.

**2.5.4. Zeta potential of milk formulations.** Zeta potential measurements of the model milk samples were made using a Zetasizer (Nano series, Malvern Instruments) at 25 °C. The zeta potential values help to understand the stability of the milk emulsions where  $\pm 0$ –10,  $\pm 10$ –20,  $\pm 20$ –30, and  $\pm 30$  mV are considered to be unstable, relatively stable, moderately stable, and highly stable, respectively.<sup>28</sup> For these measurements, samples were diluted 100 times using Milli-Q water and placed into folded capillary cell (DTS1070). The instrument calculates the zeta potential by determining the electrophoretic mobility and then applying the Henry equation which fits the Smoluchowski model. The refractive indices for fat and SMUF were taken as 1.46 and 1.334, respectively.<sup>29</sup>

## 2.6. Microstructure of the formulated milk

To understand the fat and protein distribution within the coarse emulsion and the model milk emulsions, their microstructure was observed using a light microscope under 40 $\times$  magnification to assess droplet size and phase separation, and a confocal laser scanning microscope (CLSM) (A1 HD25, Nikon Instruments Inc., Japan) under 100 $\times$  magnification with oil immersion to examine fat and protein distribution. These measurements provided valuable information on the droplet size, concentration, aggregation nature, and nature of the oil-in-water emulsion of the model milk samples. For CLSM, protein in the model milk was stained with fast green and was excited at 635 nm. The fat component was stained with Nile red excited at 488 nm. A 200  $\mu$ L sample was stained with 4  $\mu$ L of Nile red (1 mg/1 mL) and fast green (1 mg/1 mL) each and mixed well. Samples were placed on a glass slide and observed using CLSM while maintaining a dark environment to protect samples from photobleaching.

## 2.7. Particle size distribution of milk formulations

Particle size distribution of the fat globules was used to evaluate the stability of model milk for 15 days.<sup>29</sup> Particle size of the fat globules of the formulations was measured by laser diffraction method using Mastersizer 3000 attached to a HydroMV sample handling unit (Malvern Mastersizer liquid cell-3000-ATA scientific instruments, Australia). The refractive indices of milk fat and the dispersant (SMUF) were considered as 1.460 and 1.334, respectively, at 25 °C.

## 2.8. Techno-functional properties

**2.8.1. Emulsion stability of milk formulations-creaming index.** The observation for phase separation was made for a storage period of 15 days at 4 °C as this temperature is commonly used to refrigerate milk. For this, 15 mL of each milk sample was poured into glass tubes of equal diameter, and the height of the cream (phase separated) layer was measured as a percentage of initial height. Sodium azide (0.04 g/100 mL) was added to the samples to avoid microbial growth during storage.<sup>30</sup> Creaming index was determined by  $100 \times (H_S/H_E)$  expression, where  $H_E$  is the total height of the emulsion in the tube and  $H_S$  is the height of the creamed layer.

**2.8.2. Thermal stability.** The thermal stability of the MPC-based milk, SPC-based milk and protein concentrate powders (SPC and MPC) were evaluated using a differential scanning calorimetry (DSC Q-2000, TA Instruments, New Castle, DE, USA). A sample of 5 mg was sealed in aluminium pans (Perkins Elmer volatile sample pans) and an empty pan used as a reference (air as the reference). Samples were heated from 20 °C to 200 °C at a heating rate of 5 °C min<sup>-1</sup> under nitrogen gas flush at 50 mL min<sup>-1</sup> purge rate. A modulated amplitude of  $\pm 0.54$  °C for 40 s was used throughout. The denaturation temperature ( $T_d$ ), onset temperature ( $T_{on-set}$ ), off-set temperature ( $T_{end-set}$ ), peak temperature ( $T_{peak}$ ) and the enthalpy change ( $\Delta H$ ) during heating process were determined using UniversalTM software (TA Instruments, New Castle, DE, USA).

**2.8.3. Rheological properties during heat induced gelation.** The rheological properties of milk formulations during heating and cooling were determined using Discovery HR3 Hybrid Rheometer (TA instruments, Australia) equipped with a 60 mm measuring plate with a 2° angle cone head. Measurements were made at a frequency of 1 Hz and 0.1% strain. The oscillatory storage modulus ( $G'$ ) was measured during heating (20 °C to 95 °C) and cooling (95 °C to 20 °C) at a rate of 1 °C min<sup>-1</sup>.<sup>31</sup> TRIOS software was used in these analyses. Results were used to determine heat-induced gelation of milk formulations.

## 2.9. Determination of changes of protein conformation

Fourier transform infrared spectrometric spectra (FTIR) were recorded in 4000–400 cm<sup>-1</sup> range and at a resolution of 2 cm<sup>-1</sup> of all milk formulations using a FTIR spectrometer (PerkinElmer, CT, USA). The spectra in the amide I region (1700–1600 cm<sup>-1</sup>) were analyzed to determine the changes in the protein's conformation. Milli-Q water was used to acquire reference (background) spectra. Sixty-four scans were taken and averaged for each sample.

## 2.10. Statistical analysis

All measurements were made in triplicate in terms of sample preparation and tests unless otherwise specified above. Results are presented as mean  $\pm$  standard deviation (SD). The analysis was performed using MINITAB 17 software. One way ANOVA test (Tukey test) was performed to determine the significant difference. The significant difference between any two mean values were determined at 95% confidence level ( $p < 0.05$ ).



### 3 Results and discussion

#### 3.1. Proximate composition

An amount of 24.37 g of SPC was extracted from 100 g of *Spirulina* biomass powder. The extracted SPC contained 81.53% crude protein (dry basis), 7.80% moisture, 1.90% lipid, and 7.81% total ash. The mineral content of SPC and MPC was compared to assess the impact of mineral salts in milk-based systems. Notably, SPC showed significantly higher sodium levels ( $p < 0.05$ ) than MPC (Table 2), likely due to the use of sodium hydroxide during the extraction process. Elevated sodium levels in SPC can impact the flavor profile of HMFs, potentially leading to undesirable levels of saltiness and reduced consumer acceptability. For reference, bovine milk typically contains 39.1–64.4 mg/100 g of sodium and 121.2–168.1 mg/100 g of potassium, which together contribute to its naturally balanced salty taste.<sup>32</sup> Beyond sensory effects, high sodium intake is linked to adverse health outcomes, including hypertension and kidney strain.<sup>33</sup> Sodium content in SPC can be reduced through ultrafiltration, diafiltration, or dialysis. Additionally, increasing both the number of washing steps and the volume of washing solution used during centrifugation has been shown to effectively lower residual sodium levels. MPC had higher levels of magnesium, calcium, and potassium (Table 2), with these differences being statistically significant ( $p < 0.05$ ). These variations highlight the influence of composition and processing methods on the mineral composition of protein concentrates. Mineral composition of SPC is rarely found in literature despite its importance and this information can broaden its application, for example, to enrich iron (Table 2). SMUF was used in this study to replicate the ionic environment of bovine milk, as the mineral profile plays a crucial role in the buffering capacity of milk systems.<sup>34</sup> This is because minerals like ionic calcium and phosphate release hydrogen ions during heat treatment, causing a decrease in pH.

