Original Article

The Quality of The UHT Skim Milk as Affected by Addition of Rennet Skim Milk

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A B S T R A C T

Background and Objectives: Consumption of whole dairy products has declined due to the awareness of possible harmful effects of fat on consumers' health. The purpose of the present paper was to investigate the possibility of substituting the Ultra-high temperature processing (UHT) whole milk with partially hydrolyzed κ-casein to manufacture a UHT skim milk.

Materials and Methods: UHT skim milk samples were prepared using 0.2, 0.4, 0.6 and 0.8 % (w/w) of rennet followed by storage at 60°C for 15 minutes to inactivate the enzyme. The Chemical, physical and sensory characteristics of control and treated samples were evaluated. Statistical analysis was performed by SPSS.

Results: The results showed that a higher concentration of rennet increased and decreased b^* (blue–yellow) and a^* (green–red), respectively ($p < 0.05$). The regression profiles of viscosity plotted against heat stability of samples show a polynomial relationship ($R^2 = 0.96$). The zeta value of treatment samples was significantly lower than the absolute value of CUSM (Control UHT skim milk with 0.3% fat). There is a correlation between the appearance viscosity and average particle size ($R^2 = 0.92$). The skim milk consisting of 0.6% of rennet skim milk did not have any significant regarding aroma, taste and texture from those of the control UHT whole milk ($p < 0.05$).

Conclusions: The result showed that, RSM_4 (Renneted UHT skim milk with 0.3% fat and 0.8 % (w/w) rennet) has similar characteristics to whole milk and it is also clear in viscosity and sensory evaluation among other samples. It has been noted that non-dairy components were not used in RSM_4 and the new production can be a very functional and safe fat mimetic by merely changing the structure of casein micelles.

Keywords: UHT milk, Rennet skim milk, Fat mimetics, Healthy food

Introduction

Milk is a colloid of fat globules in a water-based solution that consists of dispersed carbohydrates and protein aggregating with minerals (1). The stability of colloidal system of milk strongly depends on casein micelles stability. Casein micelles in milk consist of 92-94% protein, 6% small ions (Ca, Mg, phosphate, citrate) and 2 to 4 g water/1g protein (2). Milk gelation may take place through heat-acid combination, acidification or selective enzymatic hydrolysis of kappa casein (3). Coagulation of casein micelles is achieved by adding particular proteolytic enzymes, rennet, to the milk. Rennet is specifically suited to hydrolyzation of the peptide bond in Phe105- Met106 of the κ-casein, and is considered to be the most efficient protease for the cheese-making industry (4). The activity of rennet can be impacted by various factors: appropriate temperature, pH, ionic strength and calcium activity. Aggregation will lead to milk gel formation (5). Aggregation of casein micelles begin when about 60% of the rennet-clotting time has passed and is completed after 85 to 90 % of κ-casein is hydrolyzed. In the textured milk, however, limitation of rennet takes place after 20 to 60% removal and the activity of rennet is limited by heat treatment before a gel is formed from the casein aggregates (6).

An increase in the temperature of sterilized milk results in a greater decrease in bacteria and heat

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resistant enzymes compared to pasteurized milk. Long-life sterilized milk, such as UHT milk, is treated by heat at temperatures of 135 to 150 ℃ for only a few seconds in order to minimize heat damage (7). Stability of micelles can be affected by reactions occurring during UHT processing (8). It appears that heat treatment could limit rennet activity, while increasing pasteurization temperature could result in the denaturation of whey proteins and their interaction with casein micelles (9).

