Commercial manufacturing of dairy products involves the addition of dairy ingredients (such as nonfat dry milk and milk protein concentrates), as well as non-dairy additives (such as gums, stabilizers, emulsifiers, and texture modifiers) to get the best product appearance, maintain the product quality, and extend shelf life. Though these nondairy additives are not harmful, consumers do not prefer them in dairy food formulations. Therefore, the dairy industry is working on improving the inherent functionality of dairy ingredients using different processes. Recently, fibrillation emerged as a new technique to convert globular proteins such as whey proteins into fibrils, which provide enhanced viscosity, foaming, and emulsification capacity. Therefore, skim milk was subjected to microfiltration followed by ultrafiltration of microfiltration permeate to fractionate whey proteins. Then, whey proteins were selectively fibrillated and mixed back with other streams of microfiltration and ultrafiltration to get fibrillated skim milk. Fibrillated skim milk was spray-dried to get fibrillated nonfat dry milk (NDM). Visible whey protein fibrils were observed in reconstituted fibrillated NDM, which showed survival of fibrils in fibrillated NDM. Fibrillated NDM showed significantly higher viscosity than control NDM. Fibrillated NDM also showed higher emulsification capacity, foaming capacity, and stability than the control NDM but lower gel strength. Considering the improved functionality of fibrillated NDM, they can be used in product formulations such as ice cream mix, where the thickening of a solution, good emulsification, and foaming properties are required.

Key words: whey protein fibrils, functional modification, ingredient functionality

Milk is an important source of nutrients, including EAA, fatty acids, and minerals. Milk is converted into a variety of dairy products, such as yogurt, ice cream, and cheese, which increases milk consumption and overcomes the limitation of lower shelf life of milk. However, these products must have desirable properties for customer acceptance, such as yogurt with a thick gel and no syneresis (Peng et al., 2009), drier ice cream with optimum meltdown (Yan et al., 2021), and cheese and cheese spread without excessive oiling off (Kindsstedt and Rippe, 1990). Therefore, different additives such as texture modifiers, emulsifiers, stabilizers, and gelling agents are added to the product formulation at a commercial scale to meet customer acceptance and retain product quality throughout the supply chain (Baba et al., 2018; Khubber et al., 2021). The additives used in the formulations are generally recognized as safe for human consumption. However, the demand for natural and clean label food formulations continues to grow and necessitates the omission of unfamiliar additives (Maruyama et al., 2021). In milk solids, milk proteins have inherent functional properties that can be used to meet consumer demand.

Regarding the inherent functionality of milk proteins, milk proteins can provide gelation by forming a 3-dimensional network (Donato et al., 2007) and interfacial properties such as emulsification and foaming properties by absorbing at the oil-water and air-water interface, respectively. Therefore, manufacturers are adding additional protein to the formulation using protein-rich ingredients such as milk protein concentrate (MPC), NDM, and micellar casein concentrate. However, milk proteins are costly ingredients in milk product formulation; therefore, the addition of milk protein ingredients will increase the overall cost of manufacturing. Further, the additional TS from proteins will also change the physical properties of the product, which might create difficulties in processing the product. Therefore, processing interventions are needed to enhance functionality with similar protein content. Recently, fibril-
ulation emerged as a new technique to modify globular proteins such as whey proteins into fibrils. The fibrillar structure of a protein is known to provide more enhanced functional properties, such as gelling, foaming (Oboroceanu et al., 2014), emulsification (Mantovani et al., 2018), and viscosity (Loveday et al., 2012), relative to native globular proteins (Loveday et al., 2017). Recently, Meng et al. (2022) reviewed the benefits of food fibrils and their potential use as color, flavor, and iron carriers with improved sensory attributes by masking off-flavors. However, the underlying mechanism is not fully understood and requires further research (Yue et al., 2022).

Recently, Rathod and Amamcharla (2021) reported a process to manufacture fibrillated MPC by selective conversion of globular whey proteins into fibrils, which showed improved functional properties over the control MPC. Nonfat dry milk is a widely used ingredient in milk product formulation (Sharma et al., 2012). Therefore, improving the functionality of NDM can help to reduce the use of nondairy ingredients. So, the current study was focused on improving the functionality of NDM using selective fibrillation of whey proteins. Hence, skim milk was subjected to microfiltration and UF to fractionate whey proteins. Then, fractionated whey proteins were subjected to fibrillation followed by mixing back with other membrane-fractionated constituents to get fibrillated skim milk. This fibrillated skim milk was spray-dried to get fibrillated NDM. Then, the fibrillated NDM was checked for the survival of fibrils and other functional properties, such as thickening, gelling, emulsification, and foaming after reconstitution.

**MATERIALS AND METHODS**

No human or animal subjects were used, so this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board.