#### 3.2. Physicochemical properties of HMFs

**3.2.1. Color of the milk formulations.** Color of a food product is an important sensory attribute which determines the

consumer acceptability. Overall color and whiteness of HMFs were compared as milky-white color is preferred by consumers in bovine and other milks. The  $L^*$  and whiteness indices of HMFs significantly decreased upon SPC incorporation ( $p < 0.05$ ; Table 3), indicating a darker appearance compared to the control. SPC addition also caused a color shift from white to a greenish-brown hue, as reflected by a decrease in greenness (less negative  $a^*$ ) and an increase in yellowness ( $b^*$ ) values (Table 3). Three main pigments—chlorophyll, phycocyanin, and carotenoids—are present in *Spirulina* biomass.<sup>35</sup> Some portion of each of these pigments was retained in the SPC. After alkalization and centrifugation, the supernatant appeared dark brown, but during acidification, its color shifted to green. This color change is due to the conversion of chlorophyll to pheophytin in the presence of iron ions and alkalinity, which initially gives the brown color.<sup>36</sup> Protein extraction method is also known to affect the color of microalgal protein. Silva *et al.*<sup>37</sup> extracted *Spirulina* protein from de-fatted biomass using ultrasound assisted alkaline solubilization at pH 9 followed by precipitation at pH 3.0. They observed that the aqueous solution (0.5% w/v) of the extracted protein displayed a blue color, which darkened as the pH increased. This variation was attributed to the presence of C-phycocyanin pigment bound to the *Spirulina* protein. In our study, a brown color was observed instead of blue when the pH was adjusted to 12.0 during protein extraction. However, the dark color of HMFs, especially at higher SPC content, may negatively affect consumer perception.

**3.2.2. Zeta potential of different milk formulations.** Zeta potential, which shows the magnitude of charge present on a colloidal system, can indicate the stability of casein micelles and milk fat in bovine milk.<sup>28</sup> Zeta potential is impacted by pH, ions and temperature.<sup>28</sup> Previous studies have reported that the zeta potential of casein micelles and reconstituted milk at pH 6.7 was  $-22$  mV, and  $-34.6$  mV, respectively.<sup>38</sup> As shown in Table 3, the SPC and the SPC-containing milk formulations had higher negative zeta potential. It is reported that microalgal surfaces bear a negative charge due to the presence of carboxylic and amine groups in the cell membrane.<sup>39</sup> The negative charge (negative zeta potential value) of HMFs increased with the incorporation of SPC (Table 3). Because of the negatively charged surface of *Spirulina* protein particles, they repelled each other and readily adsorbed onto the oil/water interface of the milk emulsion, helping to form a stable SPC-fat emulsion in the model milk formulation. Production of HMFs through microfluidization is advantageous as temperature could be controlled, and it can produce stable emulsions in a short processing time.<sup>40</sup> The pressure and turbulence created during microfluidization is also expected to be conducive in producing stable HMFs.

**3.2.3. Viscosity of the milk formulations.** When SPC was dissolved in SMUF (before microfluidization), the solution became more viscous compared to MPC. This increase in viscosity was likely due to the interaction between *Spirulina* protein and various ionic groups present in SMUF.<sup>5</sup> Polar amino acids in *Spirulina* protein can increase the absorption of water which forms a viscous solution.<sup>5</sup> The viscosity of *Spirulina* protein varied with protein solubility, and at higher pH levels

Table 2 Mineral composition of SPC and MPC powder<sup>a</sup>

Mineral	SPC freeze dried powder	MPC powder
<b>Macro (mg/100 g)</b>		
Na	15.22 ± 0.10 <sup>a</sup>	0.64 ± 0.03 <sup>b</sup>
Ca	0.26 ± 0.15 <sup>a</sup>	10.98 ± 0.41 <sup>b</sup>
Mg	0.14 ± 0.07 <sup>a</sup>	0.62 ± 0.11 <sup>b</sup>
K	0.62 ± 1.30 <sup>a</sup>	1.91 ± 0.05 <sup>b</sup>
<b>Micro (mg/100 g)</b>		
Fe	2.50 ± 0.05 <sup>a</sup>	<0.001 <sup>b</sup>
Zn	<0.001	<0.001
Cu	<0.001	<0.001

<sup>a</sup> Results are represented as an average of triplicates ± SD. Different letters (superscript) indicate significant differences within a row ( $p < 0.05$ ).



Table 3 Color parameters and zeta potential parameters of milk formulations<sup>a</sup>

Sample	$L^*$	$a^*$	$b^*$	Whiteness index	Zeta potential (mV)
100% MPC	$87.11 \pm 0.06^a$	$-3.00 \pm 0.15^a$	$4.66 \pm 0.07^a$	$85.97 \pm 0.27^a$	$-35.2 \pm 0.3^a$
25% SPC	$55.41 \pm 0.10^b$	$-2.07 \pm 0.04^b$	$24.64 \pm 0.45^b$	$49.01 \pm 0.55^b$	$-35.8 \pm 0.1^b$
50% SPC	$44.84 \pm 0.23^c$	$-1.63 \pm 0.01^c$	$21.94 \pm 0.03^c$	$40.61 \pm 0.83^c$	$-37.4 \pm 0.7^c$
75% SPC	$38.26 \pm 0.03^d$	$-1.17 \pm 0.20^d$	$19.67 \pm 0.11^d$	$35.19 \pm 0.08^d$	$-37.5 \pm 0.5^d$
100% SPC	$32.52 \pm 0.01^e$	$-1.18 \pm 0.05^e$	$15.96 \pm 0.34^e$	$30.65 \pm 0.72^e$	$-40.2 \pm 0.3^e$

<sup>a</sup> Results are represented as an average of triplicates  $\pm$  SD. Different letters (superscript) indicate significant differences within a column ( $p < 0.05$ ).

(>8), it decreased as protein solubility increased.<sup>41</sup> In 100% SPC, a significantly higher viscosity was observed compared to other formulations, as shown in Fig. 2B, the 100% SPC showed much higher viscosity across all shear rates. The viscous nature of SPC-based milk could be attributed to the water absorption capacity of protein.<sup>5,42</sup> Water absorption capacity/water binding ability of proteins is an important factor in food formulations where imbibing water is required to achieve a desired viscosity without solubilizing protein.<sup>42</sup> Chen *et al.*<sup>5</sup> reported that the presence of phosphate groups can increase the water absorption capacity of proteins. It was also observed that the particle size distribution of 100% SPC shifted towards larger particle sizes, indicating an increase in viscosity. The ionic environment

of SMUF affected the amphiphilic nature of *Spirulina* protein and altered its surface-active properties. It also led to an increase in viscosity in 100% SPC. Calcium and phosphate ions interact caseins in milk proteins to form casein micelles and these micelles combine with fat droplets to form a stable emulsion. As shown in Fig. 2A, the color of the solutions became darker with increasing SPC content, reflecting its unique composition.

### 3.3. Microstructure

Freshly prepared five milk formulations were observed under light microscope (40 $\times$ ) and CLSM (100 $\times$  oil immersion). The

