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Characterizing the changes of bovine milk serum proteins after simulated industrial processing

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ABSTRACT

This study investigated the changes and lactose glycosylation of milk serum proteins under simulated industrial processing conditions, including raw milk (R), holder pasteurization (L), high temperature short time pasteurization (H), extended shelf life (E), ultra-high temperature sterilization (U) and spray-drying (S). Through label-free proteomics, 433 proteins were identified in the samples. Several immune-related proteins, such as lactoferrin, complement C3, lactadherin, cystain, and lactoperoxidase, decreased in abundance after severe thermal treatments, while the abundance of caseins increased. No immunoglobulins and xanthine oxidase could be detected in milk after E, U or S treatments while 30%–60% of immunoglobulins was retained after pasteurizations. In detail, lactoferrin showed a better retention in H treatment while IgG showed a better retention in L treatment. UPLC-MS results showed that a slight lactose glycosylation occurred to α-LA and β-LG after severe thermal treatments (E, U and S). In addition, the results of LC-MS/MS based proteomics were verified by determining the lactoferrin and IgG content using ELISA. The observations here would update current information on the changes of milk proteins during traditional thermal processing and help to optimize current dairy processing.

1. Introduction

Milk has a complex biological composition of nutrients and bioactive components, and consuming milk and dairy products has been associated with physical development and health promotion (Tunick et al., 2016; Walzem, Dillard & Germanet, 2002). The main nutrients of milk are fat, proteins, lactose, minerals and vitamins (Li, Li, Wu, et al., 2020). A wide variety of immune-related proteins in milk, such as lactoferrin, lactoperoxidase, and immunoglobulins, are beneficial for the immune system development as well as fighting against pathogen invasion, epistemic development, and regulating intestinal microbes of infants (Brick et al., 2017; Lu et al., 2016; Ma, Zhang, Wu, & Zhou, 2019; Yang et al., 2018). However, occurrence of pathogens, aflatoxin and other biochemical contaminations in milk remain to be a risk for health (Ketney, Santini, & Oancea, 2017), thus industrial treatments and quality control of raw milk play vital roles in dairy industry. Common industrial treatments include homogenization, holder pasteurization (62.5 °C for 30 min) or HTST (72 °C for a minimum of 15 s), UHT (135–150 °C for 4–7 s), and spray-drying. These treatments are usually applied in dairy industry to ensure the microbial safety and extend the shelf life of dairy products; otherwise, these treatments would result in changes of milk components and protein quality, especially for the immune-related proteins in milk serum. Recently, Manzi and Durazzo (2017) compared the antioxidant properties of UHT, microfiltered and pasteurized milk, and found that UHT milk showed the highest values in all assays, probably due to the development of antioxidant compounds during severe heating treatment. Moreover, most proteins in milk would undergo various structural modifications depending on heating time, temperature, water activity and other factors. These changes can either adversely impair the functional properties (Bogahawaththa, Chandrapala, & Vasiljevic, 2017; Escuder-Vieco, Espinosa-Martos, Rodríguez, Fernández, & Pallás-Alonso, 2018; Manzo, Nicolai, & Pizzano, 2015; Xiong, Li, Boeren, Vervoort, & Hettinga, 2020) or enhance the bioavailability of milk proteins, such as its digestibility (Ye, Cui, Dalgleish, Singh, & 2016; Ye et al., 2019). It is therefore of significance to quantify the changes and identify the potential chemical modifications from the point of view of nutritional and immunological quality control of dairy products and milk.