In the past, whole milk was more favorable than skim milk because of taste, texture and appearance. Recently, consumption of whole dairy products has declined due to the awareness of possible harmful effects of fat on consumers' health. Unfortunately, absence of functional properties of removed fat can lead to a less organoleptic quality of products. Due to necessity of fat reduction, some food hydrocolloids are widely utilized as food ingredients (1). Ever since many alternative compounds have been used to improve the properties of low fat dairy products, Dave and Shah reported that the use of hydrolyzed casein increased the viscosity of gel yoghurt (10). Fat replacers, Litesse and Simplesse, generally increased the concentration of volatiles found in the headspace of ice cream compared with milk fat or cocoa butter (11). In another case, thise results pointed toward a Simplesse® D-100 (protein-based fat replacers) and Raftiline® HP (carbohydrate-based fat replacers) can improve the texture and sensory properties of low-fat fresh kashar cheese (12). Adding gum tragacanth to the low-fat cheeses increased the moisture content and improved the sensory properties, but only to 0.75 gr of gum/kg of milk (13). Tobin *et al* reported that the addition of modified konjac increased the viscosity and stability to sedimentation during simulated storage of UHT skim milk (14). The current study showed controlled cleavage of κ-casein of skim milk by rennin, produced renneted skim milk (RSM) consisting of new type of fat mimetic in yoghurt (6). Addition of inulin to low fat ice cream caused the adhesiveness to decrease compared to low fat ice cream with no inulin added (15).

The aim of the present study was to investigate the possibility of substituting the UHT whole milk with partially hydrolyzed κ-casein to manufacture a UHT skim milk and consider the effect of this substitution on the physicochemical, rheological and sensory attributes of the UHT milk.

Materials and Methods

Liquid rennet (40 mg/L) was prepared by dissolving 1 g of fungal rennet (Rennilase®, 55 International Milk Clotting Unites (IMCU) /mL, DSM, France) in 100 mL distilled deionized water. As a control commercial UHT whole milk with 3 % fat (CUWM) and skim milk with 0.3 % fat (CUSM) samples were purchased randomly from large supermarkets. UHT skim milk samples were prepared using 0.2 (RUSM$_1$), 0.4 (RUSM$_2$), 0.6 (RUSM$_3$) and 0.8 (RUSM$_4$) % (w/w) of rennet kept at 60 °C for 15 min to inactivate the enzyme, and then kept overnight at 4 ℃ (Table 1). Controls and treated samples were prepared in triplicate.

Table 1. Codes of different skim milks present in this paper

Milk code	Treatment
CUSM	Control UHT skim milk with 0.3% fat
RUSM$_1$	Renneted UHT skim milk with 0.3% fat and 0.2 % (w/w) rennet
RUSM$_2$	Renneted UHT skim milk with 0.3% fat and 0.4 % (w/w) rennet
RUSM$_3$	Renneted UHT skim milk with 0.3% fat and 0.6 % (w/w) rennet
RUSM$_4$	Renneted UHT skim milk with 0.3% fat and 0.8 % (w/w) rennet
CUWM	Control UHT whole milk with 3% fat

Chemical composition: A digital pH meter (Metrohm, Herisau, Switzerland) was used to measure the pH. Fat content was done by the Gerber method (ISO 2446:2008/IDF 226). Total solid contents were calculated by heating process (ISO 6731:2010/IDF 21). Crude protein content was determined by counting the total nitrogen using the Kjeldahl method (ISO 8968-3:2004/IDF, 20), (16).

Rheological measurements: The rheological experiments were performed by a Brookfield viscometer (model LV-DVIII, Brookfield Programming Rheometery, Inc., USA) with LV$_2$ spindle at 60 rpm (shear rate 10 s^{-1}) at 19 ± 1 ℃. The viscosity meter was calibrated with Brookfield calibration liquids. The spindle was selected based on the torque measurement ranging between 10 to 100 % as suggested by the manufacturer (17).

Color parameters: The colorimetric properties of samples were determined using a Hunterlab

40

colorimeter (D25 DP9000, Hunter Associates Laboratory, Inc., Reston, USA). The colorimetric was calibrated with a calibration plate. Samples were placed into a glass cell and scanned of L^* (whiteness), a^* (green–red), b^* (blue–yellow), (6).

