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Improving the microbial and nutritional quality of skim milk using microfiltration combined with thermal and nonthermal techniques

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ABSTRACT

The effectiveness of microfiltration (MF), alone or combined with high temperature short time (HTST) pasteurisation, ultraviolet-C (UV-C), and ultrasonication (US) in improving the microbial and nutritional quality of skim milk was evaluated in comparison with commercial ultra-pasteurisation (UP). Compared with untreated skim milk, MF combined with UV-C and US retained more bioactive proteins, which made these techniques superior to MF combined with HTST. Almost no bioactive proteins survived after UP. MF alone reduced the bacterial load by 2.5 log; however, MF combined with HTST (MH), UV-C (MUV), or US (MUS) can reduce the bacterial load to an undetectable level and extend the microbial shelf-life of skim milk to 40 days. In addition, MUV and MUS did not induce significant protein oxidation. Even though the skim milk after MUV or MUS showed only minor microbial growth, the shelf-life was limited by proteolysis through plasmin activity.

1. Introduction

How to guarantee the microbial safety and retain bioactive components in milk, such as immunoglobulins, enzymes, and lactoferrin (LTF), is still a challenge for the dairy industry all over the world. Thermal treatments, such as UHT (135 °C, 5 s) and ultra-pasteurisation (UP, 125 °C, 5 s), a common extended shelf life (ESL) treatment in dairy processing, can realise a long shelf-life; however, the milk flavour (Al-Attabi, D'Arcy, & Deeth, 2008; Valero, Villamiel, Miralles, Sanz, & Martínez-Castro, 2001) and thermosensitive bioactive components (Liu, Zhang, Zhang, Hettinga, & Zhou, 2020b) are usually severely compromised and damaged. Studies have demonstrated that low-temperature pasteurisation (high temperature short time, HTST, and Holder pasteurisation) is able to retain more bioactive components while it cannot provide a longer shelf-life (7–10 days) even when storing in the refrigerator. To avoid microbial spoilage and retain bioactive proteins, many studies have been conducted on alternative approaches, such as microfiltration (MF), ultraviolet-C (UV-C), and ultrasonication (US) techniques, high-pressure processing (HPP) (Soni, Samuelsson, Loveday, & Gupta, 2021). The effects of UV-C and ultrasonication on milk quality and microbial safety have been described by us previously (Liu et al., 2020a). The results showed that UV-C and US would not decrease the native milk serum protein concentration and could retain the immunoglobulin G (IgG) and lactoferrin (LTF) by ~90% and ~80%, respectively.

To further extend the shelf life of fluid milk, other novel combinations of techniques have also been reported recently. Wang, Fritsch, and Moraru (2019) reported a combination of cold MF (1.4 μm pore size) and HTST pasteurisation and found that HTST, cold MF alone or combined with HTST reduced the microbial load by 2 log, 3.4 log and the near-complete elimination of vegetative microflora in skim milk, respectively, without compromising the sensory quality. In an earlier study, Fernández García and Riera Rodríguez (2014) firstly submitted defatted cow milk to different thermal treatments (73–130 °C, 2–15 s) and then combined these with MF (1.4 μm pore size) at 50 °C to extend the shelf-life, and reported a maximum shelf life of 74 days (room temperature) and 33 days (4–6 °C) after heating at 125–130 °C and 90 °C, respectively. These reported studies mostly concentrate on extending the shelf life of milk while ignoring the retention its bioactive components. In our previous studies, we separately reported the effects of a combination of MF and UV-C/US on the microbial quality and bioactive proteins and found that these two combinations were efficient in removing bacteria while retaining bioactive proteins (Zhang et al., 2021a,b). However, a comprehensive evaluation or

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direct comparison of such combined approaches on improving milk quality in comparison with current thermal ESL treatment (UP) has not been reported yet.

The objective of this study was therefore to evaluate and compare the effects of MF alone, or combined with thermal and nonthermal techniques, and current ESL treatment on bioactive proteins, enzymes, as well as the microbial and proteolytic shelf life of skim milk. The results of this study can help to improve current processing conditions for high-quality milk products.

2. Material and methods

2.1. Raw milk collection and MF treatment

Fifty litres of fresh raw milk was purchased from a local dairy plant and skimmed with a disc milk fat separator (CLARA 20, Alfa Laval, Sweden) and marked as non-treated skim milk (S) which was the control group. MF was operated with a lab-scale GCM-C-03 MF device (Guochu Technology, Fujian, China) equipped with three tubular ceramic membranes (TAMI Industries, Nyons, France) according to the method described by Zhang et al. (2021b). The obtained samples (abbreviated as MF) were collected in sterilised containers for the next treatments and analysis.

2.2. UV-C, ultrasonication, and HTST treatment

The MF sample obtained was subsequently subjected to UV-C and US treatments by an in-house prepared UV-C device and ultrasonication device (shown in Fig. 1). The UV-C and US treatment dosage were set as 39.3 mJ cm^{-2} and 1296 J mL^{-1}, respectively according to our previous studies (Zhang et al., 2021a,b). The milk samples obtained were abbreviated as MUV and MUS, respectively. Another MF sample was submitted to HTST treatment (72 °C for 15 s) with a UHT/HTST 20 heat exchanger system (Power Point International, Toda-Shi, Japan) and abbreviated as MH.