**Experimental Approach**

Pasteurized skim milk was subjected to microfiltration (MF) to fractionate casein in MF retentate and whey proteins in MF permeate. Subsequently, MF permeate was subjected to UF to further increase the whey protein concentration in UF retentate. The UF retentate was subjected to a fibrillation process to convert whey proteins into fibrils and mixed with casein-rich MF retentate and UF permeate, keeping the ratio of all the constituents similar to skim milk and termed as fibrillated skim milk. Fibrillated skim milk was spray-dried to obtain fibrillated NDM and characterized for their functional properties. The whole experiment was replicated with 3 independent lots of skim milk.

**Preparation of Control and Fibrillated NDM**

Control and fibrillated NDM were prepared as shown in Figure 1. Briefly, pasteurized skim milk was collected from the Davis Dairy Plant, South Dakota State University (Brookings, SD). Pasteurized skim milk was warmed to 23.3°C and subjected to MF (concentration factor-3×) to get casein-rich MF retentate (expected whey protein removal 60–70%) and whey proteins in UF permeate. Microfiltration was performed using 0.1 µm ceramic membranes (EP1940, Membralox GP, Pall Corporation, Port Washington, NY). Microfiltration was performed at 23.3°C using a transmembrane pressure of 86.2 kPa (34.5 kPa inlet and 103.4 kPa differential). Microfiltration retentate was cooled, divided into 2 parts, and kept overnight in cold storage (4°C). Microfiltration permeate was subjected to UF (concentration factor 4.5–5×) to fractionate and concentrate whey proteins in UF retentate and nonprotein components in UF permeate. Ultrafiltration was performed using two 10 kDa polyether sulfone spiral wound membranes (SD3838-BS, Domnick Hunter Filtration Division–North America, Parker Hannifin Corporation, Oxnard, CA) arranged in parallel. Ultrafiltration was performed at 23.3°C using a 276 kPa transmembrane pressure (207 kPa inlet and 138 kPa differential). Ultrafiltration retentate was diluted to 2% protein using UF permeate and divided into 2 parts. The remaining UF permeate was also divided into 2 parts and kept in cold storage (4°C). The first half was used as a control and stored in cold storage (4°C) without giving any further treatment and termed as control UF retentate. Another half was acidified to pH 2.0 using 6 N HCl and heated to 80°C for 14 h under constant stirring, followed by immediate cooling to 4°C using an ice bath to get fibrillated UF retentate. Fibrillated UF retentate was neutralized to pH 6.7 using 3 N NaOH and stored in cold storage. The MF retentate, control UF retentate, and UF permeate were mixed, keeping the mixture composition similar to skim milk and termed control skim milk. Similar to control skim milk, the remaining part of MF retentate, fibrillated UF retentate, and the remaining part of UF permeate were mixed and termed fibrillated skim milk. Control and fibrillated skim milk were dried using a 2-stage spray-drier with an external vibrating fluidized bed and termed control NDM and fibrillated NDM, respectively.
**Proximate Analysis**

Total solids were measured by gravimetric method and protein content was measured by micro-Kjeldahl analysis using the standard methods as described by Hooi et al. (2004).

**Confirmation of Fibril Formation at Different Stages of Production of Control and Fibrillated NDM**

Representative samples were taken at each manufacturing stage (stage 1, UF retentate; stage 2, fibrillated UF retentate at pH 2; stage 3, fibrillated UF retentate at pH 6.7; stage 4, fibrillated skim milk; and stage 5, fibrillated NDM powder) as shown in Figure 1 to confirm fibril formation and survival. The fibrillated NDM sample was reconstituted to 10% (wt/wt) TS in distilled water (45°C) with constant stirring for 30 min using an overhead stirrer (Caframo, Ontario, Canada) at 500 rpm, followed by overnight storage in the refrigerator (4°C) to ensure complete rehydration. A reconstituted fibrillated NDM sample was ultracentrifuged (90,000 × g) for 1 h at 20°C and the supernatant was collected. The presence of fibrils was confirmed by thioflavin T fluorescence (ThT) value and transmission electron microscopy (TEM), as suggested by Rathod and Amamcharla (2021). Tricine–SDS-PAGE was used to determine the conversion of whey proteins into fibrils. The viscosity of control and fibrillated skim milk and reconstituted NDM (10% TS) were checked by the method suggested by Rathod and Amamcharla (2021).

**Tricine–SDS-PAGE**

Samples collected at different stages (Figure 1) were diluted to 0.5% protein content using distilled water and vortexed. Diluted samples were mixed with Tris-Tricine sample buffer in a 1:2 ratio and vortexed. The mixture was incubated at 37°C for 15 min. Then, a 15-µL mixture was loaded in the 16.5% precast Tris-Tricine gel (Bio-Rad Laboratories, Hercules, CA) fitted into an electrophoresis assembly (Bio-Rad Mini Gel system) filled with 1× Tris-Tricine sample buffer (Bio-Rad Laboratories) prepared from 10× Tris-Tricine buffer by mixing sample buffer and distilled water in a 1:9 ratio. The gel was run at 20 mA for 15 min for stacking, then adjusted to 25 mA for 90 min (Schägger, 2006). When the dye reached the bottom line, the gels were removed from the casting tray. The protein bands in the gel were fixed in 10% trichloroacetic acid solution for 1 h. The gel was removed and dipped into a tray filled with staining solution (Coomassie brilliant blue
Acid Gelation

The acid gelation study was conducted by the method suggested by Rathod et al. (2022). Briefly, both reconstituted NDM dispersions (10% wt/wt TS) were heated to 90°C for 10 min, then immediately cooled to 4°C, and kept at refrigerated temperature (4°C) overnight before further analysis.

