Ultrasonication retains more milk fat globule membrane proteins compared to equivalent shear-homogenization

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ABSTRACT

Ultrasonication, like common shear homogenization, can reduce the milk fat globule size and may change the milk fat globule membrane (MFGM). This work compared the effect of ultrasonication to equivalent shear homogenization on MFGM proteins and lipid-derived volatile components. Results showed that treating milk with ultrasound at 35 kJ/L would realize a similar size distribution of the milk fat globules as shear-homogenization at 20 MPa. Proteomics analysis revealed that in total 192 MFGM proteins were identified and quantified and a number of these proteins were lost after both treatments; however, more MFGM proteins remained after ultrasonication than after shear-homogenization. SDS-PAGE results showed that milk plasma proteins, and especially caseins, were absorbed on the milk fat globules after both treatments. In addition, the amount of the volatile free fatty acids increased after both treatments.

Industrial relevance: Ultrasonication, as an innovative food processing technology, in comparison to traditional homogenization, was shown to equally efficiently decrease the MFG size, but lead to less damage to native MFGM proteins, which may be due to its longer homogenization time window. These results increased knowledge on the biochemical changes of milk fat globules after their size reduction and showed that ultrasonication could be used as a novel approach to improve dairy product quality.

1. Introduction

The lipids in milk are present in the form of milk fat globules (MFG), consisting of a triglyceride core, surrounded by a thin membrane, called the milk fat globule membrane (MFGM) consisting of an inner mono-layer and an outer bilayer (Dewettinck et al., 2008). MFGM contains polar lipids, cholesterol, and membrane-specific proteins, acts as an emulsifier for milk fat, and protect the MFG from enzymatic degradation and coalescence (Holzmüller & Kulozik, 2016; Zheng et al., 2020). A large variety of bioactive proteins were observed to be present in MFGM, such as xanthine oxidase/dehydrogenase (XO/XDH), butyrophilin (BTN) and Periodic acid Schiff base 6 and 7 (PAS6/7) (Spitsberg, 2005), and several hundreds of low abundant proteins with diverse functions (Yang et al., 2018). Although MFGM proteins only account for 1–2% of total milk protein, their nutritional and technological properties have

attracted considerable research attention (Lu et al., 2016). The MFGM fraction of bovine milk was generally similar in composition to that of human milk and has been industrially isolated and added to infant formula (Lee et al., 2021). Clinical data suggested that MFGM plays an important role in neurodevelopment and cognitive development (Brink, Gueniot, & Lönnerdal, 2019). However, separation of MFGM proteins is still a challenge. Cream washing and ultracentrifugation, or filtration of buttermilk, are the most common methods to isolate MFGM material. These isolation procedures will inevitably lead to a loss of native MFGM proteins (Holzmüller, Müller, Himbert, & Kulozik, 2016).

Emerging processing technologies, such as ultrasonication, can change the properties of milk, such as stabilizing the raw milk by increasing the zeta-potential of MFG (Abesinghe et al., 2020), and improving rennet gelation properties (Chandrapala et al., 2016), although its application in milk is currently relatively limited. It has

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been reported that ultrasonication can efficiently reduce the size of MFG by shearing, pressure fluctuations, and turbulence (Abesinghe et al., 2020; Nguyen & Anema, 2017). When ultrasound travels through a liquid medium, it generates numerous microbubbles by "acoustic cavitation". The collapse of these microbubbles induces localized violent shear forces and turbulence, thereby breaking the MFG into smaller droplets (Ashokkumar et al., 2010). Although there are several reports that focused on applying ultrasonication in dairy processing (Nguyen & Anema, 2017; Van Hekken, Renye Jr., Bucci, & Tomasula, 2019), studies related to investigating the effects of ultrasonication on MFGM proteins have not been performed before. In addition, this technique also inactivates bacteria in a lower temperature and better retains immune-active milk serum proteins (Drakopoulou, Terzakis, Fountoulakis, Mantzavinos, & Manios, 2009; Liu et al., 2020). Besides the beneficial effects for milk quality, owing to the generation of extremely strong localized temperatures and pressures, free radicals and other reactive species may be generated from water vapor, such as H· and OH· radicals, and reactive oxygen species (ROS) (Riener, Noci, Cronin, Morgan, & Lyng, 2009). These ROS species, such as H_2O_2, can induce redox reactions with the lipid phase and affect the volatile components in milk (Juliano et al., 2014; Reis et al., 2020). Generally, the carbonyl compounds pentanal, hexanal and heptanal, being secondary products of free radical-induced lipid oxidation resulting from the decomposition of unsaturated fatty acid hydroperoxides, are used as markers for the extent of lipid oxidation (Riener et al., 2009). In addition, structural damage to the MFGM may also lead to increased lipase activity, resulting in the release of free fatty acids (Deeth, 2006).

Although the homogenizing effect of ultrasonication in milk has been widely studied and reported, the information of ultrasonication on the proteins located in MFG have not been widely investigated, especially compared to well-known shear homogenization. Changes to the MFGM proteins may not only depend on how small the droplets are after homogenization, but also how frequent break-up and re-coalescence of droplets happens (Jafari, Assadpoor, He, & Bhandari, 2008). Ultrasonication would produce a constant acoustic cavitation which may avoid the frequent break-up and re-coalescence as occurring during common shear homogenization, which may thereby lead to differences in the MFGM proteins between ultrasound and shear homogenization. This study aimed to investigate the effects of ultrasonication, compared with shear-homogenization, on MFGM proteins by label-free LC/MSMS-based proteomics. In addition, their effect on generation of lipid-derived volatile components in milk was investigated by GC–MS.