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With the help of proteomics technology, hundreds of low-abundant milk proteins have been identified in milk, which provided a better and further insights into the biological functions of milk proteins (Li, Li, Cao, et al., 2020; Lu et al., 2018; Qu et al., 2019; Yang et al., 2016). Recently, several studies have investigated the quantitative changes of milk serum proteins during different heating process (Brick et al., 2017; Xiong et al., 2020; Zhang et al., 2016), showing a decrease in protein abudance due to denaturation and aggregation of these proteins. In our previous study, the changes in bioactive milk serum proteins was also tracked and characterized by mimicking the process of milk powder by spray-drying (Liu, Zhang, Han, Zhang, & Zhou, 2020). However, information on qualitative and quantitative analysis of milk serum proteins in different dairy products is still quite rare, especially after being treated under simulated industrial processing conditions. Besides loss of function through denaturation and aggregation, it was reported that heat processing and improper storage (high temperature and relative humidity) of dairy products might also cause other chemical modifications, such as Maillard reactions (Le, Bhandari, & Deeth, 2011; Fenaille et al., 2006).

This study not only applied a proteomics approach to investigate the changes in milk serum proteins in several common thermal treatments, but also verified the results of proteome by determining the lactoferrin and immunoglobulins content using ELISA. In addition, the degree of glycation of α-LA (α-lactalbumin) and β-LG (β-lactoglobulin) after different heating treatments was also checked by UPLC-MS as well as the xanthine oxidase activity. We hope the results of this study would provide some more detailed information on changes in active milk serum proteins during industrial processing, which may be useful for developments of dairy industry.

2. Materials and methods

2.1. Raw milk collection and processing

Fresh raw milk was purchased from a local dairy plant and was immediately shipped to the laboratory with an ice-box in 2 h. Fresh milk was homogenized by JHG-Q54-P110 homogenizer (Pulisheng Co., Ltd., Shanghai, China) at 25 MPa and then divided into 6 groups, namely the raw milk (R), holder pasteurized milk (L), HTST milk (H), ESL treated milk (E), UHT treated milk (U) and spray-dried milk powder (S). In detail, the holder pasteurized milk was processed at 63 °C for 30 min in a water bath and quickly cooled to below 10 °C with ice-water mixture; the HTST, ESL and UHT milk was processed at 72 °C for 15 s, and 125 °C and 135 for 5 s, respectively with a UHT/HTST 20 heat exchanger system (Power Point International, Toda-Shi, Japan). The milk powder was prepared by heat-treatment at 90 °C for 15 s to inactivate lipase as well as most bacteria (Sun, Wang, Wang, & Guo, 2018; Liu et al., 2020), then spray dried with a Mini Spray Dryer B-290 (Buchi Laboratoriums-Technik AG, Flawil, Switzerland) with an inlet temperature at 170 °C and outlet temperature at 85 °C. Then the obtained milk powder was dissolved into warm deionized water (45 °C) at 1:10 (m/v) under magnetic stirring according to Zhang et al. (2016). All these treatments were finished within 2 days to avoid the microbiological spoilage of milk. The whole experimental design is shown in Fig. 1.

2.2. Separation of native milk serum samples

A part of processed milk was centrifuged at 2000 g for 15 min to obtain skim milk. To obtain the native milk serum, the pH of skim milk was adjusted to 4.6 with 1 mol/L HCl to remove the casein and denatured serum proteins (Xiong et al., 2020), then milk serum was transferred to ultracentrifuge tubes and ultra-centrifuged at 100,000×*g* for 90 min at 25 °C (Optima L-80, Beckman Coulter, USA). After the ultracentrifugation, samples were separated into three phases. Residual milk fat was in the top layer, milk serum was in the middle layer, and casein micelles with aggregated/denatured whey protein was in the bottom layer (pellet). The milk serum was carefully collected, and a portion of milk serum was used to determine its protein concentration by BCA assay (Thermo Fisher Scientific, USA). The other milk serum samples were stored at −80 °C before further analysis.