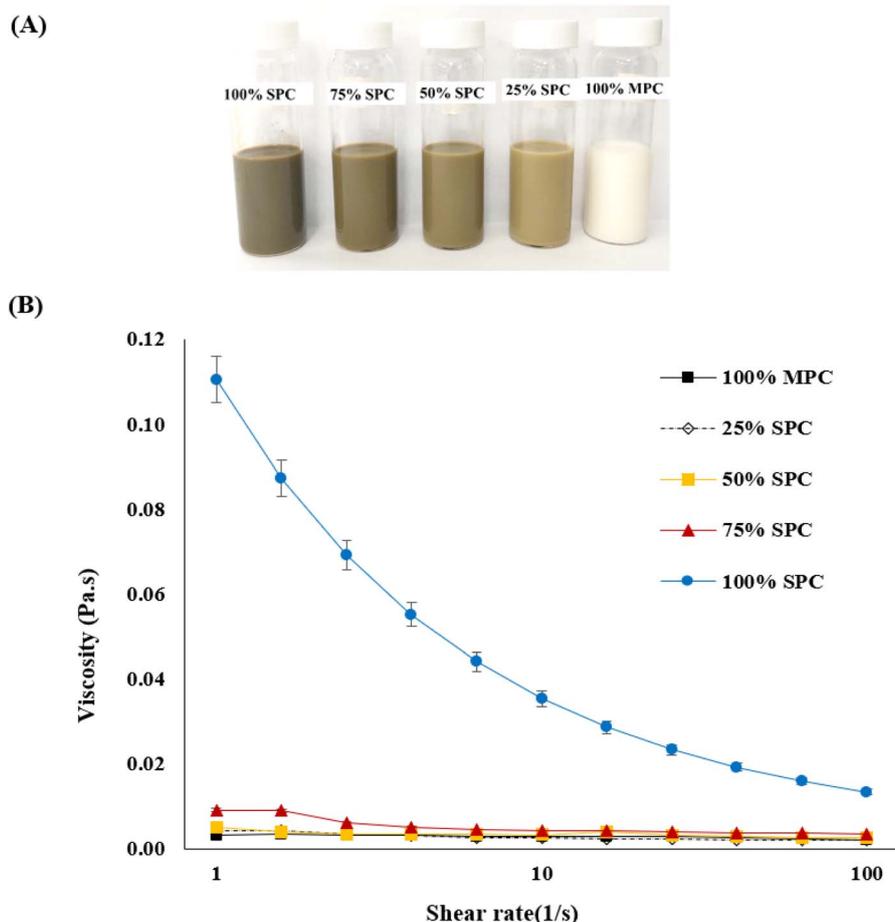


Fig. 2 (A) Milk formulations substituting bovine protein with *Spirulina* protein at 0%, 25%, 50%, 75% and 100% from right to left and (B) variation of viscosity in milk formulations at different shear rates.



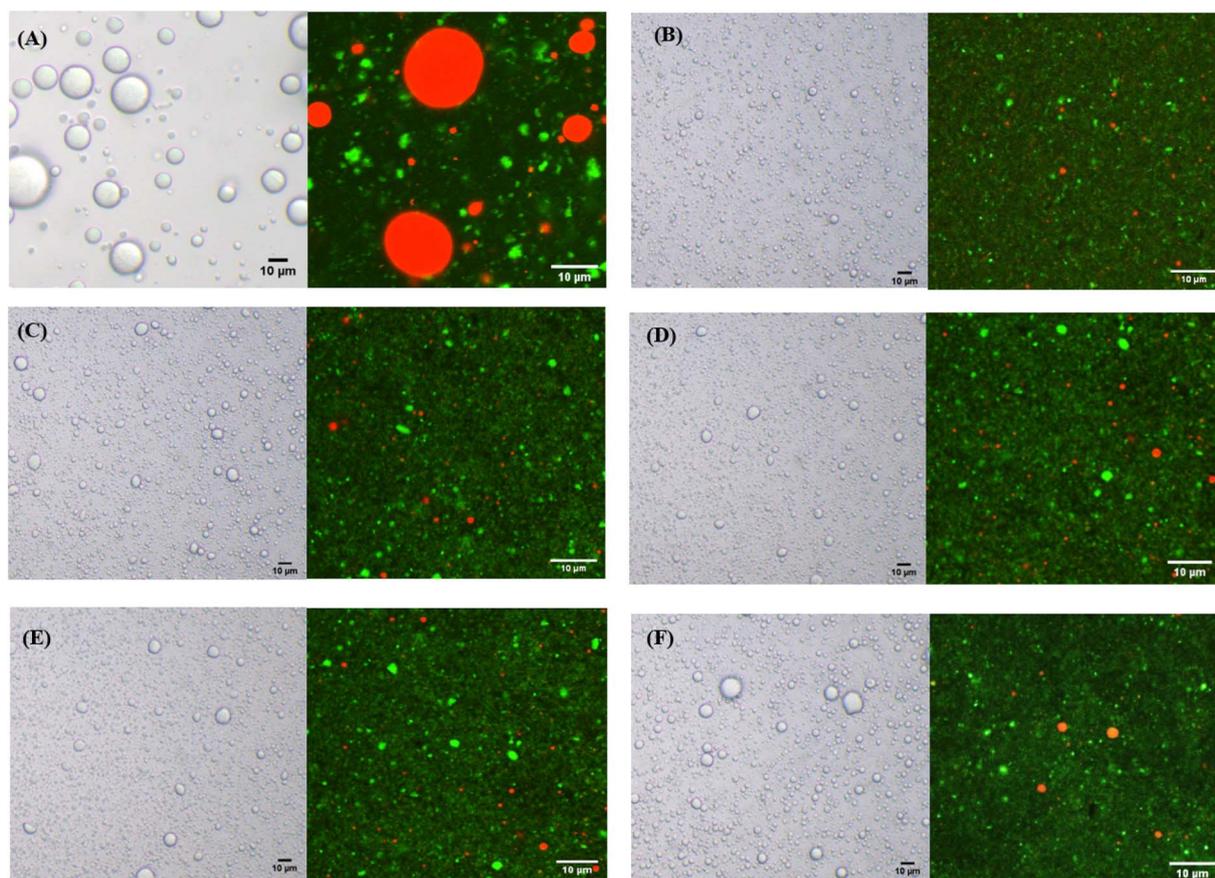


Fig. 3 Light microscopic 40 $\times$  (left side) and overlay of Nile red and Fast Green CLSM 100 $\times$  oil immersion (right side) images (scale bar: 10  $\mu$ m) of the emulsions in milk formulations. (A) Pre-emulsion of 100% SPC before microfluidization and (B) 100% MPC; (C) 25% SPC; (D) 50% SPC; (E) 75% SPC and (F) 100% SPC after microfluidization respectively.

microscopic images of preliminary emulsions in all HMFs were similar. Hence, image of preliminary emulsion of 100% SPC is presented in Fig. 3A as representative of preliminary emulsion of all milk formulations. High pressure (62 MPa) prevailing in the microfluidization promoted formation of a stable emulsion. SPC and MPC are soluble in aqueous medium at pH 6.70 at 25  $^{\circ}$  C.<sup>37,43</sup> Thus, protein component (stained with fast green) was visible in green color and was found to disperse uniformly. In both model milks produced using MPC and SPC, fat droplets (stained with Nile red) were covered by protein film or layer. The coverage by protein layer improved the stability of these O/W emulsions. The larger protein aggregates formed in the preliminary emulsion were further broken-down during homogenization as indicated by the breakdown of larger bright green areas in the pre-emulsion (Fig. 3A) to small areas in HMFs.