2. Material and methods

2.1. Milk collection and treatments

Fresh milk was collected from CARUS farm (Wageningen University Campus) from clinically healthy cows and kept at 4 °C before further treatments and analysis. To avoid microbial reproduction, 0.02% sodium azide was added into the milk.

For ultrasonication, an ultrasonicator (Branson Digital Sonifier® 450) equipped with a Branson microtip probe (60 mm*10 mm) was used for ultrasonication treatment of raw milk. To find a proper ultrasonication treatment condition that would realize an equivalent homogenizing effect as shear-homogenization, milk samples were treated at different powers and times. Before the treatments, milk was warmed up to 40 °C to make the solid fat particles melt and improve the homogenization efficiency. Then, 70 mL of milk was transferred into sterilized 100-mL glass beakers and treated separately in batch mode. The samples in glass beaker were placed in the ultrasonication cell surrounded by circulating water of 40 °C to keep the sample temperature as much as possible constant during the ultrasonication. The ultrasonicator (20 kHz) was run in pulse-pause mode (59 s on followed by 1 s pause). The treatment power was set to either 40 W or 60 W, and the treatment times used were 30, 60, 90, 120, and 180 s. According to

preliminary experiments, the temperature after each treatment was monitored, which did not exceed 52 °C. The heat-induced influence on the MFGM proteins is thus expected to be negligible.

For the homogenization treatment, one liter of raw milk was preheated at 50 °C and then homogenized at 20 MPa with a homogenizer (Labho Scope Homogenizer, Delta Instruments) for 3 times to stabilize the size distribution of MFG.

The obtained milk samples were kept in refrigerator at 4 °C for further analysis.

2.2. Measurement of milk fat globule size distribution

Size distribution of MFG was determined by a Mastersizer 3000 (Malvern Instruments, Malvern, UK), based on a previously published method (Logan et al., 2014). For this analysis, a 1-mL milk sample was mixed with EDTA/NaOH solution (35 mM, pH 7.0) at 1:1 *v/v* to dissociate casein micelles, thereby reducing the influence from casein micelles, as these are in the similar size range as homogenized fat droplets. The milk samples were then diluted 10 times with MilliQ water. After mixing, milk sample was dropwise added into the water in the measurement cell until the obscuration rate reached 6–8% and the refractive indexes (RI) for the MFG and water were 1.46 and 1.33, respectively, and the absorbance was 0.001. The volume-weighted mean diameter $d_{4,3}$ ($d_{4,3} = \Sigma n_i d_i^4 / \Sigma n_i d_i^3$) was recorded and used to calculate the specific surface area (SSA). All samples were measured in triplicates. The SSA was calculated as follows:

$$SSA\left(m^2/g\, fat\right) = 4\pi r^2 \cdot n$$

Where, r = $d_{4,3}$/2; n is the number of MFG in 1 g fat, and calculated as V $_{(fat,1g)}$/V $_{(per\, fat\, globule)}$, ρ $_{fat}$ = 930 g/cm^3.

2.3. Separation of MFGM protein

The separation of MFGM proteins was conducted by cream washing (Lu et al., 2016). Even though it is known to lead to a loss of native MFGM proteins, it should allow comparison among treatments. Homogenized and ultrasonicated milk samples were centrifuged at 3000g for 20 min at 4 °C to obtain milk cream (top layer). One mL cream was mixed and washed with 10 mL PBS (pH 6.80) and centrifuged again. This washing of cream was repeated 3 times to remove remaining caseins and whey proteins. After that, 1 mL 0.4% SDS was added to the washed cream and this was sonicated for 90 s at room temperature. After centrifugation, the bottom MFGM-enriched fraction was collected and filtered through a 1 μm membrane to remove remaining fat particles. The MFGM protein concentration was determined using the BCA method (Thermo Scientific Pierce BCA protein assay kit, USA).

2.4. Gel electrophoresis

Gel electrophoresis was performed to check the effects of ultrasonication and homogenization on major MFGM proteins, including milk plasma proteins associated with the MFG after the treatments, according to the method of Liu et al. (2020). In brief, 5 μg MFGM protein was mixed with appropriate NuPAGE™ LDS sample buffer (NP0007, Thermo Fisher Scientific, Massachusetts, USA) and reducing reagent (NP0009, Thermo Fisher Scientific, Massachusetts, USA). After heating at 75 °C for 15 min and centrifugation, samples were loaded in a 12% Bis-Tris gel (NP0341BOX, Thermo Fisher Scientific, Massachusetts, USA) in NuPAGE™ MOPS SDS running buffer (NP000102, Thermo Fisher Scientific, Massachusetts, USA). The voltage was set at 120 V and run for 80 min to determine the protein patterns. The gel was then stained with Coomassie Brilliant Blue R-250 for 45 min and rinsed with 10% ethanol and 7.5% acetic acid in MilliQ water while mildly shaking until the protein bands became clear.