Acid-Base Titration Curve

Acid-base titration profiles of reconstituted control and fibrillated NDM (10% wt/wt TS) were carried out using the method described by Rathod et al. (2022). Briefly, both control and fibrillated NDM were reconstituted at 10% (wt/wt TS). The following day, samples were tempered to 30°C using a temperature-controlled water bath and used for analysis. Samples were titrated from their initial pH of 6.7 to pH 3.0 by adding 0.2 mL of 0.5 N HCl in increments under constant stirring and constant temperature of 30°C using a temperature-controlled water bath (Cole Parmer, Vernon Hills, IL) attached with a magnetic stirrer. The pH of the sample was recorded using the Accumet AP110 Portable pH Meter (Fisher Scientific, Waltham, MA) after every 0.2 mL incremental addition of HCl. Once the pH of the sample reached 3.0, the same sample was back titrated to pH 7.0 by 0.2 mL incremental addition of 0.5 N NaOH under constant stirring. The pH was also recorded after every addition of 0.2 mL sodium hydroxide.

Acid Gel Rheology

The reconstituted NDM dispersions were tempered to 30°C using a temperature-controlled water bath. Then, glucono-δ-lactone (GDL; Fisher Scientific) was added to reconstituted NDM (2 g per 100 mL) and mixed using a magnetic stirrer for 2 min. Immediately, a part of the GDL-mixed reconstituted NDM was transferred to a temperature-controlled water bath (Cole Parmer) maintained at 30°C for continuous pH measurement using Accumet AP110 Portable pH Meter (Fisher Scientific). Simultaneously, the remaining GDL-mixed reconstituted NDM was used to monitor rheological characteristics during acid gelation using a stress-strain-controlled rheometer (MCR-92, Anton Paar) with a cup and bob geometry consisting of a coaxial cylinder (bob length: 60 mm; bob diameter: 38.7 mm; and cup diameter: 42 mm). The cup and bob geometry was preheated to 30°C. During acid gelation, the sample oscillated at a constant frequency of 1 Hz with an applied strain of 0.5%, which caused minimal disruption of acid gels during the development of the gel network (Peng et al., 2009). Measurements were taken every 5 min until the pH reached 4.6.

Acid Gel Microstructure

Reconstituted NDM (30 mL) was transferred to a beaker and tempered to 30°C. Then, the pre-estimated quantity of GDL was added to reconstituted NDM, which was enough to reach the final acid gel pH of 4.6. The mixture was stirred using a magnetic stirrer for 3 min to ensure complete mixing of GDL. Then, 45 µL of the sample-GDL mixture was transferred on the glass slide having a silicon spacer (0.5-mm height and 9-mm diameter) and covered with a coverslip. Then, the glass slide with the sample-GDL mixture and the leftover sample with GDL in the beaker was incubated at 30°C for 4 h, then kept in the refrigerator overnight. The next day, the pH of the acid gel formed in the beaker was measured for confirmation of a pH drop to 4.6. The coverslip on the glass slide was gently removed, and the acid gel on the slide was stained with 10 µL of Fast Green FCF (5 mg of dye in 5 mL of water; Sigma-Aldrich, St. Louis, MO) for 5 to 10 min in darkness and covered again with the coverslip. The stained gels were viewed under a confocal laser scanning microscope (LSM 5 Pascal, version 3.2 SP2, Zeiss, Thornwood, NY). A Plan-Neofluar 10×/0.3 and Plan-Neofluar 40×/1.3 Oil DIC objective were used for image acquisition. A 543-nm Helium-Neon laser line and a primary dichroic Haupt-Farb-Teiler (HFT; a dichroic beam splitter) 543 to excite Fast Green FCF. The emission signal was collected with channel 2 using a secondary
dichroic NFT 635 and a long pass (LP) 650-nm filter to detect Fast Green (green; Gandhi et al., 2017). Images were analyzed with ImageJ software (version 1.53f, ImageJ, National Institutes of Health, Bethesda, MD) with a color pixel counter-plugin to measure the green color area.

**Surface Tension and Interfacial Tension**

The surface tension and interfacial tension of reconstituted control and fibrillated NDM (10% wt/wt TS) were measured using an automatic surface tensiometer (model BZY102, Anhui, China) as per the manufacturer’s protocol (Yusoff et al., 2021). Vegetable oil (Great Value, Walmart, Bentonville, AR) was used as an oil layer for checking interfacial tension.