### 3.4. Particle size

The bimodal particle size distribution of preliminary emulsions was changed to monomodal distribution in milk formulations after third pass of microfluidization. As observed in Fig. 4A, bimodal particle size distribution is observed in the preliminary-emulsion and also in all milk formulations after

first and second pass of microfluidization. Hence, microfluidization at 62 MPa and three passes were found to be optimum homogenisation conditions to produce milk formulations. Microfluidization helps to form stable emulsions by minimizing the oil droplet size and facilitating their coverage with proteins or emulsifiers.<sup>40</sup> The average particle size (volume-weighted mean diameter,  $D[4, 3]$ ) of fat globules in the pre-emulsions was fairly consistent in these formulations, ranging from 27.5  $\mu$ m (in 100% MPC) to 29.5  $\mu$ m (in 100% SPC). After microfluidization (62 MPa, 3 passes), the globule size was significantly reduced to the nanoscale, with mean diameters remaining between 359 nm (in 100% MPC) and 411 nm (in 100% SPC) as the diameter increased with higher level of SPC. The  $D[4, 3]$  values of all HMFs increased over the storage period. By the 15th day, the 100% MPC (circled area in Fig. 4B) exhibited a bimodal particle distribution, indicating the presence of aggregates. In comparison, the fat particle size distribution of HMFs containing SPC had a much wider span than that of 100% MPC. The nature of protein adsorption influences the particle size distribution of fat in oil/water emulsions.<sup>44</sup> The particle size results suggested that the interaction between milk protein-fat and *Spirulina* protein-fat was different and indicated that this protein-fat interaction is an important factor in



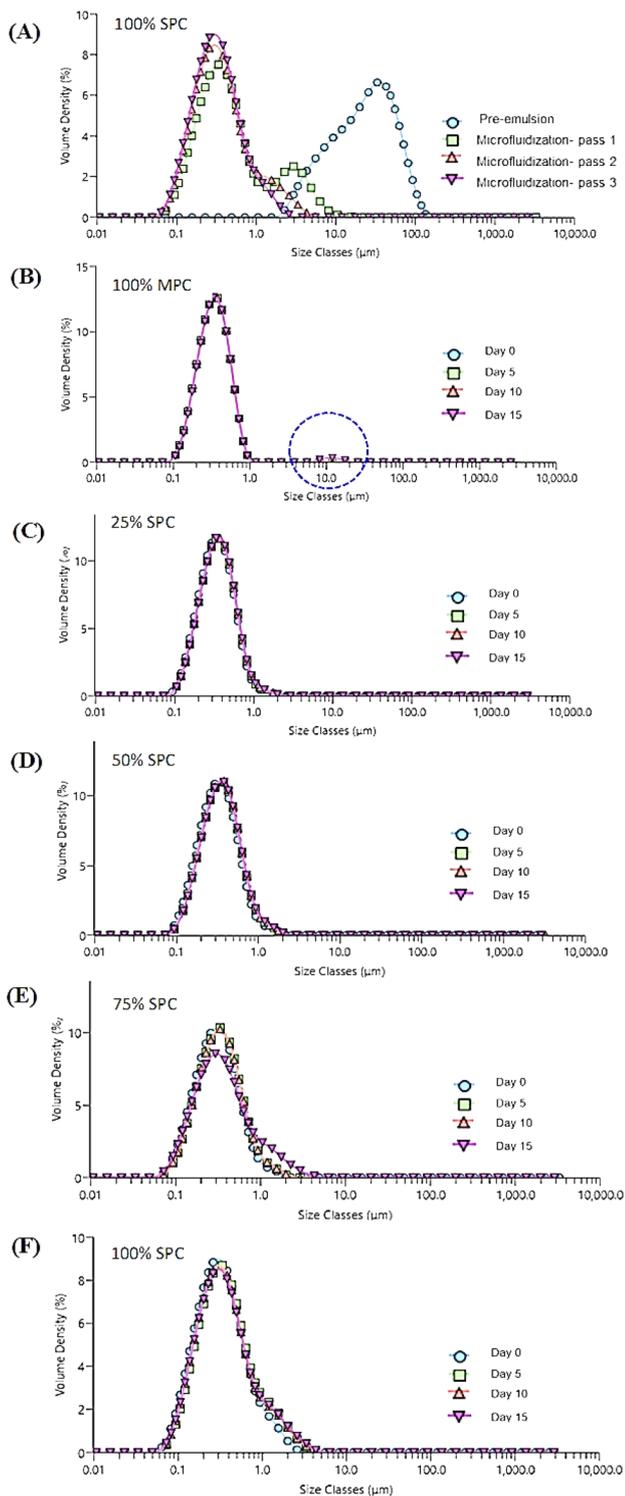


Fig. 4 (A) Particle size distribution of the fat globules ( $\mu\text{m}$ ) of 100% SPC between pre-emulsion and three passes during microfluidization; (B)–(F) represent particle size distribution of the fat globules ( $\mu\text{m}$ ) of 100% MPC, 25% SPC, 50% SPC, 75% SPC and 100% SPC HMFs during storage period respectively.

determining the particle size of the emulsions.<sup>45</sup> *Spirulina* protein showed a lower ability to adsorb to fat droplet surfaces compared to milk protein, likely due to its lower solubility at pH

6.0–7.0 and greater susceptibility to unfolding under pressure. Consequently, the particle size distribution span of 100% SPC was broader than that of 100% MPC. Despite the differences in particle size distribution, the emulsion stability of 100% SPC was comparable to that of 100% MPC throughout the storage period, as indicated by the creaming index results (Fig. 4A). Both 100% MPC and 100% SPC exhibited low creaming compared to hybrid milk (HMFs). In HMFs, the interfacial protein layer appeared more heterogeneous and discontinuous, with regions of incomplete coverage exposing the fat core. This disruption was particularly evident at intermediate SPC levels (25–75%), where the reduced continuity of the interfacial layer likely contributed to increased creaming due to insufficient droplet stabilization. However, as the SPC proportion increased—reaching 100% in the SPC-only formulation—the interfacial layer became more uniform and continuous, indicating improved coverage of fat droplets. This enhanced interfacial stability in the 100% SPC formulation corresponded with its lower creaming index compared to the HMFs (Fig. 5A). In 100% MPC, casein micelles form a stable network to envelop fat droplets, facilitated by calcium and phosphate ions in SMUF and contribute to the emulsion's stability.<sup>46</sup> This would also be one major reason due to which the size of fat droplets in 100% MPC was smaller compared to that in 100% SPC. In contrast, the interaction between *Spirulina* protein and fat droplets is less defined because it lacks this robust enveloping capability or is influenced by other factors, such as mineral salts.

### 3.5. Creaming index and pH of HMFs during storage

The pH of HMFs was measured and adjusted at different stages of their preparation. The pH of SPC solutions was higher than that of MPC solutions and was adjusted to 6.67 (milk pH) before mixing with melted ghee. The slightly lower pH in MPC can be attributed to its higher calcium ion content, which lowers the pH. However, the pH of all HMFs increased slightly after microfluidization (Fig. 5B; day 0), which is likely due to the unfolding of the protein structures. During this process, the milk and *Spirulina* proteins in the HMFs (25% SPC, 50% SPC, and 75% SPC) were expected to interact, forming an envelope around the fat droplets to stabilize the emulsion. The strength of these protein–protein interactions depends on factors such as temperature, pH, ionic strength, and the availability of free thiol groups.<sup>1</sup> Notably, the solubility of milk and *Spirulina* proteins differed at pH values between 6.0 and 7.0, which is close to the natural pH of milk. This reduced solubility of *Spirulina* protein likely contributed to the partial settling observed in the hybrid formulations after being left overnight for hydration. In hybrid protein formulations, *Spirulina* protein showed lower solubility in SMUF compared to milk proteins, leading to partial settling. The protein content in SPC was 81.53%, with the remaining portion consisting of non-protein components, which could influence the overall solubility. During storage, the pH of all milk formulations gradually decreased, although the pH of the 100% MPC formulation consistently maintained a slightly higher pH than the SPC-based HMFs.