2.5. LC-MS/MS based proteomic analysis

To compare the effects of ultrasonication with shear homogenization on MFGM proteins, we only chose the samples with similar MFG size distribution for further analysis. The sample preparation for LC-MS/MS was performed according to the methods of Xiong, Li, Boeren, Vervoort, and Hettinga (2020). Briefly, 100 μg MFGM protein was added into a low binding tube (Eppendorf, Hamburg, Germany) and then reduced by 10% (volume) DTT (150 mM). After incubation at 45 °C for 30 min, 40-ug reduced sample was pipetted into the middle of 136 μL 8 M urea in 100 mM Tris/HCl (pH 8.0) and mixed. Then, 20 ul acrylamide (200 mM) was added and incubated for 15 min at room temperature. Subsequently, 150 ul alkylated sample (30 μg protein) was transferred to a Pall 3 K omega filter (10–20 kDa cut-off, OD003C34) and centrifuged at 14000 g for 30 min. Then, 160 μL NH_4HCO_3 (50 mM) was added to the filter and centrifuged again. After that, the filter was transferred to a new 2-ml low binding Eppendorf tube, followed by the addition of 100 μL trypsin in 50 mM NH_4HCO_3 (5 ng/μL) and incubation overnight while mildly shaking. This was followed by another centrifuge at 14,000 g for 30 min, after which 100 ul HCOOH (1 mL/L) was added and centrifuged again. Finally, 10% trifluoroacetic acid was added to adjust the pH to ~3.0 and samples were frozen at −20 °C until LC-MS/MS analysis.

Digested peptide (ca 200 ng) was injected into a 0.10 * 250 mm ReproSil-Pur 120 C18-AQ 1.9 μm beads analytical column with maximum pressure at 800 bar. An acetonitrile gradient (from 9% to 35% acetonitrile in water with 0.1% formic acid) at a flow of 500 nL/min was used to elute the peptides in 50 min. Sample were analyzed by a Q Exactive Mass Spectrometer (Thermo Electron, San Jose, CA, USA) at spray voltage of 3.5 kV, and MS scan range from 380 and 1400 m/z.

For the proteomics data analysis, the obtained raw MSMS files were analyzed and searched against the uniprot-Bos taurus protein database (Uniprot UP000009136) using MaxQuant (1.6.3.4). Trypsin was chosen as the specific enzyme. Oxidation of methionine, N-terminal acetylation and deamidation of asparagine or glutamine were chosen as variable modification; carbamidomethylation of cysteines was set as a fixed modification. Maximum two missed cleavages were allowed, 20 ppm and 4.5 ppm tolerance for the peptide MS peaks during the first and main search, respectively. Proteins identified by more than 2 peptides (of which at least 1 unique and 1 unmodified) were considered as reliable and used for analysis. The obtained iBAQ (intensity based absolute quantification) intensity was processed by Perseus software, and the proteins identified in three replicates was used for subsequent analysis. The heatmap was plotted using TB tools software and PCA was performed using Origin 2020.

2.6. Volatile components analysis by GC–MS

The volatile components in milk after the treatments was determined using headspace solid-phase microextraction (SPME)-GC/MS according to a previous method (Quintanilla, Hettinga, Beltrán, Escriche, & Molina, 2020). First, 3-mL milk samples were placed in 10-mL vials and sealed with a 20-mm silicone/PTFE cap (Grace, Albany, OR). Volatile components were obtained from the headspace for 5 min with a 75-μm PDMS-carboxen SPME fiber (Supelco, Bellefonte, PA) at 45 °C for 40 min using an autosampler. Then, volatile components were released from the fiber by heating in a Best PTV injector (Thermo-Finnigan, San Jose, CA) at 230 °C for 10 min. After each run, the fiber was cleaned by heating at 290 °C for 10 min. Separation of the volatile components was conducted with a Trace gas chromatographic (GC) connected to a DSQ mass spectrometer (Thermo-Scientific) with MilliQ water as blank control. Volatile components were separated in an apolar BPX-5 column (30 m length, 0.15 mm i.d., 0.25-μm film thickness, Thermo, Austin, TX). The temperature in oven was maintained at 40 °C for 3 min, increased to 230 °C at 20 °C/min, followed by 1 min holding. The MS interface and the ion source were kept at 250 °C, and helium (0.8 mL/ min) was used

as the carrier gas. The MS scans were collected in full scan mode using m/z from 33 to 250 and electron impact mode at 70 eV. Each compound was identified using the Chromeleon and AMDIS software (NIST, Gaithersburg, MD) and an in-house library (Hettinga, 2009).

3. Results and discussion

3.1. Size distribution of milk fat globules

Ultrasonication has been considered as an effective system for homogenizing MFG through cavitation effects, which refers to formation and violent collapse of bubbles (Ertugay & Şengül, 2004). To find the appropriate ultrasonication treatment dosage that would have a similar homogenization effect compared with shear homogenization, we treated the milk at different treatment intensities and times (40 W and 60 W for 30, 60, 90, 120, and 180 s). As shown in Fig. 1, the size distribution of milk fat globules was changed after all ultrasonication treatments. The average MFG size in raw milk was around 3–5 μm, and shear-homogenizing decreased the average MFG size to around 1 μm, which is in agreement with previous reports (Ye, Cui, Dalgleish, & Singh, 2017). Fig. 1a and b display the size distribution of the MFG after ultrasonic treatments at 40 W and 60 W respectively. The results show that ultrasonication treatment at 40 W and 60 W both reduced the MFG with prolonged treatment time. Recently, Van Hekken et al. (2019) also found that the size distribution of MFG decreased with ultrasonication exposure time and an increased treatment temperature would be more efficient in decreasing the size of MFG. In addition, the size distribution of MFG after treatment at 40 W for 60 s is close to that of shear-homogenization. Fig. 1c and d show the SSA of MFG after treatments at 40 W and 60 W, respectively. As expected, the SSA was increasing with decreasing MFG size, as a result of more and smaller MFG after homogenization. As shown in Fig. 1c, the SSA of MFG after 40 W for 60 s is close to that of shear-homogenization. In addition, taking into account the actual temperature during treatment, we chose the sample treated at 40 W for 60 s for the further experiments.