Heat stability: The heat stability was measured as described by Fuller (2015). Aliquots (1 mL) of samples were placed in sealed glass tubes and were immersed in an oil bath thermostatically controlled at 140 ±1 ℃. The inquired time for the first visible aggregates to appear was recorded as the HCT (heat coagulation time), (18).

Non-protein nitrogen (NPN) content: NPN content (gr of nitrogen /kg of protein) was achieved by using the Kjeldahl method according to ISO 8968-3:2004/IDF, 20 and after precipitation at 12% (v/v) trichloroacetic acid (16).

Particle size distribution and zeta potential measurements: Tackling the Stabisizer® (PMX 200CS, Particle Metrix, Germany) and Zetaview® (Particle Metrix, Germany) with a 5mw He Ne laser operation at a wavelength of 780 nm, the particle size distribution and zeta potential of the samples were measured at 25±1 ℃. The used milk dilutions were 1:50 and 1:200 for particle size distribution and zeta potential measurements, respectively (19).

Sensory analysis: Sensory evaluations of samples were estimated according to the recommended protocol by the ISO 22935-2 (16). Seven trained and familiarized with dairy products panelists were recruited from Agricultural Engineering Research Institute, Karaj-Iran. The assessors evaluated the following attributes: aroma (milky, intensity, grassy and non-dairy), appearance (yellowish, creamy, sediment and streaky), taste (cooked, bitter, watery and undesirable taste) and texture (viscosity, flocks and consistency) (20). Approximately 100 mL of samples were put in random numbers clear glasses under normal light in the air-conditioned sensory laboratory and served for judgment free from external aromas, noise, and distraction. Panelist used distilled water and unsalted plain crackers to clean their mouths before testing samples. Five-point hedonic scale was used with the 5 structure level from 1 to 5 (1 = liked least, 5 =liked most).

Statistical analysis: The experiments were repeated three times. Data was subjected to *t* test and analysis of variance (ANOVA). The *t* test for independent samples was used to estimate the statistically significant difference for sensory evaluation. ANOVA was performed to examine the effect of other experiments. The means of treatments were examined by Duncan's multiple range test (significant level $p < 0.05$). All statistical analyses were performed using the statistical program SPSS 21 (SPSS INC., Chicago, IL, USA).

Results

Chemical characteristics of samples (pH, fat content, total solid and crude protein) are shown in Table 2. All samples did not differ in pH value and crude protein content ($p \geq 0.05$), but CUWM had the highest total solid and fat content.

The findings in present study indicate that the viscosity of samples change significantly after adding rennet compared to control UHT skim milk, while the highest viscosity of treated samples were found in a RUSM₃, where as it is lower than whole milk sample (Table 2). The plotted profiles of viscosity against renneted skim milk concentration (% w/w) showed a polynomial relationship ($Y_{Viscosity} = -0.03X^2_{concentration\ of\ rennet} + 0.23X_{concentration\ of\ rennet} + 1.29$, $R^2 = 0.98$). The sample containing 0.6 % (w/w) rennet (RUSM₃) had the nearest viscosity to whole milk (CUWM).

Table 2. Means (± SD) of the chemical characteristics and apparent viscosity of milk samples

Samples	pH	Fat (%)	Total Solid (%)	Crude Protein (%)	Viscosity (mPa.s)
CUSM	6.55ᵃ±0.04	0.38ᵃ±0.45	9.03ᵃ±0.24	3.36ᵃ±0.03	1.51ᵃ±0.03
RUSM1	6.59ᵃ±0.05	0.34ᵃ±0.17	9.06ᵃ±0.14	3.35ᵃ±0.05	1.64ᵇ±0.05
RUSM2	6.60ᵃ±0.01	0.33ᵃ±0.35	9.07ᵃ±0.28	3.40ᵃ±0.08	1.73ᶜ±0.02
RUSM3	6.58ᵃ±0.03	0.37ᵃ±0.22	9.05ᵃ±0.39	3.34ᵃ±0.02	1.78ᵈᶜ±0.04
RUSM4	6.56ᵃ±0.02	0.35ᵃ±0.32	9.09ᵃ±0.17	3.32ᵃ±0.04	1.72ᶜ±0.06
CUWM	6.57ᵃ±0.06	3.0ᵇ±0.20	12.12ᵇ±0.23	3.44ᵃ±0.02	1.83ᵈ±0.07