**Emulsification Capacity**

Emulsification capacity was measured as suggested by the Zaitoun et al. (2022) method. Briefly, reconstituted NDM (10% wt/wt total solids) samples were diluted to 0.05% protein. Then, 120 mL of diluted reconstituted NDM sample was taken in a 600-mL beaker at room temperature. The handheld homogenizer (CAT Scientific X120, PolyScience, Niles, IL) and conductivity meter electrode (Accumet AP75, Fisher Scientific) were placed in the beaker containing the diluted sample. Vegetable oil (Great Value, Walmart, Bentonville, AR) was filled in a 125-mL separatory funnel and placed just above the beaker. Then, the vegetable oil addition was started by opening the 125-mL separatory funnel valve fully open with continuous blending. Oil addition was stopped when the emulsion breakpoint was detected. The emulsion breakpoint was marked when conductivity became zero due to an increase in electric resistance upon emulsion collapse. Emulsification capacity was expressed as the total grams of oil emulsified per milligram of soluble protein (Acton and Saffle, 1972).

**Foaming Capacity and Foam Stability**

Foam capacity and foam stability were measured according to the method described by Sinha et al. (2007). Briefly, reconstituted NDM (10% wt/wt TS) samples were diluted to 3% (wt/wt) protein with distilled water. Then, 100 mL of diluted reconstituted NDM sample was taken into a 400-mL beaker. The diluted reconstituted NDM sample was whipped for 3 min at high speed using a handheld homogenizer (CAT Scientific X120, PolyScience). The foam was poured immediately into a 250-mL measuring cylinder, and the total volume of the foam was measured immediately after 30 s. The foam stability was determined by measuring the fall in the volume of the foam after 30 min. All the experiments were performed in triplicate, and the results are the average of 3 values.

**Heat Coagulation Time**

Heat coagulation time was performed by the method suggested by Rathod and Amamcharla (2021). Briefly, reconstituted NDM samples (10% wt/wt TS) were adjusted to pH 6.9 and kept overnight in a refrigerator. Then, 2 mL of reconstituted NDM sample was filled in heat-resistant screw cap test glass vials (8 mL, 17-mm diameter × 63-mm height; DWK Life Science, Millville, NJ). The glass vials were inserted into a stainless-steel rack. The rack, along with the glass vials, was immered in a mineral oil bath (Narang Scientific Works Pvt. Ltd, New Delhi, India) maintained at 140°C and placed on the rocker. Heat coagulation time was noted as the time in seconds elapsed between dipping the glass vial with reconstituted NDM samples in the hot oil bath and the onset of visible clots or flakes in the glass vial.

**Protein Oxidation**

Protein oxidation was analyzed according to the method given by Scheidegger et al. (2010) and Keppler et al. (2019). Briefly, reconstituted NDM (10% wt/wt TS) samples were diluted to 0.1% protein content using distilled water followed by vortex mixing. Protein oxidation was measured in terms of decay in 2 AA, namely, tryptophan and tyrosine, and their respective oxidation products are N-formylkynurenine and dityrosine. Diluted reconstituted NDM samples were analyzed in quartz cuvette using a spectrofluorometer (LS-55, Perkin Elmer, Waltham, MA) using right-angle assembly for tryptophan (excitation and emission wavelength 294 and 340 nm, respectively) and L-tyrosine (excitation and emission wavelength 274 and 310 nm, respectively) using 1% attenuation filter with 10-nm excitation slit width and 20-nm emission slit width. Similarly, oxidation products N-formylkynurenine (excitation and emission wavelength 325 and 435 nm, respectively), and dityrosine (excitation and emission wavelength 284 and 415 nm, respectively) were also measured. The fluorescence intensity was recorded to compare the concentration of a specific component in control and fibrillated NDM.

**Statistical Analysis**

Statistical analysis was performed using Excel (Microsoft Corp., Redmond, WA). One-way ANOVA and Tukey’s multiple range test were done using SAS Version 9.4 (SAS Institute Inc.).
RESULTS AND DISCUSSION

Confirmation of Fibril Formation and Viability During the Manufacture of Fibrillated NDM and Comparison with Control NDM