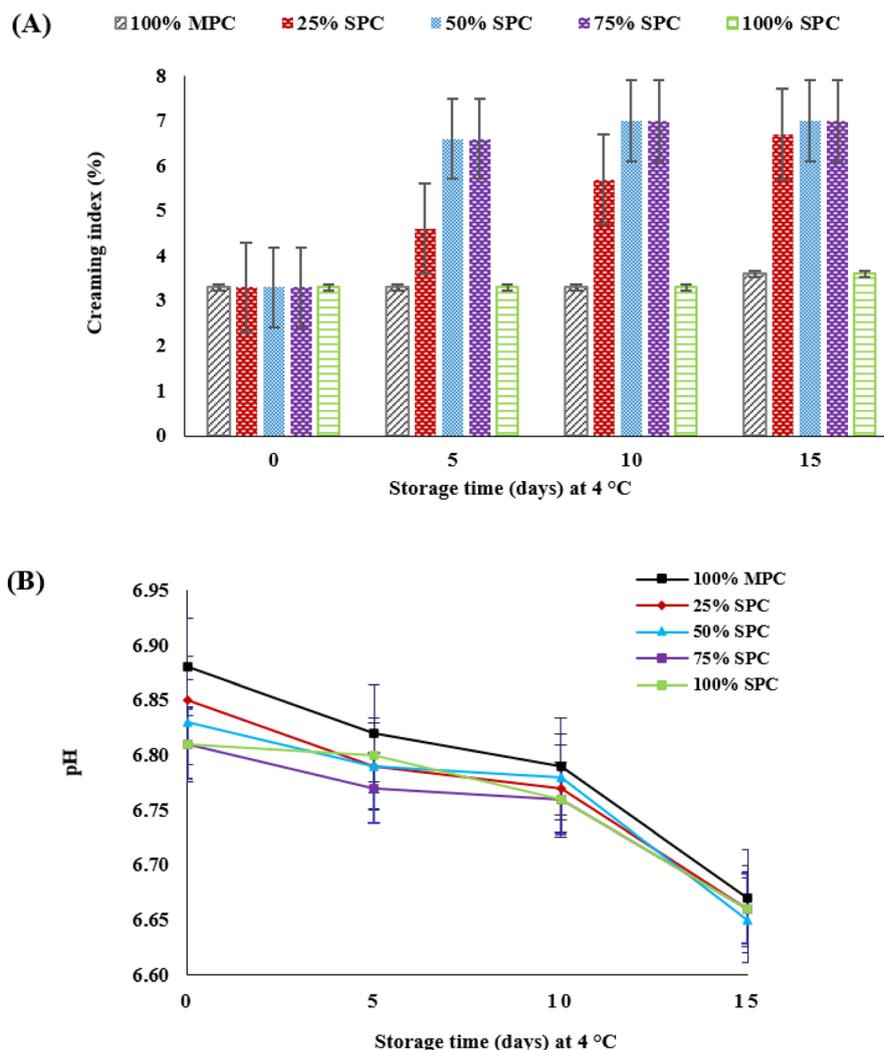


Fig. 5 (A) and (B) represent the creaming index and pH changes of milk formulations during storage (mean  $\pm$  SD;  $n = 3$ ).

The creaming index measures the gravitational separation of dense and light phases in an oil-in-water emulsion.<sup>47,48</sup> The creaming index of 25% SPC, 50% SPC, and 75% SPC increased during storage (Fig. 5A). For the 100% SPC and 100% MPC formulations, the creaming index remained stable until the 10th day, while a noticeable increase was observed by the 15th day (Fig. 5A). Microfluidization enhances emulsion stability by reducing oil droplet size and helping to increase the coverage of fat globules by proteins, both of which help prevent phase separation. However, cooling the emulsions can lead to partial crystallization of the fat phase, promoting phase separation. The presence of these two different protein types in the formulations appeared to increase the tendency for phase separation due to the aggregation of unabsorbed proteins.

### 3.6. Techno-functional properties

**3.6.1. Thermal stability.** The thermal stability of proteins is crucial for the functionality of dairy products; for instance, thermal denaturation induces gelation. Studies have shown that the extent of whey protein denaturation during heat

treatment also depends on the prevailing pH.<sup>49</sup> The thermal stability of HMFs is influenced by the nature of their components and interactions, such as protein-protein, protein-lactose, and protein-fat.<sup>50,51</sup> The Maillard reaction, which occurs between amino groups of proteins and reducing sugars, can alter protein functionality. Previous research showed that the Maillard reaction between SPC and maltodextrin can occur at a moderate temperature of 60 °C.<sup>19</sup> Therefore, glycation between lactose and proteins is likely to occur during heat treatment of the milk formulations.

The protein denaturation temperature serves as an indicator of thermal stability/instability; thus, the denaturation temperatures of SPC and MPC were assessed to evaluate the thermal behavior of milk formulations (Fig. 6). The thermal analysis of SPC at pH 7 (Fig. 6A) revealed two distinct endothermic events within the 20–200 °C range, indicating multistage decomposition due to the presence of multiple protein fractions with different thermal stabilities. The first broad peak occurred between 58.3 °C (onset) and 97.5 °C (end-set), with a peak temperature ( $T_{\text{peak}}$ ) of 89.9 °C and an enthalpy change ( $\Delta H$ ) of



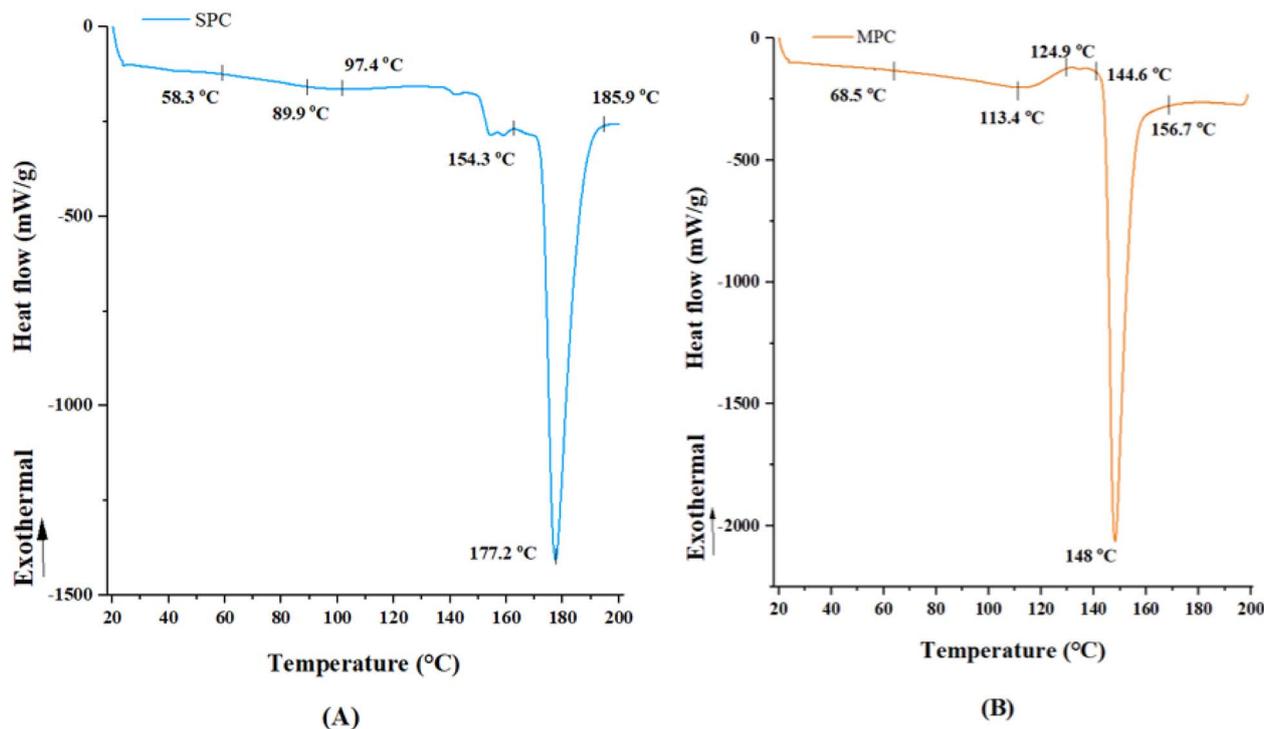


Fig. 6 Thermograms of (A) SPC and (B) MPC obtained from differential scanning calorimetry by heating in the range 20–200 °C at 5 °C min<sup>-1</sup>.