ᵃ, ᵈ Means within the same column with different superscript differ significantly (*p*<0.05)

The colorimetric characteristics (L^*, a^* and b^*) of control and RUSM samples were prepared by different concentrations of rennet that have been summarized in Figure 1. The results revealed that $RUSM_2$ and $RUSM_3$ had the highest whiteness ($p<0.05$) and the nearest lightness to CUWM sample.

Figure 1 showed that a higher concentration of rennet increased and decreased b^* and a^*, respectively. In our study it is noticed that concentration of rennet was significantly correlated with a^* and b^* of samples ($Y_{b*} = 0.37X$ concentration of rennet $+ 9.33$, $R^2 = 0.92$ and $Y_{a*} = 0.29X$ concentration of rennet $- 2.68$, $R^2 = 0.94$).

The results for UHT treated skim milk containing 0.2 up to 0.8% (w/w) renneted skim milk (Table 3) showed that the presence of this substitution significantly ($p<0.05$) declined the rate of movement

of the dispersed phase at addition levels up to 0.6% (w/w), from a rate of 4.31 min for CUSM to 5.58 min for samples containing 0.6% (w/w). The regression profiles of viscosity plotted against heat stability of samples show a polynomial relationship ($Y_{Viscosity} = -0.06X^2$ heat stability $+ 0.82X_{heat\ stablity} -0.84$, $R^2= 0.96$).

The UHT skim milk samples were chosen for the NPN analysis and contents were 1.40, 1.46, 1.52, 1.64 and 1.78 (gr/kg), respectively (Table 3). The NPN fractions increased with the rise of concentration of renneted skim milk and significant differences were observed between samples ($p<0.05$).

The zeta value of control sample was around -20.50 mV and it was significantly lower than absolute value of CUSM (Table 3). There is a correlation between the NPN values and zeta potential with a correlation coefficient of $R^2 = 0.94$.
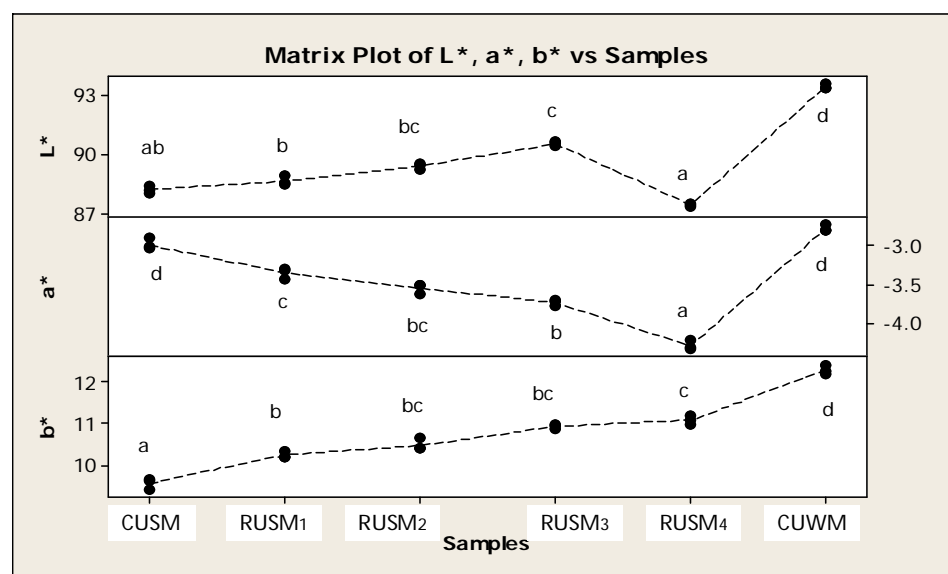
**Figure 1.** Matrix plot of L^*, a^* and b^* of milk samples
[a, d] Means within the same column with different superscript differ significantly ($p<0.05$)