3.2. Gel electrophoresis analysis

Fig. 2 displays the MFGM protein patterns after shear-homogenization and ultrasonication, both under reducing and non-reducing conditions, which demonstrates that the major proteins in the isolated MFGM are xanthine dehydrogenase/oxidase (XDH/XO), CD36, butyrophilin (BTN), adipophilin (ADPH), and PAS-6/7. The applied washing procedure for MFGM isolation would have resulted in a loss of MFGM protein, although comparison among samples should be possible as the same isolation procedure has been applied to all samples. Both shear-homogenization and ultrasonication changed the MGFM protein composition, where some bands corresponding to MFGM proteins disappeared while some plasma proteins bands (e.g. caseins) appeared after both treatments. Compared with ultrasonication, shear-homogenization induced a more extensive change of the MFGM proteins. This can be seen by the disappearance of the protein band corresponding to XHD/XO and the relatively faint bands corresponding to CD36 and BTN, suggesting extensive loss of these proteins after shear-homogenization. Ultrasonication at both 40 W for 60 s and 60 W for 30 s better retained these proteins. As mentioned above, the bands corresponding to caseins appeared both after shear-homogenization and ultrasonication treatments, which can probably be attributed to the interaction between MFG and caseins during both these treatments. During these treatments, the native MFG are disrupted and become smaller, while the SSA of MFG would be enlarged. During these changes to the MFG, native MFGM proteins may be lost from the MFG surface whereas simultaneously caseins and whey proteins migrate to the newly formed MFG surface, forming a casein/whey-based surface layer (Lee & Sherbon, 2002). It was once reported that these newly created surfaces are covered preferentially by casein micelles over whey proteins
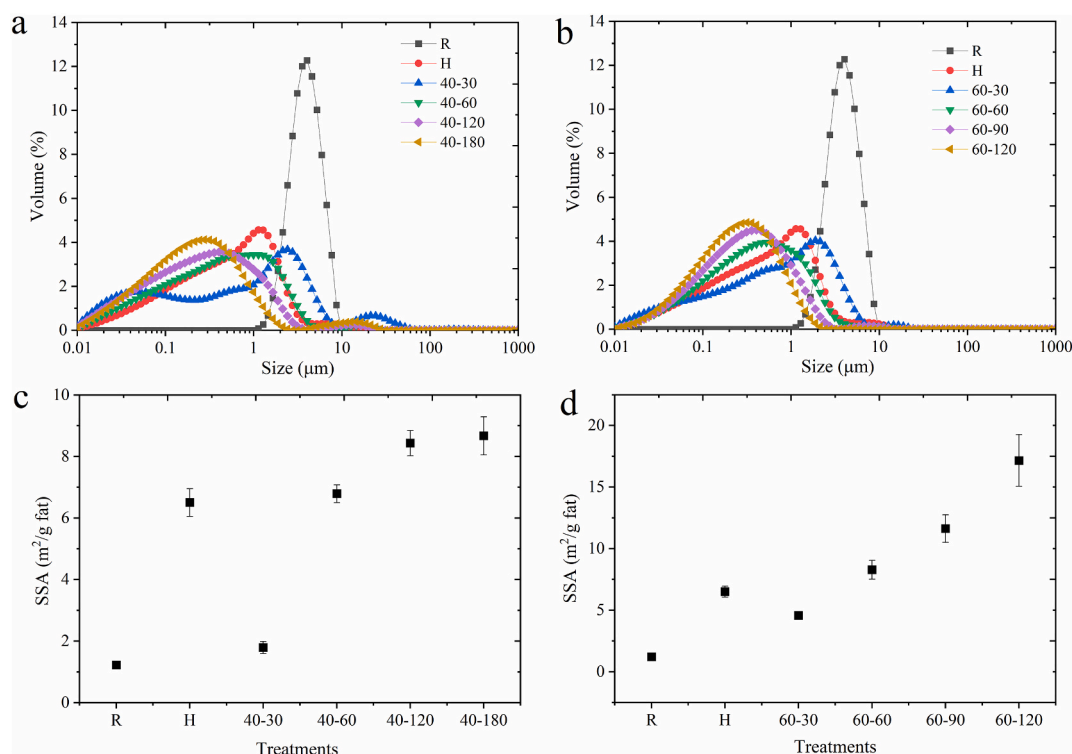
Fig. 1. Size distribution and SSA of milk fat globules after different treatments; a and b, size distribution of MGF after ultrasonication at 40 W and 60 W, respectively; c and d, SSA of MFG after ultrasonication at 40 W and 60 W, respectively; R, raw milk; H, shear-homogenization; The ultrasonication treatment is described by two numbers, first the power (40 W or 60 W) after the hyphen followed by the duration of the treatment (30, 60, 90, 120 or 180 s).
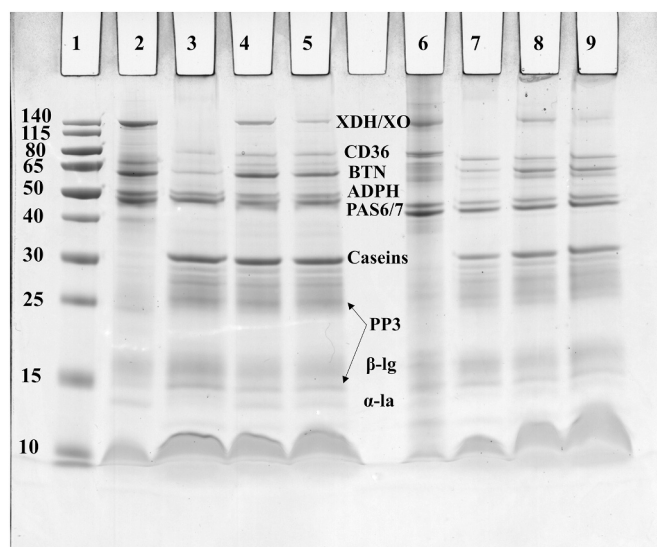

Fig. 2. SDS-PAGE of MFGM proteins after treatments; lane 1–5: 1) protein marker, 2) raw milk, 3) shear-homogenized milk, 4) 40 w for 60 s, 5) 60 w for 30 s, all under reducing conditions; lane 6–9: 6) raw milk, 7) shear-homogenized milk, 8) 40w for 60 s, and 9) 60 w for 30 s, all under non-reducing conditions.