6.1 J g<sup>-1</sup>. A second, sharper peak was observed from 154.3 °C (onset) to 185.9 °C (end-set), with a  $T_{\text{peak}}$  of 177.2 °C and  $\Delta H$  of 114.7 J g<sup>-1</sup>. This dual-stage behavior was found in the studies of Ramírez-Rodríguez *et al.*<sup>52</sup> and Chronakis,<sup>10</sup> who reported dual peaks at 89 °C and 173 °C, and 67 °C and 109 °C, respectively, in SPC, indicating the presence of proteins with varying molecular weights and structural properties that contribute to different levels of thermal resistance. The thermal stability of proteins is influenced by the nature of bonding, purity, extraction method, and pH.<sup>10,52</sup> The purity of the *Spirulina* protein extracted in the current study showed higher purity (81.5% dw) than the previously reported (76.1% (dw) and 78.6% (dw)).<sup>10,52</sup> Ramírez-Rodríguez *et al.*<sup>52</sup> reported that SPC produced by alkaline solubilization, and isoelectric precipitation had molecular weights ranging from 12 to 100 kDa. Additionally, phycocyanin, a phycobiliprotein in *Spirulina*, was found to have molecular weights of 17 kDa and 21 kDa. These variations in molecular weight help explain the multiple decomposition (denaturation) peaks observed in the thermograms of the current study (Fig. 6). In the thermograph of MPC (Fig. 6B), containing casein and whey proteins at a ratio of 80:20, two endothermic peaks were observed within the 20–200 °C range. The first peak occurred between 69 °C (onset) and 125 °C (end-set), with a  $T_{\text{peak}}$  of 113.4 °C and an enthalpy change ( $\Delta H$ ) of 26.4 J g<sup>-1</sup>. This peak could be attributed with the unfolding of protein, protein aggregation and moisture lost from the sample.<sup>53</sup> A previous study reported that MPC with the same protein content (85%) began to undergo protein denaturation at a lower temperature (63 °C) to that observed in the current study.<sup>53</sup> The second peak appeared from 145 °C (onset) to 157 °C (end-set), with a  $T_{\text{peak}}$  of 148 °C and  $\Delta H$  of 146.4 J g<sup>-1</sup> that may occur with the

denaturation of protein and sample decomposition.<sup>53,54</sup> Ptiček Siročić *et al.*<sup>54</sup> determined the thermal behavior of casein by scanning between 25 and 600 °C at 10 °C min<sup>-1</sup> and observed transitions around 40–150 °C for the first stage and 150–250 °C for the second stage. This revealed that different casein fractions exhibit different thermal stabilities. MPC has a heterogeneous structure due to the presence of caseins and non-casein proteins. The casein component is composed of  $\alpha 1$ ,  $\alpha 2$ ,  $\beta$ , and  $\kappa$ -casein, which extend the thermal stability up to 150–250 °C.<sup>54</sup> In contrast, whey proteins, including  $\beta$ -lactoglobulin ( $T_d$ : 88.5 °C),  $\alpha$ -lactalbumin ( $T_d$ : 56.7 °C), and immunoglobulins ( $T_d$ : 73.3 °C), have lower thermal stability.<sup>50,54</sup> Therefore, the thermal stability of MPC depends on the casein-to-whey ratio.<sup>53</sup> In the current study, the  $\Delta H$  and  $T_{\text{peak}}$  values of the first peak indicate that SPC (Fig. 6A) has comparatively lower thermal stability than MPC (Fig. 6B).

DSC analyses of milk formulations were performed to examine thermal transitions resulting from the physicochemical interactions of the milk components during heating. A pronounced endothermic peak was observed in all milk formulations between 125 and 161 °C along with distinct thermal transitions occurring at different temperatures across the five formulations (Table 4). The endothermic peak could be attributed due to dehydration<sup>55–57</sup> and interaction between lactose and amino group of proteins.<sup>57</sup> Maillard browning is known to occur during heating of protein–lactose mixtures.<sup>58,59</sup> It is initiated by a reaction between reducing sugar (lactose) and amino acid (lysine) leading to the formation of various compounds that influence the color, flavor, and texture of the food. The DSC analysis of the Maillard reaction has not been extensively studied in recent years. A study revealed the reaction



between glucose and lysine is characterized by an endothermic peak between 100 and 150 °C, followed by a second endothermic peak at 200–260 °C upon further heating.<sup>60</sup> Maillard reaction is influenced by factors like nature of the heat treatment, pH, salts (metal ions), water activity, sugar ratio, amino acid type and emulsion structure.<sup>61</sup> The HMFs in this study form complex systems with various milk components, making it challenging to fully understand their thermal behavior. The thermal transitions of lactose are known to occur at different temperatures according to its isomeric properties. For example, DSC analysis of 96% w/w  $\alpha$ -lactose monohydrate powder revealed two endothermic peaks at 146.9 °C and 219.8 °C, corresponding to water loss from  $\alpha$ -lactose and the melting point, respectively.<sup>55</sup> The reduction in water content lowers the water activity in milk formulations, creating ideal conditions for the Maillard reaction.<sup>62</sup> Physical treatments such as high pressure applied to milk can influence the protein–protein and protein–lactose interactions.<sup>40</sup> In particular, the application of high pressure (62 MPa) during the formulation of HMFs may have affected the structural changes in proteins, which, in turn, impacted lactose solubility. In SPC based milk formulations, higher energy was required for the dehydration due to the resistance of water molecules bound to the milk system with high water-binding capacity of SPC compared to MPC. Additionally, SPC increased the viscosity in HMFs, thereby restricting water mobility. This effect explains the observed increase in enthalpy at higher SPC levels (Table 4). The lower thermal stability of SPC compared to MPC (Fig. 6) is a key factor contributing to structural changes in SPC-based milk systems at lower temperatures. This effect was evident in the thermal transitions observed in the DSC analysis of milk formulations, where the onset occurred at lower temperatures as the SPC-to-MPC ratio increased (Table 4).

In the dairy industry, milk undergoes various heat treatments, such as pasteurization, sterilization, and drying, which can alter its physicochemical properties due to changes in molecular structure and interactions among the components, especially at high temperatures (>100 °C).<sup>59</sup> These conditions can cause protein denaturation and increased interactions with fat, lactose, and calcium phosphate. Therefore, understanding heat-induced effects, such as denaturation, gelation, and crystallization of SPC based HMFs, is important for optimizing the process parameters of HMF production.