2.3. UP treatment

To compare the shelf life and milk quality with common industrial UP milk, 5-L skimmed milk was directly heated at 125 °C for 5 s (Liu, Zhang, Zhang, Hettinga, & Zhou, 2020c) with the same heat exchanger system and marked as UP. All these treatments were finished within 2 days to avoid microbial spoilage and stored in a refrigerator before further analysis.

2.4. Microbial quality and shelf-life study

Total bacteria count (TBC) and coliform count of the six samples obtained (S, MF, UP, MH, MUV, MUS) were determined using aerobic count plate (Aerobic Count Plate, 3M Petrifilm™, America) according to the method of AOAC (2002). Bacterial spores in milk samples were determined according to a reported method (Fritsch & Moraru, 2008). For the shelf-life study, 30 mL milk samples were placed in sterilised centrifuge tubes and were stored at 4 °C. During refrigeration, three samples from each group were selected randomly every 3 days for TBC analysis until the shelf-life study ended.

2.5. Total protein and native whey protein concentration determination

Total nitrogen (TN), non-protein nitrogen (NPN), and true protein (TP) were determined according to the method of Crowley et al. (2014). Noncasein nitrogen (NCN) was determined according to the method of Santos, Ma, and Barbano (2003), and all protein concentration results were calculated using a conversion factor of 6.38. TP and CN were calculated by (TN-NPN) × 6.38 and (TN-NCN) × 6.38, respectively, and the value of the change in CN/TP (%, change in casein as a percentage of true protein, CN/TP) were calculated and used as an index of proteolysis during storage. To study the effects of processing on whey protein denaturation, the native whey proteins after treatments were separated by acid

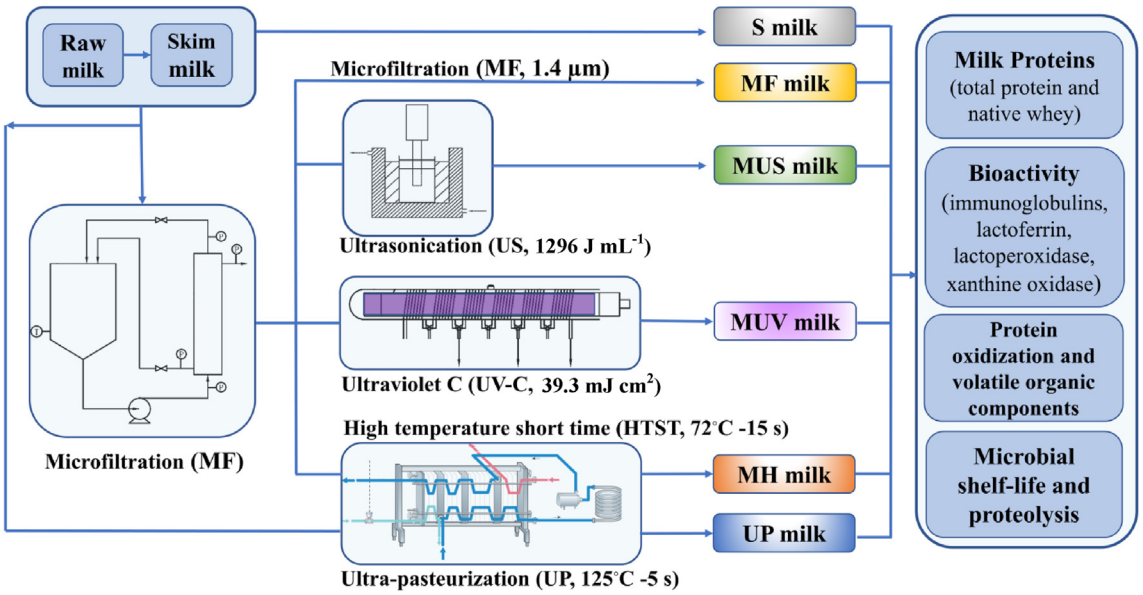

Fig. 1. Research diagram of this study.

precipitation and ultracentrifugation, then determined by BCA assay (Thermo Fisher Scientific, USA) according to reported methods (Liu et al., 2020b).

2.6. SDS-PAGE of native whey proteins

Native whey was diluted 10-fold with Milli-Q water to reach approximately 1 mg mL^{-1} and mixed (1:1, v/v) with 10-μL 2 × concentrated loading buffer (Thermo Fisher Scientific, Massachusetts, USA), and heated at 70 °C for 10 min. Samples were loaded onto 10% bis-Tris gels (NP0301BOX, Thermo Fisher Scientific, Massachusetts, USA) and run in MES buffer (NP0002, Thermo Fisher Scientific, Massachusetts, USA) at 120 V for approximately 2 h at room temperature (XCell SureLock Mini-Cell, Thermo Fisher Scientific). After that, gels were stained by Coomassie Brilliant Blue R-250, destained with 10% ethanol and 7.5% acetic acid, and analysed by a gel scanner (ChemiDoc XRS, Bio-Rad, Hercules, CA, USA).