2.3. SDS-PAGE of milk serum proteins

SDS-PAGE was used to characterize the effect of thermal processes on the major milk proteins under reducing and non-reducing conditions. Milk serum was diluted to 2 mg/mL with MilliQ water and mixed with equal loading buffer (25 mmol/L Tris-HCl, 2% SDS (w/v), 10% glycerol (v/v), and 0.1% Bromophenol blue (w/v)). Milk serum samples (10 μL)

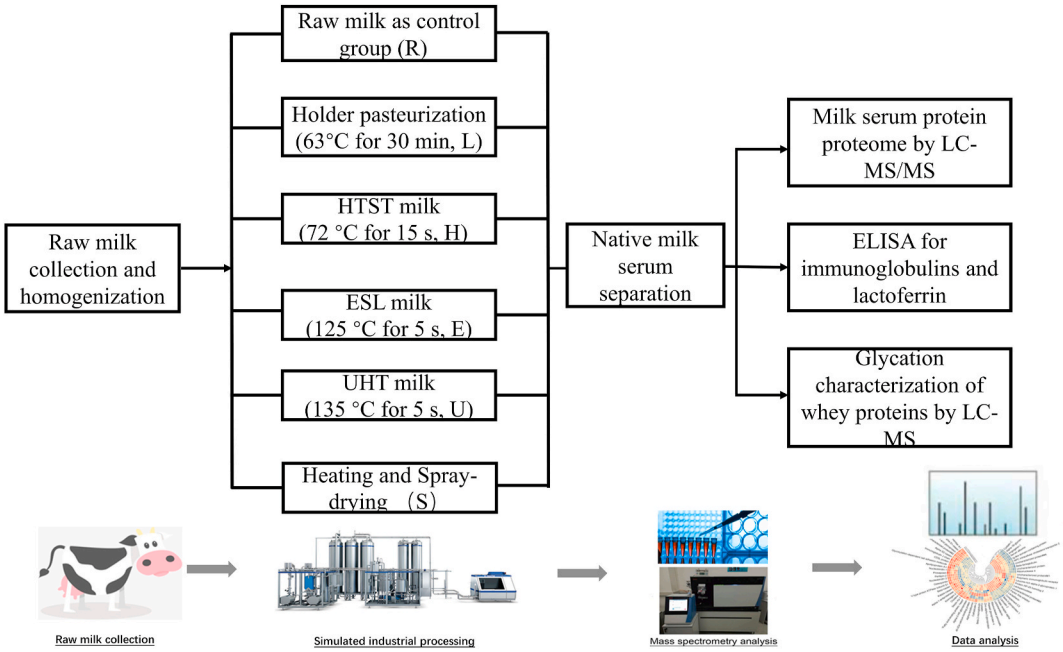

Fig. 1. Experiment design and workflow of this study.

was loaded in each lane. The gels were firstly run at 40 V for the stacking gel and then 80 V for the separating gel until ending the experiment. Coomassie Brilliant Blue (R-250) was used to stain the protein bands for 1 h, then distained by 10% ethanol and 7.5% acetic acid in MilliQ water overnight.

2.4. Determination of immunoglobulins and lactoferrin by ELISA

The content of lactoferrin and immunoglobulins (IgG, IgA and IgM) in milk serum were determined by commercial enzyme-linked immunosorbent assay (ELISA) test kits from Bethyl Laboratories, USA (Cat. No. E10-126, 118, 131, and 101) according to the provided protocol. Briefly, milk serum samples were diluted for 500–1000 times with MilliQ water (Heidebrecht & Kulozik, 2019). 100 μL sample or standard was added into a 96-well plate. After blocking, incubation, washing and TMB coloring, the absorbance of three replicates was recorded by a Multiskan FC plate reader (Thermo Fisher Scientific, Karlsruhe, Germany) at 450 nm. The standard curves were generated as a 4-parameter curve fit using Soft-Max Pro. The final concentrations of immunoglobulins and lactoferrin were expressed as the average of three replicates.