**Table 4** Thermal properties of milk formulations with different bovine protein and *Spirulina* protein ratios<sup>a</sup>

Sample	$T_{\text{on-set}}$ (°C)	$T_{\text{off-set}}$ (°C)	$T_{\text{peak}}$ (°C)	$\Delta H$ (J g <sup>-1</sup> )
100%MPC	148.0 ± 0.3 <sup>a</sup>	161.0 ± 1.4 <sup>a</sup>	151.0 ± 0.6 <sup>a</sup>	2425 ± 0.7 <sup>a</sup>
25%SPC	145.6 ± 0.1 <sup>b</sup>	160.5 ± 0.1 <sup>b</sup>	149.6 ± 0.5 <sup>b</sup>	2921 ± 4.5 <sup>b</sup>
50%SPC	128.4 ± 7.2 <sup>c</sup>	140.0 ± 8.6 <sup>c</sup>	137.1 ± 0.1 <sup>c</sup>	3011 ± 0.1 <sup>c</sup>
75%SPC	129.7 ± 2.4 <sup>d</sup>	136.2 ± 4.1 <sup>d</sup>	133.5 ± 0.8 <sup>d</sup>	3127 ± 0.3 <sup>d</sup>
100%SPC	125.0 ± 0.1 <sup>e</sup>	140.1 ± 0.8 <sup>e</sup>	130.1 ± 0.2 <sup>e</sup>	3678 ± 0.2 <sup>e</sup>

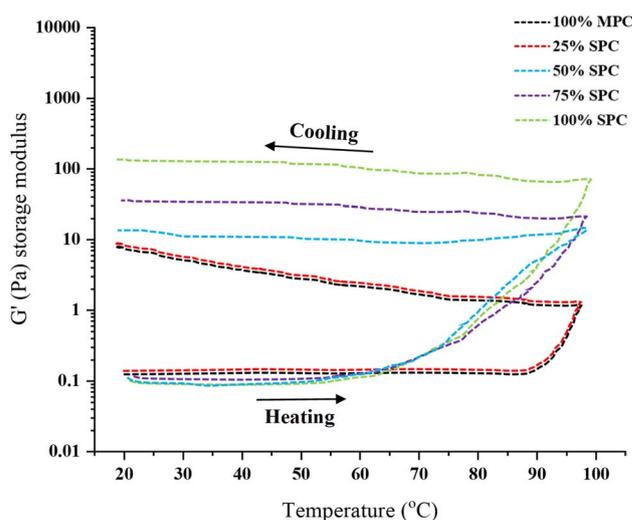
<sup>a</sup> Results are represented as an average of triplicates ± SD. Different letters (superscript) indicate significant differences within a column ( $p < 0.05$ ).

### 3.7. Heat-induced gelation

Understanding the rheology and gel formation of formulated HMFs during heating and cooling is essential for controlling the fluidity and texture of products that may be produced from it (e.g., yogurt, cheese, ice cream). The heat-induced gelation curves (Fig. 7) show that replacing bovine proteins with *Spirulina* protein affects the rheology of the HMFs, as indicated by changes in the storage modulus ( $G'$ ) of the gel network formed during heat-induced gelation.

Heating was followed by cooling to assess the stability of the gel. The lower thermal stability of SPC HMFs, observed in the DSC thermogram, was also reflected in the heat-induced gelation curves, where SPC HMFs showed a rapid increase in  $G'$  at lower temperatures compared to 100% MPC. During heating, both bovine and *Spirulina* proteins in the HMFs unfolded from their native state, forming an intermolecular network with water, sugar, and fat. Gelation occurred earlier in HMFs with higher SPC content (50–100% SPC) compared to those with lower SPC content (25% SPC) and 100% MPC. The gelling points of milk formulations decreased as MPC was increasingly replaced with SPC, with 50% and 100% replacement showing gelling points of 62.5 °C and 56.0 °C, respectively, while 100% MPC had a gelling point of 90.1 °C.

These results show that SPC and MPC have different gelling characteristics, likely due to the differing influence of secondary protein structure, pH (release of hydrogen ions) and mineral salts (release of calcium ions).<sup>34,63,64</sup> This explains why HMFs with higher SPC concentrations (50–100%) gelled at lower temperatures compared to 100% MPC. The findings suggest that SPC could be useful in developing low-temperature gelled products. At the same time, formulations with lower SPC content (up to 25%) retained thermal stability comparable to the 100% MPC control, indicating that functional integrity can be maintained at modest substitution levels. To avoid premature gelation, low temperatures ( $\leq 63$  °C) should be used to



**Fig. 7** Comparison of rheological behavior in milk formulations during heating and cooling.



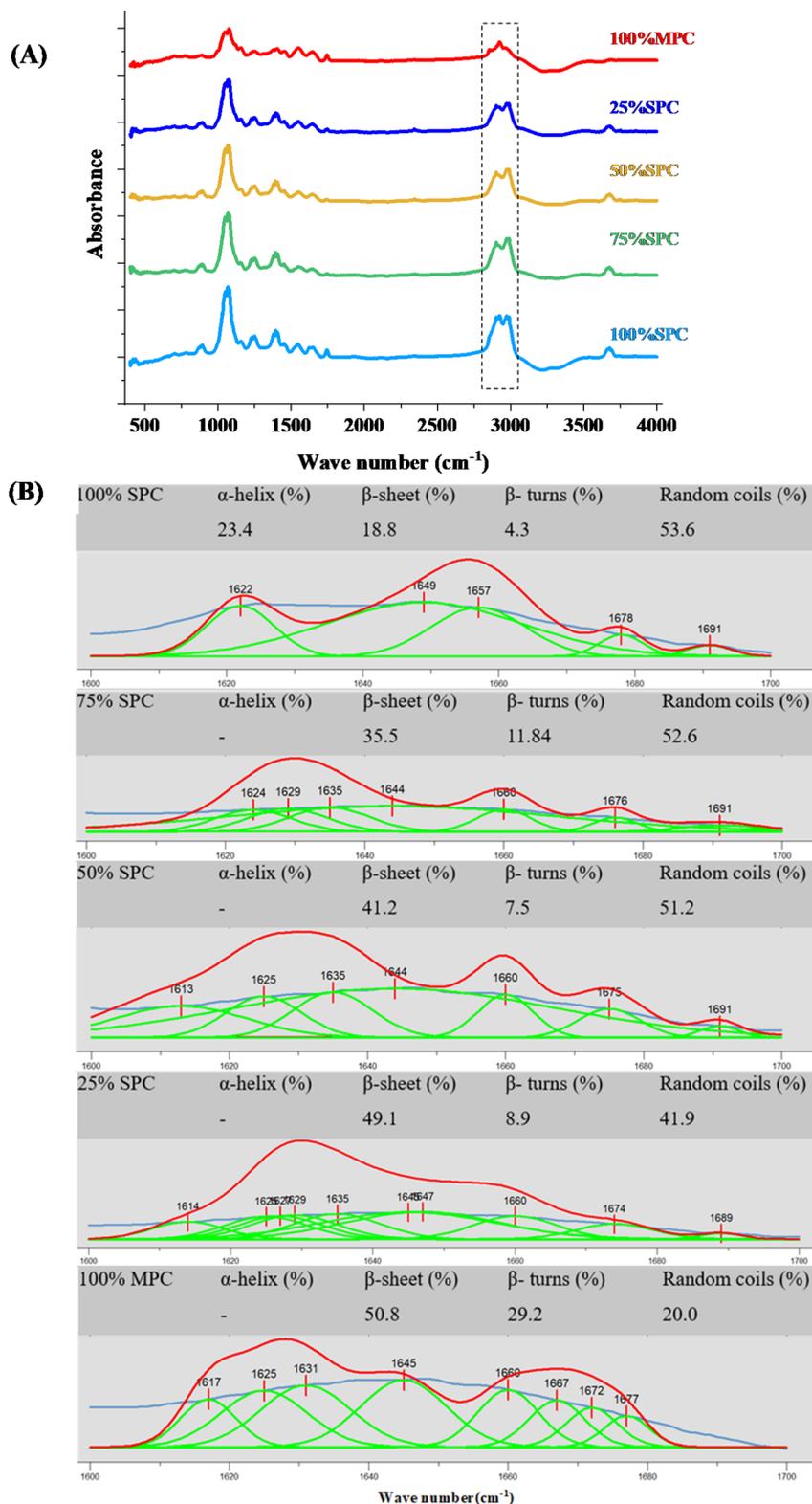


Fig. 8 FTIR analysis of milk formulations; (A) full range spectra of the HMFs and (B) second derivative, deconvolution of secondary structural changes with respective concentrations (%) of  $\alpha$ -helix,  $\beta$ -sheet,  $\beta$ -turns and random coils.