Fibrillated NDM was produced as per the process shown in Figure 1. Fibril formation was confirmed at all 5 stages of production using the ThT value, transmission electron microscopy, and Tricine-SDS-PAGE. Table 1 shows that ThT values of fibrillated UF retentate (both at pH 2 and 6.7) were significantly \((P < 0.05)\) higher than the control UF retentate. Similarly, fibrillated skim milk showed a significantly \((P < 0.05)\) higher ThT value than control skim milk. When both NDM was reconstituted, fibrillated NDM also showed a significantly \((P < 0.05)\) higher ThT value than control NDM (Table 1). The ThT value is an indirect indicator of fibril formation (Pan and Zhong, 2015); therefore, a higher ThT value indicates the presence of fibrils in fibrillated UF retentate, fibrillated skim milk, and fibrillated NDM. Several studies reported an increase in ThT values after the conversion of globular proteins into fibrillar proteins (Koudelka et al., 2012; Mantovani et al., 2016). For further confirmation of the presence of fibrils, samples were viewed using TEM (Figure 2). Visible fibrils were observed in TEM images of fibrillated UF retentate at pH 2 and pH 6.7. Similar TEM images of milk whey protein isolate fibrils were seen by Rathod and Amamcharla (2021) and other researchers for whey proteins fibrils (Loveday et al., 2012) and β-LG fibrils (Dave et al., 2013). Fibrillated skim milk and fibrillated NDM did not show visible fibrils similar to fibrillated UF retentate, which could be due to the small proportion of fibrillated whey proteins (~0.8%) in fibrillated skim milk and fibrillated NDM. Therefore, reconstituted fibrillated NDM was ultracentrifuged (90,000 \(\times\) g at 20°C for 1 h) to remove casein from the solution in form of a pellet, and the supernatant was viewed using TEM. Visible fibrils were seen in the TEM images of the supernatant of fibrillated NDM (Figure 2E), which confirmed the presence and survival of whey protein fibrils after spray-drying. However, the fibrils were short, fragmentated and aggregated compared with straight long fibrils (Figure 2A) at production. Tricine-SDS-PAGE was used as another method for confirmation of fibrillation and gels images are shown in Figure 3. Here, all the fibrillated samples showed the presence of small molecular weight (2–8 kDa) peptides, which is also an indicator of fibril formation (Akkermans et al., 2008b; Rathod and Amamcharla, 2021). During the fibrillation process, the protein solution is heated at low pH, leading to the hydrolysis of proteins to peptides, which subsequently self-assembled into an ordered fibrillar form. Due to SDS, a dissociating agent, whey protein fibrils were dissociated into peptides with different molecular weights (2–8 kDa), which can be seen in the gel image (Figure 3). Further, fibrillated skim milk showed significantly \((P < 0.05)\) higher viscosity compared with control skim milk (Table 1), and reconstituted fibrillated NDM also showed significantly \((P < 0.05)\) higher viscosity compared with reconstituted control NDM. A similar increase in viscosity due to fibril formation was reported by Rathod and Amamcharla (2021) for fibrillated model MPC. These results suggest that fibrillated samples have a presence of whey protein fibrils and whey protein fibrils survived the spray-drying condition as the presence of whey protein fibrils was confirmed in reconstituted fibrillated NDM.

Comparison of Functional Properties of Control and Fibrillated NDM: Rheology

The rheology of reconstituted control and fibrillated NDM (10%, 15%, and 20% TS) is shown in Table 2. Fibrillated NDM showed significantly \((P < 0.05)\) higher viscosity than the control NDM (Table 2). At 10% TS, the difference in viscosity between reconstituted control and fibrillated NDM was smaller. The difference in viscosity was increased with an increase in TS. Similarly, the consistency coefficient was also significantly \((P < 0.05)\) higher for reconstituted fibrillated NDM, and the difference in consistency coefficient was also increased with an increase in TS. However, the flow behavior index of reconstituted fibrillated NDM was significantly \((P < 0.05)\) lower than reconstituted control NDM. At 10% TS, both NDM showed shear-thickening behavior. However, with an increase in the TS, the solution turned shear thinning. Similarly, an increase in viscosity and consistency coefficient was seen for fibrillated model MPC by Rathod and Amamcharla (2021). Also, they have seen the shear-thinning behavior of both fibrillated and control model MPC. They reported that whey protein fibrils form a fibrillar network, which increases overall viscosity and consistency coefficient; therefore, an increase in viscosity and consistency coefficient was seen in the reconstituted fibrillated NDM samples, as compared with reconstituted control NDM. An increase in TS further increases the number of whey protein fibrils and other constituents, which cumulatively resulted in viscosity and consistency coefficient. Due to applied shear, fibrillar network breaks and fibrils align to the direction of shear forces, which resulted in the shear-thinning behavior of fibrillated samples (Akkermans et al., 2008a; Mantovani et al., 2018). However, the reason for shear-thickening behavior at lower TS still needs further investigation.
Acid-Base Titration

Both reconstituted control and fibrillated NDM (10% wt/wt TS) were analyzed for acid-base titrations. Reconstituted fibrillated NDM uses a slightly higher amount of acid (6.8 mL) to bring down pH to 3.0 than reconstituted control NDM (6.6 mL). Similarly, reconstituted fibrillated NDM used a slightly higher amount of base (7.6 mL) to neutralize to pH 7 than reconstituted control NDM (7.2 mL). This observation is similar to our earlier study on fibrillated model MPC, where fibrillated milk whey proteins and fibrillated model MPC used a higher amount of acid to bring down pH to 3 and higher alkali to neutralize pH to 7, Rathod et al. (2022). Fibrillation-mediated rearrangement in functional groups of whey proteins and overall charge distribution could be the possible reason for the requirement of higher acid and alkali requirements for fibrillated samples showing higher buffering capacity of fibrillated samples, which is well discussed by Rathod et al. (2022).