2.7. Determination of IgG, IgA, IgM, and LTF

Native immunoglobulins (IgG, IgA, and IgM) and LTF concentrations in native milk whey were determined by sandwich enzyme-linked immunosorbent assay (ELISA) test kits purchased from Bethyl Laboratories (USA) according to the method of Heidebrecht and Kulozik (2019). Each sample was measured in three replicates and the results were reported as retention (%, mean ±SD) compared with the S group.

2.8. Lactoperoxidase, xanthine oxidase, and plasmin activity

Lactoperoxidase (LPO) and xanthine oxidase (XO) are important natural antimicrobial enzymes in milk, which form the "LPO-XO system" and provide innate immune protection (Al-Shehri, Duley, & Bansal, 2020). The activity of LPO and XO was determined by a fluorescent method according to the methods of Zou et al. (2020) and Zou et al. (2021) respectively. Each sample was determined in triplicates and the results were reported as retention (%, mean ±SD) compared with the S group.

Plasmin, the principal indigenous milk proteinase, hydrolyses most milk proteins and deteriorates the sensory quality of milk. Thus, it is interesting to study the plasmin activity in milk after different thermal and nonthermal treatments. The plasmin activity was measured according to a reported colorimetric method (Pereda, Ferragut, Buffa, Guamis, & Trujillo, 2008). Each sample was determined in triplicates and expressed as the retention (%, mean ±SD) compared with the S group.

2.9. Carbonyl and sulphydryl group content

During heating, ultrasonication, and UV-C irradiation treatments, proteins are prone to be oxidised by, among others, generated reactive oxygen species (ROS). To reflect the oxidation levels of proteins after treatments, carbonyl and sulphydryl group content were measured (Feng et al., 2015).

2.10. Volatile components analysis (VOC)

The volatile components in milk after different treatments were measured by a headspace solid-phase microextraction (SPME)-GC/MS (Pegasus GC-HRT 4D+, LECO, MI, USA). Briefly, 5 mL milk samples were pipetted into 10-mL GC vials with 10 μL 10 mg mL^{-1} 2-octanol as internal standard, mixed well, and sealed. Volatile components were extracted by an SPME fibre (50/30 μm DVB/CARBOXEN-PDMS) at 60 °C for 30 min and desorbed for 10 min in the GC injection port at 250 °C. A vial filled with air was used as a blank. The initial column temperature was set at 45 °C for 3 min, followed by an increase to 230 °C at a rate of 10 °C min^{-1}, and held for 6 min. Helium was used as a carrier gas fed at a flow rate of 1.0 mL min^{-1}. The MS ion source was maintained at 250 °C. The MS scans were collected in full scan mode, using a mass range of 33–450 *m/z* with electron impact mode at 70 eV. Each component was analysed with Xcalibur software with NIST (National Institute of Standards and Technology) 2005 and Wiley 7 databases for identification, and expressed as μg mL^{-1}.

2.11. Statistical analysis and visualisation

SPSS 18.0 (SPSS Inc., Chicago, IL) was used for all statistical analysis. One-way ANOVA (Duncan test) was used to determine the significance ($p < 0.05$). All the data were expressed as the mean ± SD of three replicates and were visualised by OriginPro 2021(OriginLab, MA, USA).

3. Results and discussion

3.1. Microbiological analysis

Table 1 shows the microbial quality of the milk samples. The TBC in skim milk was 4.4 log, which meets the standards of China (2×10^6 cfu mL^{-1}) and the USA (1×10^5 cfu mL^{-1}). After MF treatment, the TBC was decreased to less than 2 log, which meets both the standards of pasteurised milk of China (5×10^4 cfu mL^{-1}) and USA (2×10^4 cfu mL^{-1}). UP killed all the bacteria in skim milk, while MF could not remove all the bacteria but could remove all the *Escherichia coli*. This is in accordance with previous reports (Fernández García, Álvarez Blanco, & Riera Rodríguez, 2013), as the size of some species of bacteria is smaller than the pore size (1.4 μm), such as *Pseudomonas fluorescens, Yersinia enterocolitica,* and *Brucella abortus.* However, when MF was further combined with either HTST, UV-C, or US, all the bacteria were killed, suggesting that these combined techniques were effective in removing bacteria equivalent to or better than commonly used UP. For heat-resistant spores, it was reported that HTST could not completely kill these spores (Zhang et al., 2021a) while almost no spores survived after UP, MF treatment alone, or when MF was combined with other treatments (shown in Table 1), suggesting that MF is efficient in removing spores. These data are in accordance with our previous reports (Zhang et al., 2021a,b).

3.2. Protein profile analysis

To check the influence of different treatments on the milk proteins, the total and native whey protein concentrations in processed

Table 1
Microbial quality of milk subjected to different processing methods.[a]

Process	Microbial levels (log cfu mL^{-1})		
	TBC	*E. coli*	Spores
S	4.4 ± 0.1[b]	1.9 ± 0.1	1.7 ± 0.1
MF	1.9 ± 0.2[a]	ND	ND
UP	ND	ND	ND
MH	ND	ND	ND
MUV	ND	ND	ND
MUS	ND	ND	ND

[a] Abbreviations are: S, skim milk; MF, microfiltration; UP, ultra-pasteurisation; MH, MUV and MUS, MF combined with high temperature short time pasteurisation, ultraviolet-C, and ultrasonication, respectively. Different letters in the same column indicate a significant difference (p <0.05), ND indicates not detectable.