2.5. Determination of xanthine oxidase (XO) activity

The activity of xanthine oxidase (XO) of milk samples was determined by a commercial assay kit from Sigma-Aldrich (Catalog NO. MAK078) according to the technical bulletin (Liu et al., 2020; Malik, Dhiman, & Khatkar, 2019). In brief, milk samples after different treatments were diluted 20–30 times with the provided assay buffer. 50 μL diluted samples were added into a 96-well plate, then another 50 μL reaction mixture (prepared according to the bulletin) was added into each well. The plate was then incubated at 25 °C in darkness and was read by a plate reader every 5 min. Each sample was determined in triplicates. The xanthine oxidase activity was calculated according to the following equation and expressed in relative retention (%) in comparison to the raw milk.

$$\text{Xanthine oxidase activity} = \frac{A \times \textit{sample dilution factor}}{(T_{initial} - T_{final}) \times V}$$

where A = the amount (nmole) of H_2O_2 generated between $T_{initial}$ and T_{final}.

$T_{initial}$ = time (in minute) of first reading.
T_{final} = time (in minute) of penultimate reading.
V volume (mL) of sample added to each well.

2.6. Sample preparation for LC-MS/MS analysis

The preparation of samples for LC-MS/MS was referred from a published method (Liu et al., 2020). In detail, milk serum (100 μg protein) was pipetted into a 3 kDa Microcon device YM-10 (Millipore), followed by a centrifugation at 12,000 g for 10 min. Subsequently, 200 μL of 50 mmol/L ABC (ammonium bicarbonate) was added to the samples and centrifuged once again. Then the samples were reduced by 10 mmol/L DTT (final concentration) at 56 °C for 1 h and alkylated by 20 mmol/L IAA (final concentration) at room temperature in darkness for 1 h, followed by another centrifugation at 12,000 g for 10 min and washing with 50 mmol/L ABC. Then, 100 μL of 50 mmol/L ABC and trypsin (Promega V 5280, Madison, WI, USA) was added to the protein at a ratio of 1:50, and the samples were incubated at 37 °C overnight while mildly shaking. After this incubation, the device was centrifuged at 12,000 g at 4 °C for 10 min. Then, another 100 μL of 50 mmol/L ABC was added to the device and centrifuged once. The obtained peptides were freeze-dried and resuspended in 50 μL formic acid (0.1% in MillQ water) before injection into LC-MS/MS system.

Of the digested serum fractions, 10 μL were injected onto a 0.3 mm × 5 mm Acclaim™ PepMap™ 100 C18 HPLC column (nano Viper™, particle size 5 μm, Thermo, USA). A pre-assembled column filled with a 0.075 mm × 150 mm Acclaim™ PepMap™ C18 analytical column (particle size 3 μm) was used to elute the peptides with acetonitrile gradient at 300 nL/min. The mobile phase was composed of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The samples were injected into a Q Exactive HF Obitrap MS/MS (Thermo Finnigan, San Jose, CA, USA). The parameters of gradients was listed: 4%–10% (v/v) solvent B for 5 min, 10%–22% (v/v) solvent B for 80 min, 22%–40% (v/v) solvent B for 25 min, 40%–95% (v/v) solvent B for 1 min, and 95% for 9 min. A mass range of 300–1650 m/z with a resolution of 60,000, an automatic gain control (AGC) target of $3e^6$, and a Maximum IT of 40 ms was set to obtain the MS spectra. Tandem mass spectrometry was set as follows: resolution of 75,000, AGC target of $1e^5$, Maximum IT of 60 ms, Top N of 20, and normalized collision energy of 27. Each sample is injected and measured in duplicates.

2.7. Characterization on glycation degree of whey proteins by UPLC-MS

A UPLC-ESI -MS instrument (LCZ/2690 XE/996, Waters Co., Milford, MA, USA) was used to characterize the glycation extent of whey proteins. Milk serum samples were diluted in MilliQ water at a concentration of 1 μg/μL according to a published method (Chen, Zhang, Bhandari, & Zhou, 2018). A 2.1 × 100 mm BEC C4 column (Ethylene Bridged Hybrid, Waters, Milford, MA, USA) packed with 1.7 μm particles was used. A gradient elution at 0.3 mL/min of formic acid (0.1%) and acetonitrile was carried out with the proportion of 0.1% formic acid from 98% to 60% in the initial 8 min and then to 20% during the next 2 min. MALDI SYNAPT Q-TOFMS (Waters Co.) was used in a positive ionization mode, with the cone voltage being 30 V and the collision energy 6 eV. The scan m/z range started at 20 and ended at 2000. The mass data were analyzed using MassLynx V4.1 software (Waters Co., Milford, MA, USA).