Acid Gelation: Acid Gel Rheology

The acid gelation behavior of both reconstituted NDM is shown in Figure 4 and Table 3. The gelation pH of reconstituted control NDM was 5.19 ± 0.06, which was higher than the reconstituted fibrillated NDM (5.12 ± 0.01). Once the gelation started, reconstituted control NDM showed higher storage modulus (G') than the reconstituted fibrillated NDM throughout the whole gelation period. Both reconstituted control and fibrillated NDM show continuous increase until gelation pH reached 4.6. Further, G' at pH 4.6 for reconstituted control NDM was 345 ± 76 Pa, which was significantly (P < 0.05) higher than the reconstituted fibrillated NDM (237 ± 8 Pa). These results are completely inverse of the results of our earlier studies on fibrillated model MPC. Some possible reasons exists for this change in gelation behavior. Compared with fibrillated MPC, fibrillated NDM has a lower amount of fibrils in TS. In addition, fibrillated NDM has a higher amount of lactose and minerals in its composition, compared with fibrillated MPC, which might interact with fibrils during preheating (90°C for 10 min) and make these fibrils unavailable to interact with caseins and form a gel network, leading to poor gelation, compared with control NDM where unmodified whey proteins can still interact with casein and take part in gelation (Gunasekaran and Solar, 2012). Another possible reason could be spray-drying-induced changes and reduction in solubility of fibrillated NDM compared with control NDM (Loveday et al., 2012; Farrokhi et al., 2018; Zouari et al., 2020). Therefore, a detailed investigation of the interaction of

Table 1. Thioflavin T fluorescence value of control UF retentate, fibrillated UF retentate (at pH 2 and pH 6.7), control and fibrillated skim milk and NDM, and rheology of control and fibrillated skim milk and NDM

<table>
<thead>
<tr>
<th>Stage</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control UF retentate</td>
<td>12 ± 1</td>
<td>519 ± 55</td>
<td>140 ± 9</td>
<td>169 ± 16</td>
</tr>
<tr>
<td>2</td>
<td>Fibrillated UF retentate at pH 2</td>
<td>549 ± 55</td>
<td>140 ± 9</td>
<td>169 ± 16</td>
<td>206 ± 29</td>
</tr>
<tr>
<td>3</td>
<td>Fibrillated UF retentate at pH 6.7</td>
<td>140 ± 9</td>
<td>169 ± 16</td>
<td>206 ± 29</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Control skim milk</td>
<td>169 ± 16</td>
<td>206 ± 29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Fibrillated skim milk</td>
<td>206 ± 29</td>
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<tr>
<td></td>
<td>Control NDM</td>
<td>169 ± 16</td>
<td>206 ± 29</td>
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<td></td>
<td>Fibrillated NDM</td>
<td>206 ± 29</td>
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</tbody>
</table>

AU = arbitrary units.

a–c Means (± SD) within a row with different superscripts differ among stages 1 to 3 and separately for stages 4 and 5 (P < 0.05); n = 3.
whey protein fibrils with casein and other constituents during spray-drying, subsequent reconstitution, and preheating before gelation is needed to understand the change in the gelation behavior of fibrillated NDM.

**Acid Gel Microstructure**

The microstructure of the acid gel of both reconstituted NDM is shown in Figure 5. At 10× magnification, both the acid gels look similar with some pores. When acid gels were analyzed using the color pixel counter-plugin in ImageJ software, the acid gel of reconstituted control NDM showed slightly lower green pixels (99.54%) than reconstituted fibrillated NDM (99.89%), which shows that reconstituted control NDM has more empty area than reconstituted fibrillated NDM. This difference can be seen at higher magnification (40×). Reconstituted control NDM showed 76.36% and reconstituted fibrillated NDM showed 87.24% green area. Connecting these results with rheology results, the protein network in acid gels of control NDM looks dense compared with fibrillated NDM, which might have provided higher gel strength to acid gels of control NDM. Comparing the gel images of NDM with gel images of the MPC study (Rathod et al., 2022), acid gel images of control NDM and control MPC showed almost similar protein networks. However, the acid gel of fibrillated NDM showed a more distributed protein network compared with the acid gel of fibrillated MPC shown in the earlier study. The reason for the dense protein network in fibrillated MPC was higher casein–whey protein interactions, which might be missing in the case of acid gel of fibrillated NDM, leading to a more open protein network and resulting in the weak acid gel of fibrillated NDM. However, the exact reason for this behavior needs further detailed study, as discussed in the acid gel rheology section.