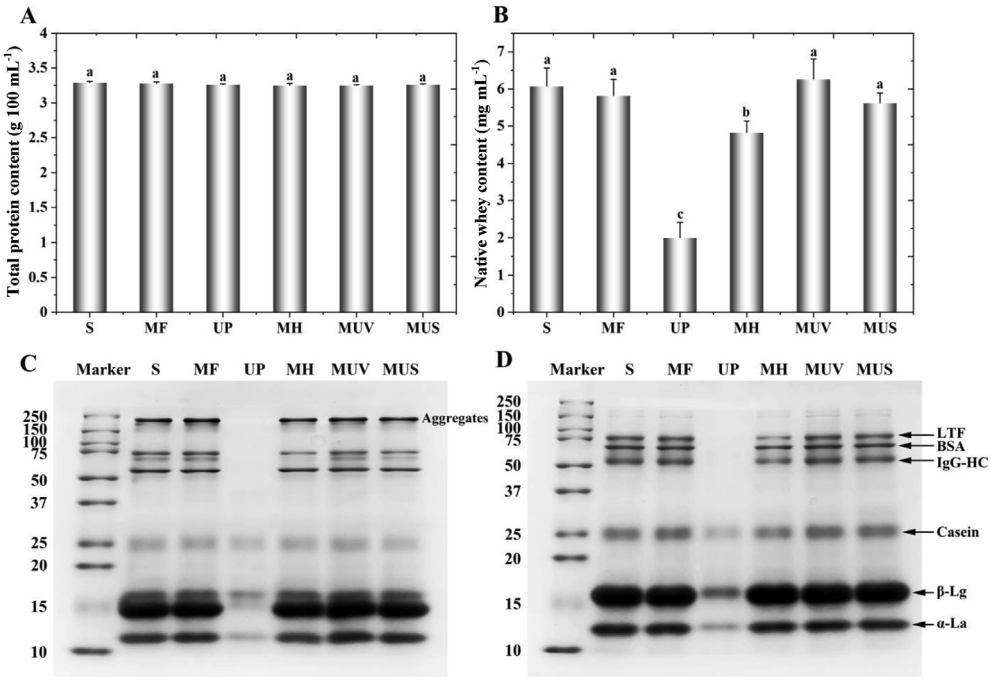

Fig. 2. Total (A) and native whey protein (B) concentration, and SDS-PAGE (C) of native whey protein under non-reducing and reducing conditions. Abbreviations are: S, skim milk; MF, microfiltration; UP, ultra-pasteurisation; MH, MUV and MUS, MF combined with high temperature-short time pasteurisation, ultraviolet-C, and ultrasonication, respectively.

milk were determined, and the results are shown in Fig. 2A and B, respectively. As shown in Fig. 2A, UP, MF alone, or its combination with other treatments did not decrease the content of total milk proteins (according to the Kjeldahl method) even though UP induced the denaturation of milk proteins. The colloidal and serum proteins in milk are able to permeate the pore size of 1.4 μm, so MF should not have influenced either of these protein fractions. However, when the milk samples were adjusted to pH 4.6 and ultracentrifuged, the concentration of native whey in UP and MH was significantly decreased, as shown in Fig. 2B. Compared with caseins, whey proteins are more susceptible to thermal treatments and may denature and aggregate with caseins (Law & Leaver, 2000). These denatured whey proteins precipitated together with caseins during pH adjustment and were removed by ultracentrifugation during sample preparation. These results obtained are in accordance with our previous studies (Liu et al., 2020c; Zhang et al., 2021b).

Fig. 2C and D display the SDS-PAGE of native whey proteins under non-reducing and reducing conditions, respectively. Under reducing conditions, it could be observed that the proteins in whey were mainly composed of β-lactoglobulin, α-lactalbumin, LTF, BSA, IgG, and remaining non-micellar casein. The intensities of whey protein bands obviously decreased after UP treatment while they were slightly decreased after MH, which is in accordance with the native whey concentration shown in Fig. 2B.

3.3. Bioactive proteins (IgG, IgA, IgM) and LPO and XO activity

To study the effects of different treatments on bioactive proteins and enzymes in skim milk, the concentrations of IgG, LTF, IgA, and IgM were determined by ELISA and LPO and XO activity were determined by fluorescence spectrophotometry. As shown in Fig. 3, different treatments had a similar effect on these bioactive proteins and enzymes, although not exactly the same. In detail, the contents of immunoglobulins and LTF and activities of LPO and XO could almost not be detected after UP treatment while MH retained 40–70% of bioactive proteins and enzyme activity, as the heat intensity of UP treatment is much higher compared with HTST.