Table 3. Means (± SD), heat stability, NPN and Z*et*a potential of milk samples

Samples	Heat stability (min)	NPN (gr/kg)	Zeta potential (mv)
CUSM	4.31[a]±0.12	1.40[a]±0.02	-20.50[a]±0.50
RUSM1	4.59[ab]±0.08	1.46[ab]±0.01	-19.42[b]±0.24
RUSM2	5.24[b]±0.07	1.52[b]±0.03	-19.01[b]±0.28
RUSM3	5.58[c]±0.04	1.64[c]±0.04	-18.19[c]±0.30
RUSM4	5.25[b]±0.03	1.78[d]±0.02	-17.40[c]±0.32

[a, d] Means within the same column with different superscript differ significantly ($p<0.05$)

According to particle size measurements, casein micelles in milk samples were affected significantly by adding partial hydrolysis of casein (Figure 2). However, the average diameter of casein micelle size of CUWM sample was the highest, following fat standardization, pasteurization and the UHT treatment. In this study there is a correlation between the appearance viscosity and average particle size (R^2 = 0.92). RSM_2, RSM_3 and RSM_4 samples had a semi similar particle distribution to that of CUWM sample.
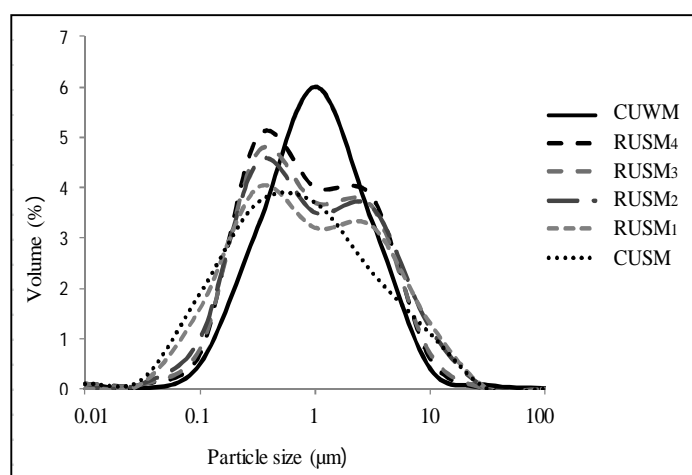
Figure 2. Particle size distribution of milk samples

Table 4 shows the scores of taste panelists for controls milk and treatments. As expected, the CUSM received the highest score for all attributes. The reductions in fat content significantly decreased the aroma, appearance, taste, and texture of samples. Results showed that supplementing the skim milk with renneted skim milk led to higher scores on the evaluated attributes in comparison with control UHT skim milk. The noted treated samples were preferred by the panelist more than free renneted skim milk. Despite this, the skim milk consisting of 0.6% of renneted skim milk did not have any significant difference in aroma, taste and texture from the control UHT whole milk ($p<0.05$).

Table 4. Means (± SD) sensory evaluation of milk samples

Samples	Aroma	Appearance	Taste	Texture
CUSM	3.11[a]±0.12	3.25[a]±0.33	2.82[a]±0.10	2.25[a]±0.30
$RUSM_1$	3.40[a]±0.08	3.40[ab]±0.14	3.06[a]±0.08	3.15[b]±0.18
$RUSM_2$	4.60[c]±0.16	3.81[bc]±0.13	3.65[b]±0.21	4.35[c]±0.21
$RUSM_3$	4.91[cd]±0.05	4.45[d]±0.04	4.71[d]±0.28	4.91[cd]±0.14
$RUSM_4$	4.41[b]±0.11	3.95[c]±0.21	4.10[c]±0.30	4.72[cd]±0.20
CUWM	5.00[d]±0.04	5.00[e]±0.13	4.90[d]±0.07	5.00[d]±0.04

[a, d] Means within the same column with different superscript differ significantly ($p<0.05$)

Discussion

The total solid contents of milk were based on percentage of fat (15). Fat globule contents existing in emulsified state, as ranging at 3.0 % in CUWM sample result in increasing the milk solid content.