**Surface Tension and Interfacial Tension**

The results of surface tension and interfacial tension of reconstituted control and fibrillated NDM are given in Table 3. The surface tension of reconstituted fibrillated NDM was significantly ($P < 0.05$) less than reconstituted control NDM, and the interfacial tension of reconstituted fibrillated NDM was less than reconstituted control NDM. However, it was not statistically significant. Both the interfacial properties are

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**Figure 2.** Transmission electron microscopy images of fibrillated UF at pH 2 (A), fibrillated UF at 6.7 (B), fibrillated skim milk (C), reconstituted fibrillated NDM (D), and supernatant of reconstituted fibrillated NDM (E). Scale: 200 nm.
important to understand functional properties, such as foaming and emulsification properties. Proteins, due to their amphiphilic nature, get adsorbed at the air-water or oil-water interface and reduce the interfacial tension between 2 immiscible phases. Compared with native proteins, fibrillated proteins reduce the surface tension by faster mass transfer on the air-water interface as fibrils are made up of small peptides and aggregates (Wan et al., 2016). Fibrillation of whey proteins unfolds whey proteins under low pH and high heat, which helps in the migration of protein to the interface (Mantovani et al., 2018). Also, unlike native whey proteins, fibrillated whey proteins have a high aspect ratio and surface hydrophobicity that facilitates adsorption on the oil-water interface, leading to a reduction in interfacial tension compared with native proteins (Fan et al., 2021). Therefore, the reduction in surface tension and interfacial tension of reconstituted fibrillated NDM was due to the presence of whey protein fibrils. The reduction in interfacial tension might positively help improve functionality, such as emulsification and foaming.

**Emulsification Capacity**

The results of emulsification capacity of reconstituted control and fibrillated NDM are shown in Table 3. The emulsification capacity (g of oil per mg of protein) of reconstituted fibrillated NDM was 3.44 ± 0.14, which is significantly \((P < 0.05)\) higher than the reconstituted control NDM (3.12 ± 0.13). In reconstituted fibrillated NDM, globular whey proteins were converted to whey protein fibrils, and this fibrillar structure shows a good emulsification capacity by providing higher viscosity and faster migration of whey protein fibrils to the oil-water interface (Mantovani et al., 2018; Jiang et al., 2022). The fibrillar structure is more open than the

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**Table 2.** Comparison of rheology (at 10%, 15%, and 20% TS) of control and fibrillated NDM

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TS (%)</th>
<th>Control NDM</th>
<th>Fibrillated NDM</th>
<th>Increase to control NDM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity at 100 s(^{-1}) (mPa·s)</td>
<td>10</td>
<td>2.28 ± 0.13(^a)</td>
<td>2.58 ± 0.09(^a)</td>
<td>13.1</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>4.14 ± 0.66(^a)</td>
<td>7.92 ± 1.04(^a)</td>
<td>91.3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>6.66 ± 1.16(^a)</td>
<td>16.93 ± 3.51(^a)</td>
<td>154.2</td>
</tr>
<tr>
<td>Consistency coefficient K (mPa·s(^n))</td>
<td>10</td>
<td>0.99 ± 0.14(^a)</td>
<td>1.38 ± 0.15(^a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>3.86 ± 1.22(^a)</td>
<td>11.12 ± 2.1(^a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>8.36 ± 3.46(^a)</td>
<td>34.54 ± 11.74(^a)</td>
<td></td>
</tr>
<tr>
<td>Flow behavior index (n)</td>
<td>10</td>
<td>1.22 ± 0.02(^a)</td>
<td>1.17 ± 0.02(^n)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1.03 ± 0.05(^a)</td>
<td>0.93 ± 0.01(^n)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.98 ± 0.04(^a)</td>
<td>0.85 ± 0.03(^n)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a,b\)Means (± SD) within a row with different superscripts differ \((P < 0.05)\); \(n = 3\).

---

**Figure 3.** Tricine-SDS-PAGE. L = ladder (2-250 kDa); lane 1, control UF retentate; lane 2, fibrillated UF retentate at pH 2; lane 3, fibrillated UF retentate at pH 6.7; lane 4, control skim milk; lane 5, fibrillated skim milk; lane 6, reconstituted control NDM; lane 7, reconstituted fibrillated NDM; lane 8, milk whey protein isolate; and lane 9, micellar casein concentrate.
globular structure and therefore more functional groups are exposed, which can interact with the aqueous phase and oil phase to form an emulsion. This could be the possible reason behind the increase in the emulsification capacity of reconstituted fibrillated NDM.