2.8. Data analysis

In this study, two replicates were used in the LC-MS/MS analysis, and proteins identified in both two replicates were used in quantitative analysis; three replicates were used in other analysis. The obtained raw MS files were used to search against the uniprot-Bos taurus protein database and analyzed using MaxQuant (1.6.2.10). The parameters were set as follows: the specificity of enzyme was set to trypsin; the maximum missed cleavages were set to 2; the precursor ion mass tolerance was set to 10 ppm, and MS/MS tolerance was 0.6 Da. Obtained LC-MS/MS iBAQ intensity of proteins were analyzed with Perseus 1.6.2.3 software (Tyanova et al., 2016). For immunoglobulins, XO activity, and lactoferrin, statistical analysis was performed using one-way ANOVA in SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Among different groups, $p < 0.05$ was considered significant difference by Duncan test. Identified proteins in all samples were used for cluster analysis by TB tools software (Chen et al., 2020) and principal component analysis (PCA) by SIMCA 14.1 software.

3. Results and discussion

3.1. SDS-PAGE of milk serum proteins

The SDS-PAGE of milk serum proteins under reducing and non-reducing conditions was shown in Fig. 2a and b respectively. Notably, the milk serum protein profiles changed after treatments compared to the raw milk, especially for the samples after E, U, and S treatments. Compared to the raw milk, the total native milk serum protein concentration was also significantly decreased ($p<0.05$) after thermal processing as shown in Fig. 2c. Compared with those severe heating treatments (E, U and S), pasteurizations (L and H) better retained the native milk serum proteins. The bands of several major milk serum