(Anderson, Brooker, Cawston, & Cheeseman, 1977). In addition, some of the plasma proteins (casein/whey) could also be washed away, and it was reported that whey proteins are more easily removed by washing than the casein components after homogenization; however, after pasteurization, whey proteins become more tightly bound and are no longer readily removed by washing (Darling & Butcher, 1978). This could be the reason why the caseins end up relatively more in the MGFM

protein fraction and were observed in the gel electrophoresis results. Our findings here also confirm previous reports of Darling and Butcher (1978), and Walstra and Oortwijn (1983). In previous studies, it was reported that high-pressure homogenization and normal homogenization would both induce adsorptions of caseins to the MFGM and a certain loss of native MFGM proteins (Kiełczewska, Ambroziak, Krzykowska, & Aljewicz, 2020; Lee & Sherbon, 2002), which is in agreement with our findings. According to above results (Fig. 1 and Fig. 2), it is worth noting that even though shear-homogenization and ultrasonication both realized a similar homogenizing effect and increased the SSA of MFG equivalently, ultrasonication would retain more native MFGM proteins. It is therefore hypothesized that the increasing SSA is not the reason why shear-homogenization resulted in a larger loss of MFGM proteins. Although the exact explanation for this effect remains unclear, it was reported that re-coalescence of emulsions droplets may happen during homogenization (Jafari et al., 2008), and shear-homogenization may lead to more extensive re-coalescence of emulsion droplets, due to its short homogenization time window, making the process more dependent on quick adsorption of emulsifier. This more frequent re-coalescence after shear homogenization may in turn have led to a more extensive loss of MFGM proteins. It remains to be further studied whether different shear-homogenization conditions, e.g. valve design and pressure, would lead to the same results. Ultrasonication would induce a more homogeneous cavitation effect in the liquid medium, and has a longer homogenization time windows, which may avoid this frequent re-coalescence (Jafari, He, & Bhandari, 2007).

3.3. Proteomics analysis of MFGM proteins after both treatments

Fig. 3 demonstrates the identified MFGM proteins after both treatments. Here, we only displayed the proteins that were identified in all three replicates, even though this reduces the number of identified proteins, as it leads to the most confident identifications and allows proper statistical analysis. In total, 192 proteins were identified in all
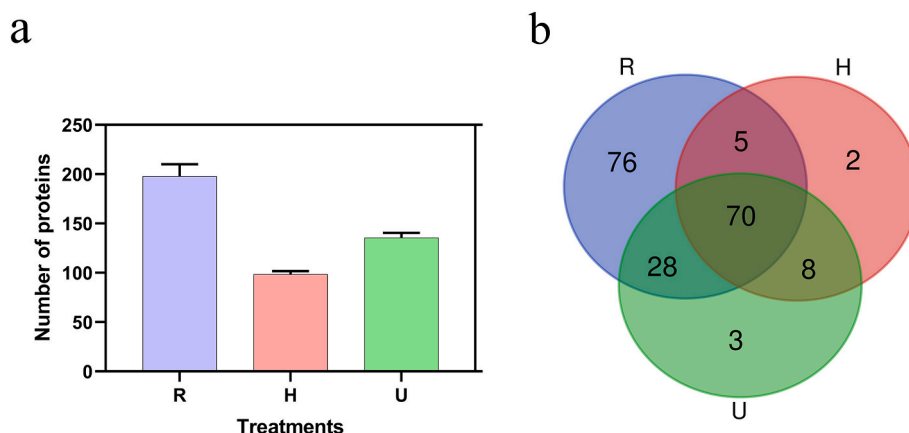
a

b

Fig. 3. Number of identified proteins after the two homogenization treatments (a) and the same data plotted as a Venn diagram to show overlap (b); R, raw milk; H, shear-homogenization; U, ultrasonication at 40 W for 60 s.

groups, of which 70 proteins were shared among the three groups. As shown in Fig. 3a, the percentage of identified proteins decreased after shear-homogenization and ultrasonication. Compared to raw milk, only around 50% and 70% of the proteins were detected after shear-homogenization and ultrasonication, respectively, suggesting that many MFGM proteins were lost during these treatments. This is in agreement with the results of gel electrophoresis, which also showed a larger effect of shear-homogenization, although here only the high abundant proteins were shown. Similar findings could be found in the studies of Lee and Sherbon (2002) and Zamora, Ferragut, Guamis, and Trujillo (2012), in which significant compositional changes took place and native MFGM proteins got lost during ultra-high pressure homogenization. Recently, Wang et al. (2019) also pointed out that common homogenization with preheating would decrease the native MFGM proteins significantly.