**Foaming Capacity and Foam Stability**

The result of foaming capacity and foam stability is shown in Table 3. A sample of reconstituted fibrillated NDM (35.3 ± 1.9) showed significantly ($P < 0.05$) higher foaming capacity than the reconstituted control NDM (31.6 ± 3.3). Similarly, reconstituted fibrillated NDM (17.0 ± 2.0) showed higher foaming stability than the reconstituted control NDM (14.3 ± 4.6), but the difference was statistically nonsignificant. When protein dispersions were whipped using a high-shear mixer, the air was incorporated in the dispersion in the form of air cells. The air cells were surrounded by liquid, and an air-protein dispersion film will be formed on the air-water interface, and finally structured as an air bubble (Damodaran, 2005). This process is influenced by how protein from the dispersion moves over the air-water interface and is adsorbed at the interface. Protein adsorption at the interface depends on the unfolding and denaturation of protein, exposure of hydrophobic residues of the protein to the air phase, and its hydrophilic segment to the aqueous phase (Oboroceanu et al., 2014). Considering the structure, whey protein fibrils have a more open structure than the native globular whey proteins. Further, whey protein fibrils at neutral pH are absorbed faster than fibrils at their formation pH (pH 2) due to the lower energy barrier to be absorbed (Wan et al., 2016). Whey protein fibrils

![Figure 4. Changes in rheological behavior during gelation of control and fibrillated NDM storage modulus (G') versus pH during gelation.](image-url)
in reconstituted fibrillated NDM were near neutral pH; therefore, they could be absorbed faster at the interface, which could be the reason behind the higher foaming capacity of fibrillated NDM. Foam stability is mainly affected by the drainage of the liquid film and bubble coalescence. Therefore, to produce a high-volume stable protein foam, drainage of liquid from between the bubbles must be minimized (Damodaran, 2005; Oboroceanu et al., 2014). Whey protein fibrils provide thickening to the solution by providing a 3-dimensional network, which can be seen by the higher viscosity of reconstituted fibrillated NDM (Table 2). Increased viscosity of reconstituted fibrillated NDM restricts the movement of the air cell, which reduces the chance of coalescence of air cells and escape of air cells, which led to an increase in foam stability.

**Heat Coagulation Time**

The results of heat coagulation time are shown in Table 3. Reconstituted fibrillated NDM showed higher heat coagulation time than the reconstituted control NDM. However, lower heat stability was reported for fibrillated samples by Rathod and Amamcharla (2021). Compared with fibrillated model MPC, fibrillated NDM has a lower concentration of fibrils and includes the presence of other nonprotein components, such as lactose and minerals. Therefore, the resultant interaction between fibrillated model MPC and NDM will vary. Heat coagulation is determined by observing visible aggregates and clots. In the case of fibrillated model MPC, where the presence of fibrils is in larger amounts, they may form a network by interacting with
each other or casein and form a gel, which could lead to early gelation and thereby lower heat coagulation time. However, in the case of fibrillated NDM, the total share of fibrils in TS is less and other constituents such as lactose and minerals are present. Therefore, fibrils may interact within, with casein, and with other constituents. But due to the lower amount of fibrils, they may not form aggregates and clots similar to fibrillated model MPC. Another possible reason is the role and state of whey proteins. During NDM manufacture, preheating milk is a common practice to denature whey proteins to prevent pseudocoagulation by crosslinking of casein micelles by denatured whey proteins, thereby increasing heat stability for further processing (Dumpler et al., 2020). In the current study, whey proteins in the control NDM were not subjected to any severe heat treatment; therefore, their presence can lower the heat coagulation time. However, whey proteins in fibrillated NDM were heated and converted to fibrils, which might positively affect heat coagulation time as similar to preheat treatment. However, the higher reactivity of fibrils with casein seen in the earlier study on fibrillated MPC (Rathod et al., 2022) cannot be ruled out without any consideration. So, the exact mechanism behind the change in the heat coagulation time needs further detailed investigation considering the interaction of fibrils with constituents other than caseins.

**Protein Oxidation**

The results of the protein oxidation study are shown in Table 4. Both reconstituted NDM showed a nonsignificant difference in tryptophan and tyrosine values. This indicates no significant loss of EAA. Oxidation products of tryptophan and tyrosine were also detected, which are N-formylkynurenine and dityrosine (Feng et al., 2015). A sample of reconstituted fibrillated NDM showed significantly (P < 0.05) higher dityrosine and N-formylkynurenine values, which is an indicator of protein oxidation (Keppler et al., 2019). However, the difference between control and fibrillated NDM for dityrosine value and N-formylkynurenine was not higher. Therefore, it could be said that the fibrillation process does not severely affect EAA content.

### CONCLUSIONS

Fibrillation of whey proteins showed improvement in functional properties such as emulsification, foaming, thickening, and gelling. Therefore, whey proteins, a globular protein in skim milk, were fractionated using the membrane process and selectively converted into the fibrillar form using the fibrillation process and mixed back into the remaining constituents of skim milk from membrane separation. The fibril-containing fibrillated skim milk was spray-dried and called fibrillated NDM. Results of ThT value, TEM, and gel electrophoresis confirmed the conversion of globular whey proteins into whey protein fibrils and survival of whey protein fibrils during further processing and spray-drying. Further, reconstituted fibrillated NDM showed higher viscosity, emulsification capacity, foaming capacity, and foam stability. Unlike fibrillated model MPC, reconstituted fibrillated NDM showed lower storage modulus than the reconstituted control NDM during gelation. The manufactured fibrillated NDM can have potential applications in products such as yogurt and ice cream and evaluate their effects on functionality of the product.

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### REFERENCES


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