Liu et al. (2020c) studied the effects of heat intensity on bioactive proteins retention and also found that UP treatment lost all these bioactive proteins while HTST retained 30–60% of bioactive proteins. Compared with the S group, MF alone had no effects on these bioactive proteins and enzymes; however, when MF was combined with HTST, the contents of bioactive proteins and enzyme activities decreased, suggesting that HTST induced heat damage resolved as protein denaturation and aggregation. It was reported that HTST decreased the LPO activity by ~20% (Griffiths, 1986), which is in agreement with our findings here.

When MF was combined with UV or US, it showed a better performance in retaining these bioactive components compared with MH but was inferior in comparison with MF alone. This suggests that these non-thermal treatments also have a slight influence on these bioactive components. In our previous study, we also found that both UVC and US decreased the concentration of LTF and IgG by ~20% and ~10%, respectively. The exact reason for UVC-induced protein damage is not known yet; however, we hypothesise that the US-induced protein damage could be related to the "side effects" from cavitation. As ultrasonic waves propagate through a liquid, they would generate water vapour bubbles. These bubbles will expand due to the pressure gradients and collapse rapidly, resulting in a local high temperature, high pressure, and strong shear force, which may denature proteins (Shanmugam, Chandrapala, & Ashokkumar, 2012). Overall, when MF is combined with these non-thermal treatments, they showed a better performance in retaining bioactive proteins and antimicrobial enzymes in skim milk than MH or industrial UP treatment.

3.4. Protein oxidation characterisation

Protein oxidation is a big concern during dairy processing, as it may negatively affect the sensory quality and native functions supporting human health (Feng et al., 2015; Rutherfurd, Montoya, & Moughan, 2014). Determination of carbonyl content is a

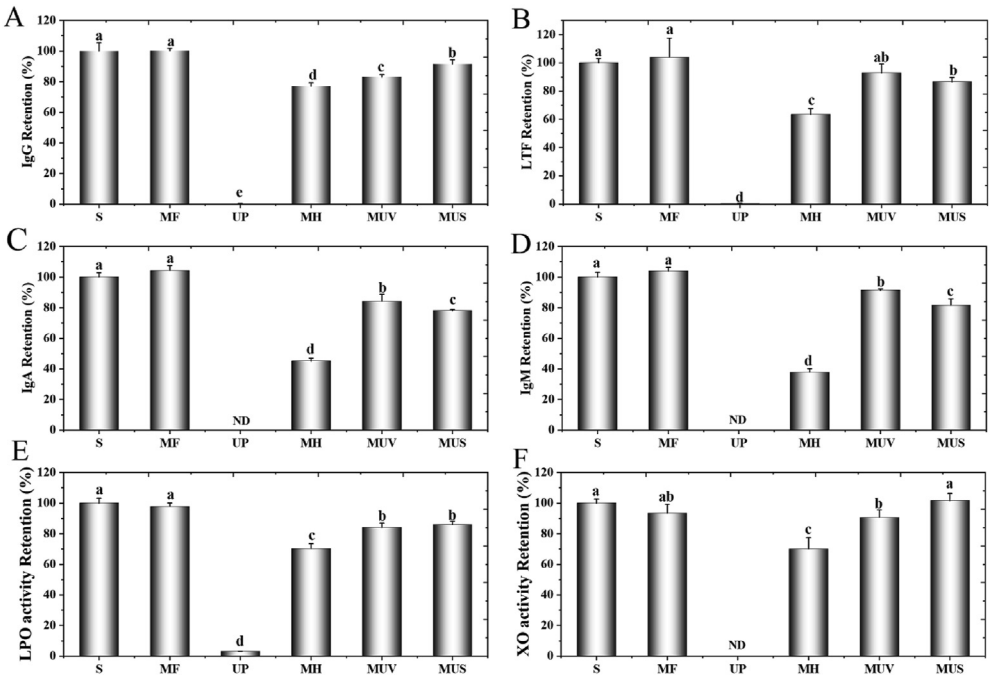

Fig. 3. IgG (A), lactoferrin (B), IgA(C), IgM (D), lactoperoxidase activity (E), and xanthine oxidase activity (F) retention in milk samples after treatments; different letters indicate a significant difference (p <0.05), and ND indicates not detectable. Abbreviations are: S, skim milk; MF, microfiltration; UP, ultra-pasteurisation; MH, MUV and MUS, MF combined with high temperature-short time pasteurisation, ultraviolet-C, and ultrasonication, respectively.

common method to evaluate the oxidation status of protein, and Fig. 4A and B display the carbonyl and sulphydryl contents, respectively, in milk protein after the different treatments. Compared with the skim milk, MF alone or its combination with HTST or nonthermal (UV and US) technologies have no significant influence on the protein oxidation as the carbonyl and sulphydryl group content did not change significantly (p >0.05); however, UP treatment resulted in a significant increase in the carbonyl content as well as a decrease in the sulphydryl content. This indicates that UP induced oxidation to milk proteins. In milk, proteins can be carbonylated by modification of the side chains of lysine, arginine, and cysteine residues through formation of either dicarbonyl compounds, such as glyoxal (GO), methylglyoxal (MGO), and 3-deoxyglucosone (3-DG), or lipid peroxidation products and heavy metal ions (e.g., iron) (Meyer, Baum, Vollmer, & Pischetsrieder, 2012). It was reported that the number of carbonylation sites increased with the harsher processing conditions, especially in UHT and infant formula products (Milkovska-Stamenova, Mnatsakanyan, & Hoffmann, 2017), which is in agreement with our findings here.