Replacement combinations are often added to solutions to enhance or control viscosity. However, such additions to milk systems can result in separation whereby a casein-enriched phase is formed; this appointed that measuring viscosity is a crucial test (14). Milk's viscosity depends on total solid, fat content, the size and shape of fat particles forces molecules and the attractions between them (21). At the first position, adding the rennet enzyme, decrease the viscosity of milk vigorously due to the release of caseinomacropeptide (CMP). The aggregation of particles started and led to a slow increase in viscosity (22). The results showed that adding various amounts of rennet had a different effect on the viscosity of samples. According to Table 2, viscosity was raised by increasing the amount of rennet as the result of controlled hydrolysis and partial accumulation of casein micelles. This result is similar to that of reported by tobin *et al* (2011) that the addition of modified konjac (0-0.12% w/w) to skim milk significantly increased the apparent viscosity to almost that of full-fat milk. Table 2 indicated that higher amount of rennet enzyme (more than 0.6 %) had a negative effect on viscosity, due to a more advanced aggregation of casein micelles and changes in the basic structure.

Color value was considered as an important parameter for consumer's acceptability as well as an indicator of system stability (23). Whiteness of milk is caused by light reflection of casein micelles, the amount of molecules of fat and calcium phosphate (24). Whiteness improvement was observed in treated samples probably due to the partial hydrolysis and incomplete coagulation of casein micelles. However in samples with higher amounts of rennet, advanced hydrolysis may lead to more aggregation of casein micelles and undesirable characteristics. As a result, aggregate may be shaped irregularly, which may decrease light scattering causing lower whiteness.

Carotene is the yellowish fat-soluble component of whole milk. Figure 1 shows that aggregation of casein micelles might have acted like a fat globule causing an improvement in yellow color formation. The result

43

of this study showed that a higher degree of κ-casein hydrolysis causes a higher release of whey protein and increases the green color seemingly due to riboflavin. Since a suitable fat mimetic in milk products should have a color similar to the color of whole milk (whiter, yellower and less green), the greenness is a negative and whiteness/yellowness is a desirable property in dairy products. Therefore, samples with highest whiteness, moderate yellowness ($RUSM_2$ and $RUSM_3$) were the most befitting samples. Mart´ın-Diana *et al* (2004) showed that no apparent color changes were observed visually in the samples due to 30 g l^{-1} whey protein concentrate (WPC) supplementation (25).

Formation and stability of milk are essentially the result of a large number of equilibria in milk including the solubility and ionization of salts, the interactions of salts with proteins and the association of proteins (26, 27). Heat stability is a well-known factor of milk capacity to resist coagulation. Having poor heat stability of UHT milk can give rise to sedimentation (28). In this study, different percentage of renneted skim milk was used and partial hydrolyzation of casein in UHT skim milk was occurred, therefore, heat stability test is one of the most important analysis. The poor heat stability of more than 0.6% (w/w) substitution is probably due to the extensive hydrolyzation of casein proteins and concomitant formation of a casein whey protein network. Uncontrolled hydrolyzed treatment of milk can also result in removal of the κ-casein brush from the micelle, reduced colloidal stability and is extremely prone to aggregation such as RSM_4 sample. This resulted in increased stability of the UHT treated skim milk towards sedimentation (Table 2), it is similar to a study by Tobin *et al* (2011) most likely due to the increase in the intrinsic viscosity of treated samples.