3.4. Quantitative and cluster analysis of MFGM proteome

Fig. 4 shows the biplot analysis of MFGM proteins in milk after different treatments. In this figure, each blue arrow represents an identified protein, showing which proteins were responsible for the differences between the sample groups and the red, blue and green spheres represent the samples. The homogenized milk was separated from the raw milk in the direction of principal component 1, whereas ultrasonicated milk was separated in the direction of principal component 2. The distribution of ultrasonicated milk is closer to the raw milk compared with the homogenized ones. The first two principal components explained 87% of the total variance in the original data set. Principal component 1 is mainly related to CD36, mucin, XO/XDH, butyrophilin, perilipin-2, ATP-binding, and lactoferrin, which are typical proteins that exist in the native MFGM (Dewettinck et al., 2008), while principal component 2 is related to κ-casein, β-lactoglobulin, and Fibrinogen, which may be due to the incorporation of plasma proteins in milk.

Fig. 5 displays the heatmap and volcano plot of the quantified MFGM proteins, to further distinguish the differences induced by the two treatments. Similar to the PCA analysis in Fig. 4, it is found that the changes of the MFGM proteins separated the different treatments into distinct clusters. According to the heatmap, the abundance of most of the MFGM proteins decreased after both shear-homogenization and ultrasonication, however, the effects of ultrasonication was smaller compared to shear-homogenization. Fig. 5b and c show the intensities of
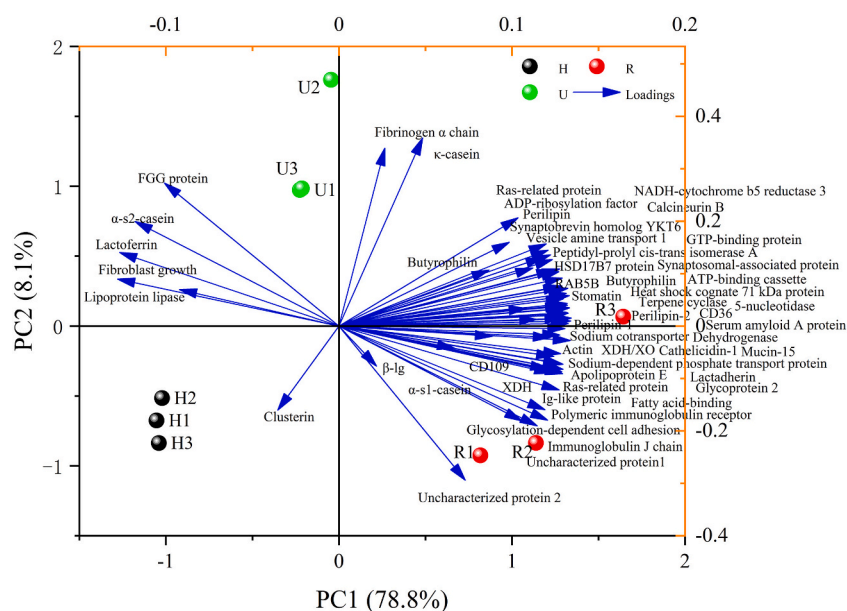

Fig. 4. Biplot of the principal component analysis (PCA) of the MFGM proteins in the different samples; R, raw milk; H, homogenization; U, ultrasonication at 40 W for 60 s. All samples were analyzed and plotted in triplicate.
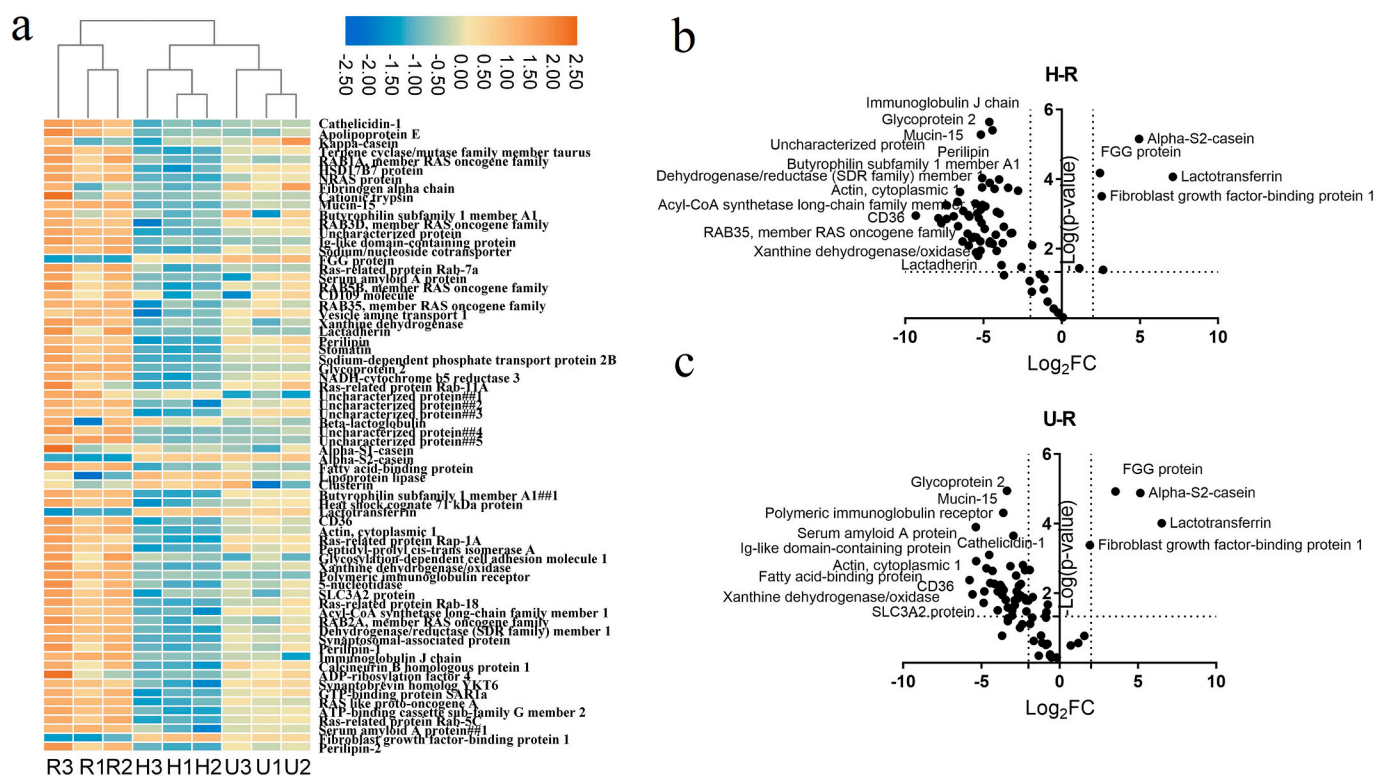
Fig. 5. Comparison of protein profiles between treatments and raw milk presented as a) heatmap, and b and c) volcano plots (b, H: homogenization vs R: raw milk; c, U: ultrasonication at 40 W for 60 s vs R: raw milk).