3.5. Volatile organic components

To characterise the effects of different treatments on the flavour of milk, SPME-GC-MS was used to analyse the volatile organic components (VOC). Fig. 5A shows the principal components analysis (PCA) of VOC, and different samples were clustered according to their treatments and chemical characteristics, suggesting that different treatments changed the native VOC of skim milk in a different way. In general, the first two principal components account for ~65% of the total variance in the data set, and untreated skim milk, and skim milk after MF and MUV formed a single cluster, suggesting that they have a similar VOC pattern. Compared with MUS and UP milk, MH milk has more similar VOC components to untreated skim milk.

The PCA score plot presented in Fig. 5A shows that UP milk was separated from skim milk along PC1 direction, whereas MUS milk was separated from other skim milk samples along PC2 direction. PC1 mainly contains heptanal, benzaldehyde, furan, octanedione, 2-nonanone, and octanal and PC2 mainly contains tetradecane, ethylbenzene, hexanal, hexanoic acid. Most of these components were also found in previous studies on milk (Amador-Espejo, Gallardo-Chacón, Juan, & Trujillo, 2017; Riener, Noci, Cronin, Morgan, & Lyng, 2009).

In detail, a total of 42 VOC were identified in all samples, including aldehydes, acids, ketones and alcohols, esters, and others (Fig. 5B). As shown in Fig. 5B, the main VOC in untreated skim milk are carboxylic acids and aldehydes; however, the total content and number of aldehydes both increased after UP treatment. Acetaldehyde, heptanal, decanal, 2-nonenal, 2-octenal, 2-heptenal were detected after UP treatment while not in skim milk and hexanal was detected in MUS milk but not in other groups (see Supplementary material Table S1). Among these increased aldehydes, nonanal had the highest relative increase after UP treatment, followed by octanal, heptanal, and decanal. It was reported that these straight-chain aldehydes are probably derived from the decomposition of hydroperoxides and autoxidation of unsaturated fatty acids through heating (Reis et al., 2020; Vazquez-Landaverde, Torres, & Qian, 2006a). Besides, the hydrocarbons increased after MUS treatment; MF, MUV, and MH had fewer effects on the VOC of skim milk.

The source of hydrocarbons in milk is complicated and is not stable; the hydrocarbons in MUS-treated milk mainly contain methylbenzene, tridecane, ethylbenzene. Riener et al. (2009) also found that ultrasonication increased the benzene, methylbenzene, and a series of aliphatic 1-alkenes, and reckoned that this is possibly induced by high local temperatures and pressure through cavitation. One volatile sulphur compound (carbon disulphide) was detected in the UP milk samples (Supplementary material Table S1), and it is considered a breakdown product of other sulphur compounds (Vazquez-Landaverde, Torres, & Qian, 2006b). However, its sensorial threshold is relatively high and this would not thus affect the aroma of heated milk, although carbon disulphide could be a good marker of heating. In general, UP treatment

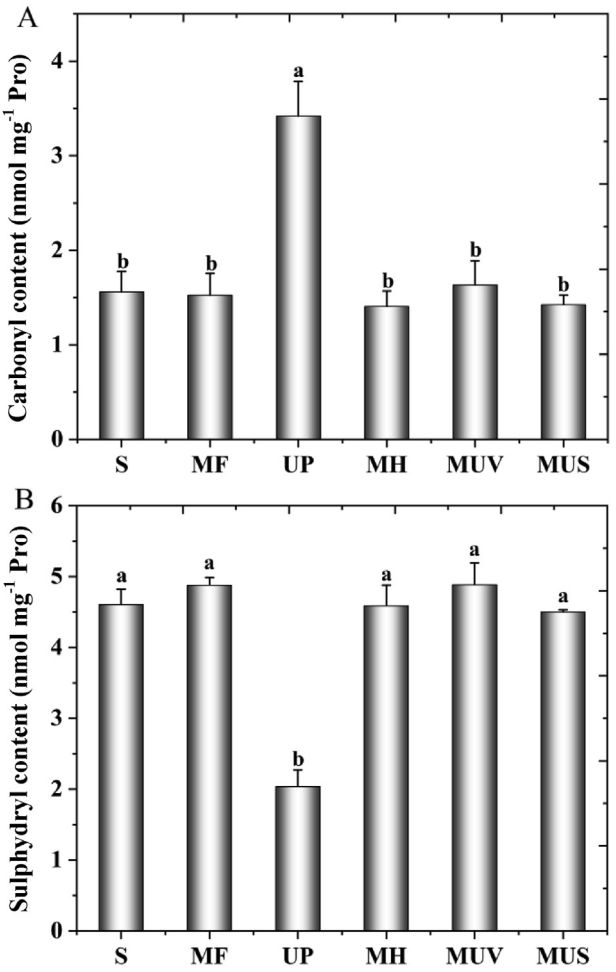

Fig. 4. Carbonyl and sulphydryl group contents in skim milk after different treatments; different letters on the columns indicate significant difference (p <0.05). Abbreviations are: S, skim milk; MF, microfiltration; UP, ultra-pasteurisation; MH, MUV and MUS, MF combined with high temperature-short time pasteurisation, ultraviolet-C, and ultrasonication, respectively.