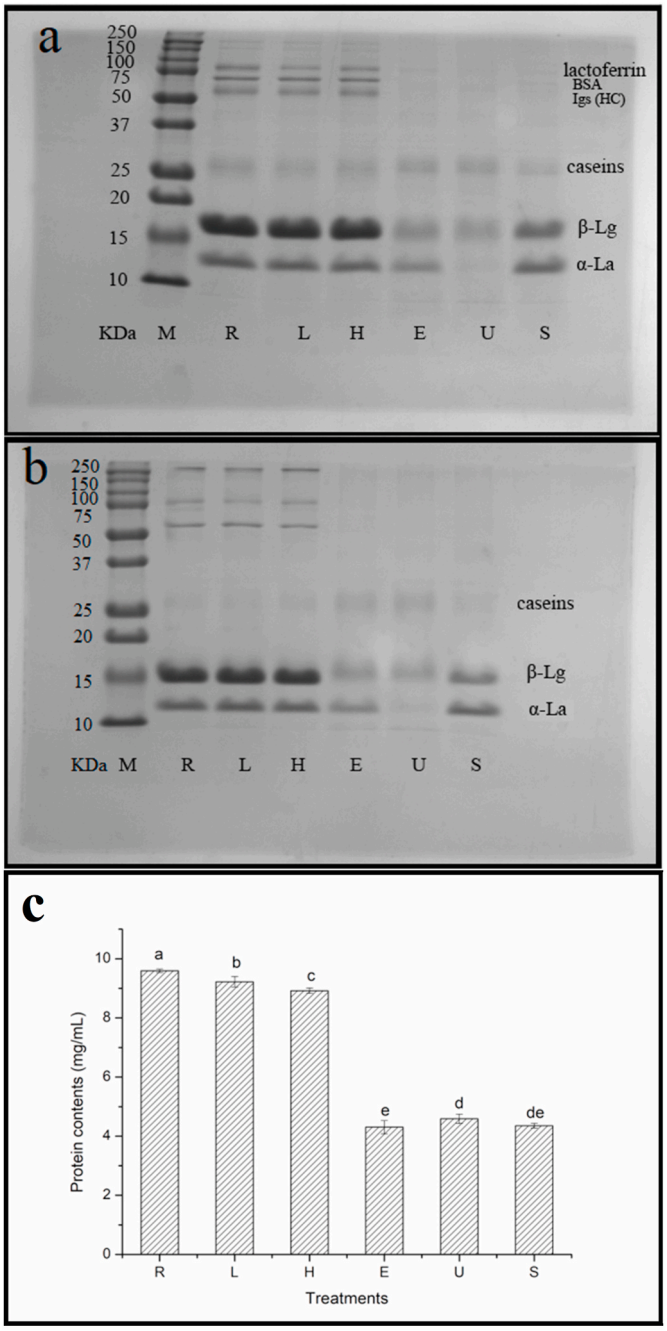

Fig. 2. SDS-PAGE of milk serum protein profiles after different industrial process under reducing (a), non-reducing (b) conditions, and milk serum protein concentration (c) by BCA.

proteins disappeared after severe heat treatments, such as lactoferrin, BSA, and heavy-chain IgG (shown in Fig. 2a) while L and H were able to keep them. SDS-PAGE showed that the decrease of total protein content is associated with denaturation of above-mentioned whey proteins. Besides, the band intensity of α-LA and β-LG in heat-treated samples also decreased obviously compared to the raw milk. This is mostly attributed to the aggregation of those heat-sensitive proteins in milk serum with the other components in the milk such as the casein micelle (Corredig & Dalgleish, 1999) and the milk fat globules (Corredig & Dalgleish, 1996), which were removed during the acidification and ultracentrifugation during sample preparations. Under non-reducing condition (Fig. 2b), some protein aggregates were observed after pasteurization, and formation of intermolecular disulfide bonds is mostly responsible for the heat-induced protein aggregates. Thiol groups of cysteine residues of the different serum proteins, which may get exposed during the denaturation step, can trigger thiol-disulfide exchange reactions forming protein aggregates (Manzo et al., 2015). Previous research has shown an important role for β-LG in this type of thiol-disulfide exchange induced aggregation (Roefs & Kruif, 1994). Recently, Xiong et al. (2020) also observed that the native milk serum protein concentration decreased significantly after thermal treatments when they investigated the changes of bacteriostatic activity of bovine milk serum after heating treatments.

In addition, Fig. 2b showed that the bands of β-LG in the ESL and UHT samples shift up compared to the other samples, suggesting that the molecular weight may increase. This can be potentially attributed to the glycation between lactose and β-LG during severe heating conditions. We therefore also characterized the glycation of the whey proteins by LC-MS (see section 3.5).

3.2. PCA analysis and milk serum proteins profiles (heatmap)

Principal component analysis (PCA) is a useful method to visualize the variation among samples. PCA-biplot analysis (Fig. 3a) of the quantified milk serum proteins demonstrated that raw and heated milk samples were classified and clustered according to their different treatments, namely the heat intensity. The PCA correlation loading plot is shown in Fig. 3a, and each point represents a protein quantified using the label-free proteomic approach. The first two PCs explained over 80% of the total variance in the original data set. According to PCA biplot, most samples were separated in the direction of PC1 while spray-dried milk was also separated in the direction of PC2. PC1 contained most of the highly variable proteins, such as caseins, complement C3, lactoferrin, osteopontin, perilipin, apolipoprotein E, and lipocalin 2, while PC2 contained α-LA, β-2-microglobulin, polymeric immunoglobulin receptor, and 45 kDa calcium binding protein. The 2-dimensional score plots showed that samples after thermal processing were separated clearly from raw milk. UHT, ESL and spray-dried milk samples formed clustered together while pasteurized milk (L and H) formed another cluster. Both pasteurized milks could not be separated from each other while severe heated samples (E, U and S) showed a relatively difference according to different treatments. In general, these results indicated that milk serum proteins were considerably changed after different thermal treatments, these milk samples formed clusters according to their different protein patterns.