pasteurize HMFs containing high levels of SPC. This implies that utilization of *Spirulina* protein as a dairy mimetic protein requires low temperature to attribute similar textures with

bovine milk in developing yogurt and cheese products. This aligns with industry trends focusing on energy efficiency, reduced processing inputs, and minimal additives. The gelation



properties of SPC-based HMFs make them suitable for producing gelled dairy products, such as cheese and yogurt, with lower energy input and without the need for added calcium ions compared to 100% MPC. Thermal processing of dairy products like drying and pasteurization accounts for physico-chemical changes like Maillard reaction (glycation between lysine and lactose), protein denaturation and aggregation, conversion of soluble calcium and phosphate into colloids, structural changes in fat globules and decrease in pH.<sup>32</sup> Thus, in the dairy industry, heat treatments are carefully designed with these considerations in mind. Similarly, HMFs developed incorporating *Spirulina* protein require thorough evaluation under various thermal processing conditions to optimize their properties. Low-temperature or moderate drying methods, such as freeze drying or vacuum drying, can be preferred to preserve the structural and functional integrity of microalgal proteins. Understanding the thermal behavior of *Spirulina* protein will provide valuable insights for designing optimal processing protocols tailored to the needs of the dairy industry. Most plant protein-based milk products incorporate stabilizers and food hydrocolloids for creating desired texture to address challenges associated with their low thermal stability.<sup>65</sup> *Spirulina* protein-based milk products, as a newer innovation, require further research on the incorporation of hydrocolloids, similar to those in plant protein-based products, to achieve optimal quality and stability.

### 3.8. Spectral properties, molecular interaction, and secondary structure of proteins in HMFs

**3.8.1. Spectral properties and molecular interactions in HMFs.** FTIR spectra (4000–400  $\text{cm}^{-1}$ ) were acquired and analyzed to gain insights on intermolecular interactions in milk formulations (Fig. 8A). All samples showed peaks between 3300 and 3000  $\text{cm}^{-1}$ , corresponding to N–H stretching vibrations of amide A (3310–3270  $\text{cm}^{-1}$ ) and amide B (3100–3030  $\text{cm}^{-1}$ ) bands, associated with the polypeptide backbone of *Spirulina* and bovine proteins.<sup>66</sup> The absorbance intensity in the amide regions was slightly higher in 100% SPC and 100% MPC compared to mixed protein HMFs. Carbohydrate-related peaks were observed around 1200–900  $\text{cm}^{-1}$ , with peaks for milk formulations containing *Spirulina* protein ranging from  $\sim 1056$   $\text{cm}^{-1}$  to 1108  $\text{cm}^{-1}$ . In contrast, the corresponding peaks for 100% MPC appeared at a higher range, from  $\sim 1079$   $\text{cm}^{-1}$  to 1159  $\text{cm}^{-1}$ . The absorption intensity of these peaks was higher in SPC-containing milk formulations than in 100% MPC (plotted area in Fig. 8A), suggesting increased intermolecular hydrogen bonding with the incorporation of SPC.

Zhao *et al.*<sup>67</sup> identified FTIR spectroscopic peaks in bovine milk at 1159  $\text{cm}^{-1}$  and 1076  $\text{cm}^{-1}$ , corresponding to the C–O–C stretching of the 1  $\rightarrow$  4 glycosidic bond and the C–OH bending of lactose, respectively. Peaks in the 1200–1000  $\text{cm}^{-1}$  range are linked to C–N bonds formed due to Maillard reactions between lysine and lactose.<sup>19,67</sup> Similar spectral features were observed in our milk formulations, with peaks at 1079  $\text{cm}^{-1}$  and 1078  $\text{cm}^{-1}$  attributed to MPC and SPC, indicating that these proteins are phosphate-containing. The peak at 1075  $\text{cm}^{-1}$  has been

ascribed to phosphate groups in casein, consistent with previous findings.<sup>68,69</sup>

**3.8.2. Secondary structure.** The amide I region of the FTIR spectrum (1700–1600  $\text{cm}^{-1}$ , corresponding to C=O stretching vibrations) was used to assess changes in the secondary structure of proteins in HMFs (Fig. 8). Major secondary structures ( $\alpha$ -helix,  $\beta$ -sheet,  $\beta$ -turns, and random coils) were determined through second derivative and deconvolution analysis of the spectra. Microfluidization is known to induce partial protein unfolding, promoting intermolecular aggregation and rearrangement, which affects viscoelastic properties.<sup>70–72</sup> These aggregates may form *via* non-covalent hydrophobic interactions and covalent disulfide bonds.<sup>51</sup> A decrease in  $\alpha$ -helices and an increase in random coils are associated with higher surface hydrophobicity.<sup>9</sup> Among the five milk formulations, the  $\alpha$ -helix structure was detected only in 100% SPC at 1657  $\text{cm}^{-1}$ . The varying amounts of random coils help explain the differences in solubility among the HMFs. As MPC was gradually replaced with SPC, the total  $\beta$ -sheet content decreased while the random coil content increased (Fig. 8). The higher proportion of random coils observed in 100% SPC HMFs could be due to a greater degree of partial unfolding of SPC compared to MPC during microfluidization. These random coils likely contributed to the aggregation of SPC, leading to increased viscosity in SPC-containing HMFs compared to 100% MPC. The  $\beta$ -sheet peaks around 1691  $\text{cm}^{-1}$  or 1689  $\text{cm}^{-1}$  were observed only in HMFs containing SPC.

## 4 Conclusion

This study explored the potential of formulating a hybrid milk using *Spirulina* protein concentrate (SPC) and milk protein concentrate (MPC). The SPC content in the formulation was progressively increased to identify an optimal balance. The results indicated that SPC significantly influenced the physicochemical and functional properties of the hybrid milk, with its impact intensifying at higher concentrations. The purity of SPC was identified as a critical factor, as residual non-protein components, including native polysaccharides and pigments, affected milk functionality. Specifically, polysaccharides contributed to viscosity and water-binding capacity, while pigments influenced color under varying pH conditions. The hybrid milk formulation containing 25% SPC and 75% MPC showed promising texture and flavor. Additionally, SPC exhibited performance comparable to MPC in terms of solubility at neutral pH (6.0–7.0) and emulsifying properties. To enhance the purity of SPC, further refinement through filtration or sequential extraction is recommended, as these methods are feasible for industrial applications. For large-scale processing of SPC-containing milk, thermal treatments should not exceed 63 °C for 30 minutes (or equivalent) to prevent heat-induced gelation.

## Ethics statement

The authors confirm that this manuscript does not include any studies involving human or animal subjects.



## Data availability

The manuscript includes all essential data supporting its findings, statements, and conclusions. Spreadsheet data can be provided upon request. Any relevant data sourced from the literature have been properly cited and acknowledged where applicable.

## Author contributions

Jayani Samarathunga: conceptualization, writing—original draft. Thi Phuong Linh Le: writing, reviewing and editing. Max Gabard: supervision. Katrina Strazdins: supervision and reviewing. Jeroen Rens: conceptualization and supervision. Benu Adhikari: conceptualization, supervision, reviewing and editing.

## Conflicts of interest

The authors have no conflict of interest to declare.

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