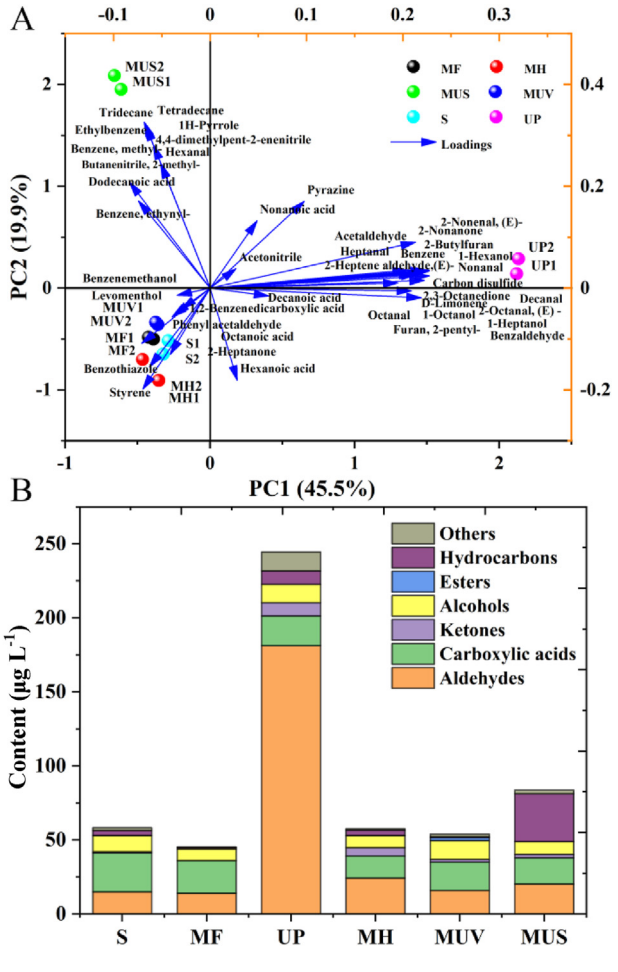

Fig. 5. PCA (A) and volatile components (B) contents in skim milk after treatments. Abbreviations are: S, skim milk; MF, microfiltration; UP, ultra-pasteurisation; MH, MUV and MUS, MF combined with high temperature-short time pasteurisation, ultraviolet-C, and ultrasonication, respectively.

had a great influence on the VOC of skim milk, and this seems mostly related to the oxidation effects of milk proteins and remaining lipids in skim milk, which is in accordance with the protein oxidation levels shown in Fig. 4.

3.6. Shelf-life and milk quality studies

Fig. 6A and B demonstrate the changes of TBC (total bacteria count) and proteolysis levels in skim milk during the shelf-life study. As shown in Fig. 6A, the microbial load in raw skim milk increased rapidly and it was no longer monitored after 8 days. All the treatments used in this study were able to realise microbial safety as the TBC decreased from ~4.5 log to below 2 log or even an undetectable level. During the shelf-life study, the TBC in skim milk after MF treatment had a slower microbial growth rate in the first 4 days, but increased rapidly after the first 4 days and exceeded the limits of China's national standard (GB) and FDA standard after 12 days storage. However, when MF was combined with either HTST, UV-C, or US, no bacteria survived. During the whole shelf-life study, no or only low TBC counts were found, realising a similar effect as UP treatment. The findings reported here agree with our previous reports (Zhang et al., 2021a,b) where MF combined with UV-C, or ultrasonication was able to extend the shelf-life of skim milk.

Despite the insignificant microbial growth in the multiple samples, proteolysis still occurred. According to the study of Santos et al. (2003), when the decrease in CN/TP (%) reached 4.76%, 50% of participants would perceive off-flavours due to proteolysis. Thus, we used this value as the threshold in this study. After ~4 days refrigerated storage, the decrease in CN/TP (%) in untreated skim milk firstly exceeded 4.76%, followed by MF milk after ~12 days, MH milk after ~20 days, and MUV and MUS milk after ~24 days. After storage for ~32 days, all the samples except UP milk exceeded the threshold. UP treated skim milk did not show obvious proteolysis during the whole refrigerated storage.