proteins after shear-homogenization and ultrasonication treatments relative to raw milk. Compared to raw milk, the abundance of most MFGM proteins decreased, including mucin-15, XDH/XO, CD36, glycoprotein 2, perilipin, actin, etc., which were also the proteins with high loadings on PC1 in the PCA analysis. Only a few proteins increased, such as lactoferrin, α-s$_2$-casein, and FGG, again corresponding to the PCA analysis. The MFGM proteins are arranged asymmetrically at the MFG. During homogenization, the native structure of the MFG is broken into smaller droplets causing some loss of native MFGM components, especially the proteins loosely attached at the outer layer of the membrane, such as MUC15, BTN, CD36 and PAS 6/7 (Lopez, Madec, & Jimenez-Flores, 2010), as expected to be decreased, as shown in our results. With a further decrease of the MFG size, the SSA of the milk fat concomitantly increases, resulting in the increased migration of plasma proteins from milk to the interface of the smaller MFG, such as caseins and whey proteins. This could be the reason why lactoferrin and α$_{s2}$ casein increased relatively (Lee & Sherbon, 2002). In general, even though a similar size distribution was found after homogenization, more proteins were lost after shear-homogenization treatment compared with ultrasonication. These findings correspond with our hypothesis that ultrasonication reduces loss of the MFGM proteins by providing a constant acoustic cavitation force thereby avoiding the frequent break-up and re-coalescence of milk fat globules as happens during shear homogenization.

3.5. Volatile components analysis

Although ultrasonication has the potential to simultaneously homogenize milk, reduce microbial load, improve its functional properties and better retain immune-active milk serum proteins (Liu et al., 2020), it may change the flavor of milk due to the extremely strong localized temperatures and pressures (Riener et al., 2009). Principal component analysis was conducted to assess the overall effect of ultrasonication and shear-homogenization on the volatile profiles of milk. Fig. 6 shows the
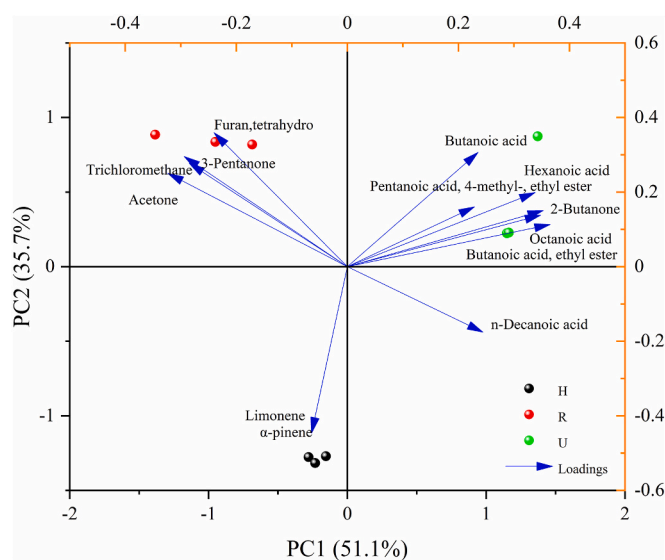

Fig. 6. PCA analysis of Volatile Components in milk; R, raw milk; H, homogenization; U, ultrasonication at 40 W for 60 s.

score and loading plots of the principal component analysis. The first two principal components explain over 86% of the variation in the data set, of which principal component 1 represents 51.1% of the variability and principal component 2 represents 35.7%. Overall, both ultrasonication and shear-homogenization had different effects on the volatile profile of milk. Compared to the raw milk, the ultrasonicated samples were separated along principal component 1, while shear-homogenized samples were separated from raw milk in the direction of component 2. Component 1 was mainly related to octanoic acid, hexanoic acid, butanone, butanoic acid (ethyl ester), acetone, and

trichloromethane; while component 2 was related to limonene and α-pinene.