Due to association of denatured whey proteins (α-lactalbumin, ß-lactoglobulin and immunoglobulins) with casein molecules during heat treatment, the NPN values of UHT milk (1-2 gr/kg) were lower than that of raw milk (7–8 gr/kg), (29, 30). Rennet coagulation of milk takes place at two steps: rennet causes proteolysis of κ-casein by breaking the Phe_{105}- Met_{106} peptide bond leading to destabilization of the casein micelle and the following of aggregation results in gel formation (19). Gel formation at normal pH of milk takes place after at least 85 to 90% κ-casein has been

hydrolyzed (31). In this paper no gel formation was observed, since rennet activity was controlled by heat treatment, it was concluded that less than 85% of the κ-casein was hydrolyzed. The changes in the proteins have been assessed by change in non-protein nitrogen (NPN), reduction of casein percentage and increases in content of high-molecular weight protein material (26). In order to investigate the above issue, changes in size distribution and zeta potential of casein micelle had been measured

The milk particles charge would be mainly related to the casein micelle and the milk fat globule membrane. Proteolysis treatment along with peptides release containing negatively charged groups such as glutamyl, aspartyl, phosphoseryl and glycosidic residues can be confirmed by the determination of a decrease of zeta potential (29). In the present paper partial proteolysis of κ-casein and CMP removal were considered as the main reasons for the reduction of the net negative charge of casein micelles up to -17.40 mV. The zeta value of samples was within the normal range of -12 to -30 mV observed in milk (30, 32).

Figure 2 shows that average size of casein micelles decreases after adding rennet and immediately increases afterwards by a hairy outer layer, it is similar to the finding of Castillo *et al* (2000). McMahon and Brown said the same concept about viscosity of milk at first and after adding rennet enzyme (22). Coalescence of micelles in treated samples may cause partial fusion of casein micelles and new interaction or weak electrostatic bond between partially hydrolyzed casein micelles and whey protein of skim milk. According to the present paper, estimating the heat stability for large and small casein micelles in milk, under the same conditions is variable. This could be as a result of distribution of casein micelles size dispersion which dictates the rate of sedimentation and diffusion (31). These results were comparable with those obtained by Lin *et al* (2016) who studied the fortification of milk protein content with different dairy protein powders and found that the aggregation behavior of protein-fortified milk samples (125 to 142 nm) is strongly influenced by the degree of mineralisation of the protein powder used in fortification, which affects the partitioning of casein and calcium in the sedimentable and nonsedimentable phases (33).

Significant difference in appearance characteristics was due to the yellowish and creamy mouth feeling

depending on the thickness and pigment of fat content. Also in treated skim milk samples a streaky appearance was created by rennet enzyme. Panelists were able to recognize differences between RSM$_3$ sample and other ones because it had more whiteness, yellowness, creamy appearance, thickness, consistency of texture and less watery and undesirable taste. Although RSM$_3$ sample had the same fat content as CSM (0.3 % fat), more intensity of sensory characteristics was observed and closely resembled the whole milk (3% fat) and it showed that RSM$_3$ can play a role of fat mimetics. Similar results were found for milk enriched with 30% CLA (microencapsulated conjugated linoleic acid) used for UHT milk, which received acceptable grading scores (34).

In conclusion, as suggested by many authors, to improve the health status of food, the amount of fat was kept similar to that of skim milk but with the consumer's acceptance and satisfaction of the new formula of skim milk. The result showed that among the samples, RSM$_4$ had similar characteristics to whole milk. Heat stability in UHT milk correlated with the viscosity and treated samples had a more stability compared to controls. It has been noted that no non-dairy components were used in RSM$_4$ and only changes to structure of casein micelles, the new production can be a very functional, safe and green fat mimetics. For industrial applications cheese production line could be applied via limited rennet activity by heat treatment beside low and non -fat UHT milk production line.

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