The proteolysis during shelf-life is mainly related to the protease in milk, including endogenous proteases and microbial proteases. Generally, the proteolysis in untreated skim milk is related to both; however, the proteolysis in treated milk samples is mainly related to the roles of endogenous plasmin as most bacteria were removed and microbial proteases are not active at refrigerator temperature. The reason for proteolysis was investigated by determining the plasmin activity, which is shown in Fig. 6C. Compared with the

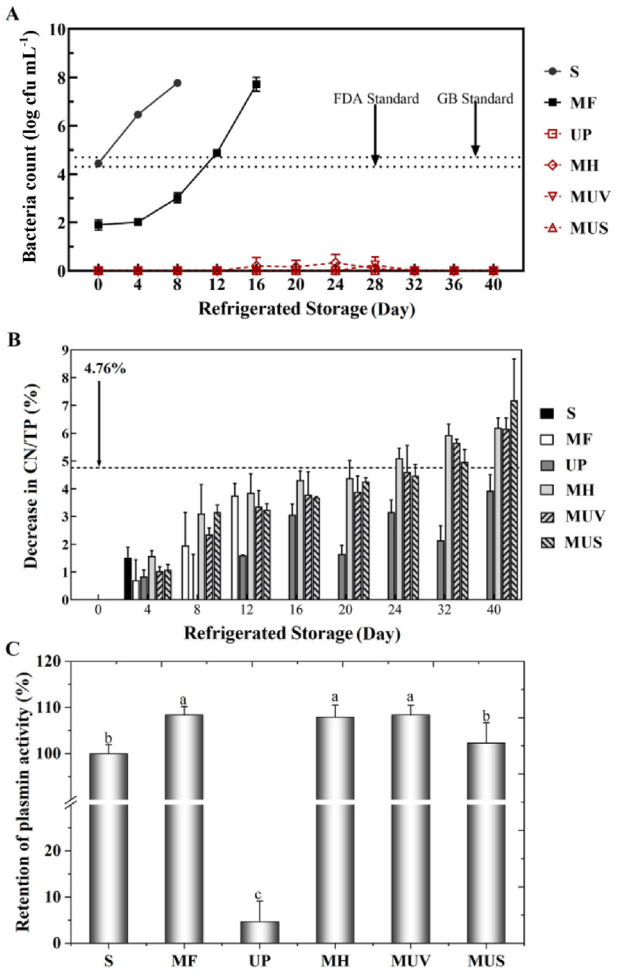

Fig. 6. Total bacteria count (A), protein proteolysis (B), and plasmin activity retention (C) of skim milk during the shelf-life study; The dashed lines in panel A represent China's national standard (GB) and FDA standard for pasteurised milk respectively. Abbreviations are: S, skim milk; MF, microfiltration; UP, ultra-pasteurisation; MH, MUV and MUS, MF combined with high temperature-short time pasteurisation, ultraviolet-C, and ultrasonication, respectively.

untreated skim milk, MF, MH, and MUV slightly increased the plasmin activity by ~10%, whereas UP significantly decreased the plasmin activity by ~95%.

Wang et al. (2019) also reported that plasmin activity in clod MF skim milk was slightly higher than in raw skim milk, but not significantly. This slight difference between these two studies is potentially attributed to temperature difference. In this study, the actual temperature during MF processing was maintained between 45 °C and 50 °C, which may inactivate the heat-labile plasmin inhibitors and enhance plasminogen activation, leading to an increase in plasmin activity. Lu et al. (2009) also found that heating between 55 °C and 75 °C for 15 s would not inactivate the plasmin, whereas heating above 85 °C would result in a decrease in plasmin and plasminogen-derived activities. UP treatment largely inactivated plasmin activity in skim milk, which agrees with previous studies (Lu et al., 2009; Rauh et al., 2014; Wang et al., 2019). Compared with MF treatment, MUS decreased the plasmin activity slightly but significantly. This could be explained by the physical effects induced from ultrasonication, including mechanical vibration, acoustic streaming, shear forces, local high pressure, and temperature, which is able to denature the enzymes, leading to impaired enzyme activity. The proteolysis of casein (Fig. 6B) was generally consistent with the plasmin activity (Fig. 6C).

In general, MUV and MUS treatments extended the microbial shelf-life of skim milk to at least 40 days, but proteolytic shelf-life was limited to 24 days by casein proteolysis through plasmin.

4. Conclusions

The current study investigated the effectiveness of MF combined with thermal and nonthermal techniques in improving the shelf-life and quality of skim milk in comparison with commercial UP treatment. The data obtained here confirmed that both MUV and MUS can remove microorganisms from skim milk more effectively than MF alone, and retain more bioactive proteins and enzymes (80% retention relative to raw skim milk) than thermal MH and UP treatments. In addition, MUV and MUS did not result in significant protein oxidation or affect the volatile components in milk compared with UP. MF combined with nonthermal techniques led to a total microbial reduction of ~4 log and microbial shelf life of >40 days during refrigerated storage. However, the shelf life of the non-thermally processed skim milk was limited to ~24 days due to plasmin proteolysis. Even though the obtained results indicate that MF combined with nonthermal techniques greatly improved milk quality, further research on implementation, e.g., equipment design, is needed to realise large-scale processes at a low cost. Besides, nonthermal techniques may not inactivate the endogenous enzymes in milk, such as plasmin and lipase, which is a concern that needs to be considered before use of such techniques for industrial dairy processing.

Credit author statement

Yaowei Liu: Conceptualization, Methodology, Writing-original draft. **Wenjin Zhang**: Conceptualization, Investigation, Validation. **Kasper Hettinga**: Supervision, Validation, Writing-reviewing and editing. **Lina Zhang**: Validation, Funding acquisition. **Peng Zhou**: Supervision, Writing-reviewing and editing, Project administration.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2022.105466.

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