Fig. 7 shows the relative abundance of volatiles identified in milk after both treatments. Seven compounds, 3-pentanone, butanoic acid, hexanoic acid, octanoic acid, acetone, trichloromethane and furan, were found in untreated milk, of which ketones, trichloromethane, acids, and α-pinene were the major compounds, as also reported in previous literature (Juliano et al., 2014; Riener et al., 2009). As shown in Fig. 7, decanoic acid was found in both ultrasonicated and shear-homogenized milk; butanoic acid ethyl ester, 2-butanone, and pentanoic acid ethyl eater were only found in ultrasonication; limonene, and α-pinene were only found after shear-homogenization. The abundance of acetone and furan decreased after homogenization and ultrasonication, respectively, and trichloromethane decreased after both treatments. Ketones are also known as secondary products of lipid oxidation, and the possible mechanism for the formation of ketones is likely to initiate with the oxidation of β-ketoacids which are then decarboxydized to the corresponding methyl ketones (Valero, Villamiel, Miralles, Sanz, & Martínez-Castro, I., 2001). The content of the free fatty acids hexanoic acid and octanoic acid (already present in raw milk) increased after ultrasonication and homogenization treatment. It was reported that homogenization would disrupt the fat globules simultaneously released the triglycerides encapsulated inside the fat globules into the surrounding medium and facilitated lipolysis by lipase, generating free fatty acids (Bermúdez-Aguirre, Mawson, & Barbosa-Cánovas, 2008; Deeth, 2006). It was also reported that these short-chain fatty acids could be produced by the degradation of triglycerides in milk which can be easily attacked by peroxides (Amador-Espejo, Gallardo-Chacón, Juan, & Trujillo, 2017). Some similar changes in volatile compounds of milk were also reported in other papers, especially after thermal and ultrasonication treatments (Hougaard, Vestergaard, Varming, Bredie, & Ipsen, 2011). In general, the changes of these volatile components in milk were potentially caused by some reactions taking place during the sonication treatment, such as the breakdown of hydroperoxides generated or sonication-induced lipid oxidation. The detailed and exact mechanism for these reactions during ultrasonication is still unclear (Juliano et al., 2014; Riener et al., 2009).

4. Conclusion

In this study, the effects of ultrasonication and shear-homogenization on milk fat globule membrane proteins and volatile components in milk was compared by LC-MS/MS-based proteomics and SPME GC–MS-based volatile analysis, respectively. The results showed that shear-homogenization induced more loss of native MFGM proteins compared with equivalent ultrasonication homogenization, which may be due to more re-coalescence and a longer homogenization time window. In addition, both ultrasonication and shear homogenization increased the content of free fatty acids, without causing the appearance of other specific off-flavors to milk. The results from this study thus suggest that ultrasonication may be a suitable tool for dairy processing, although further research on scaling-up and optimization would be required to realize a large-scale process at a low cost.
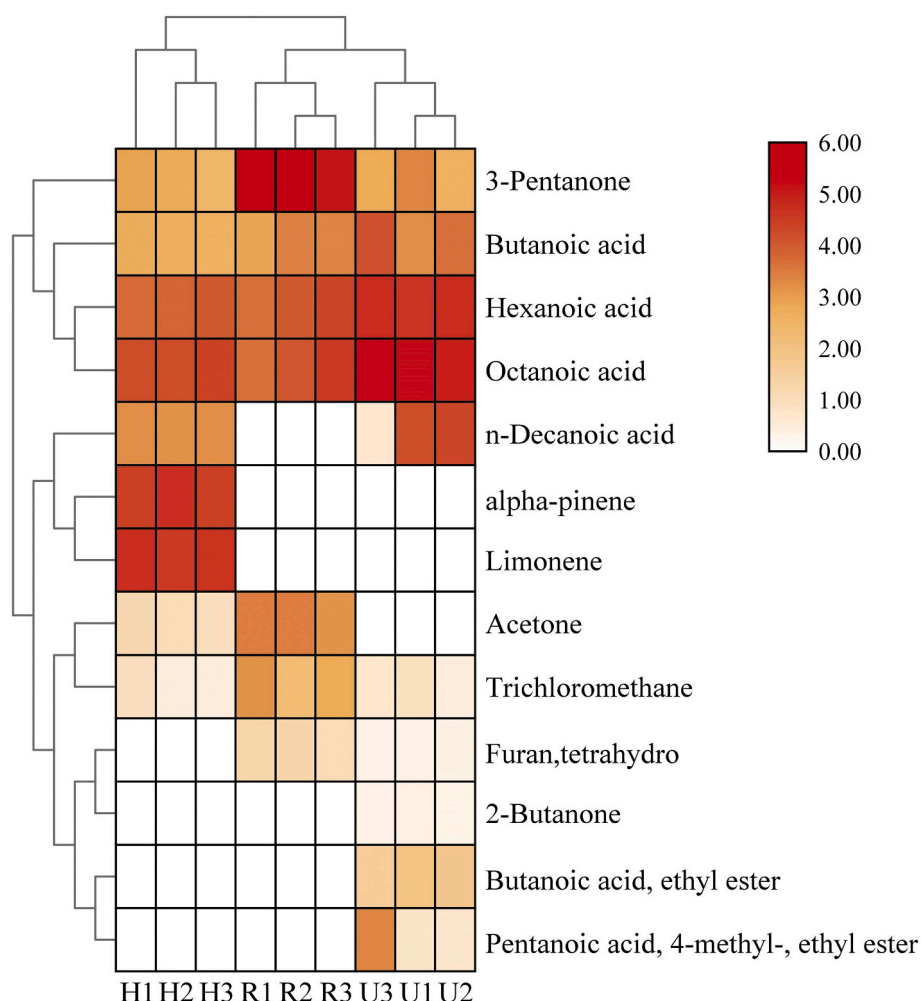

Fig. 7. Heatmap of volatile components in milk after treatments; R, raw milk; H, homogenization; U, ultrasonication at 40 W for 60 s.

Declaration of Competing Interest

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

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