To better visualize the changes and profiles of milk serum proteins after different industrial treatments, a clustering heatmap analysis was performed (Fig. 3c). Here, we only included those proteins that were quantified in all samples. In total, 433 proteins were identified in the combined samples and 54 proteins were shared in all samples. The subsequent quantitative analysis of the protein profiles was conducted by comparing the MS intensity of these proteins. It could be observed that the protein profile was altered obviously after thermal treatments. The results showed that the raw milk and the pasteurized milk (L and H) formed a sub-cluster, while the E, U and S treated milk formed the other sub-cluster, indicating that the pasteurized samples have a similar protein pattern with raw milk whereas the high temperature-treated milks (E, U, S) have different protein profiles compared with these two samples. Xiong et al. (2020) also found that milk serum heating at 75 °C for 30 min showed a significant decrease in antibacterial property and protein patterns changed a lot compared with raw milk. Many bio-active proteins in raw milk serum could exert antibacterial and immunity enhancing properties, and studies showed that consuming raw milk is able to reduce the risk of childhood asthma, atopy (Loss et al., 2011) and respiratory infections (Loss et al., 2015). In this study, the abundance of cystatin-C, serum albumin, complement C3, lactoferrin, lactadherin, and lactoperoxidase also decreased significantly in the high temperature treated samples, compared with the raw milk and low temperature-treated samples. Lactoferrin, as one of the most studied

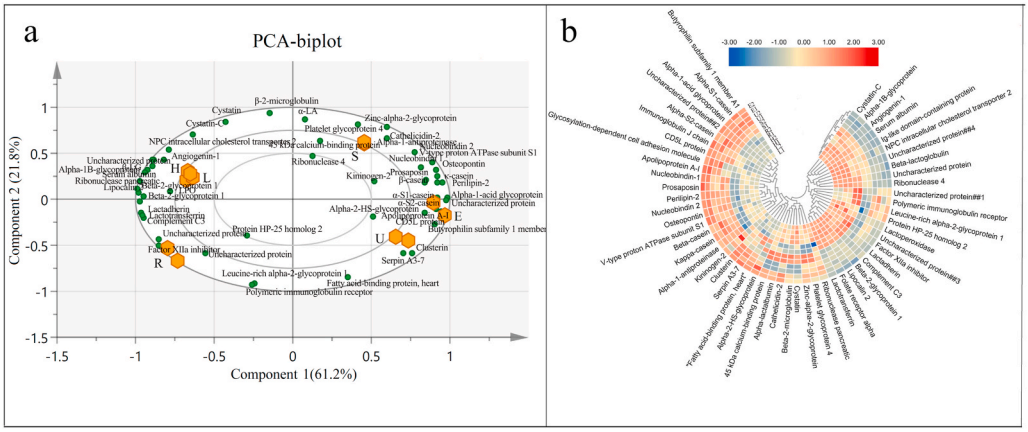

Fig. 3. PCA-plot analysis (a) of the quantified milk serum proteins from raw and heated milks (● represents quantified proteins; ⬢ represents thermal treatments); Heatmap (b) of milk serum protein profiles after different heat treatments. Rows reflect the identified proteins, and columns represent the samples. Heat map colors are bases on z-score normalized iBAQ values, combined with hierarchical clustering of samples. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

immune-related proteins, is known to stimulate and improve the immune system, by preventing pathogenic invaders, and was reported to exert a broad range of biological activities, including antibacterial, antivirus, and immunomodulatory activities (Drago-Serrano, Campos--Rodríguez, Carrero, & De la Garza, 2017). It is also widely used as functional ingredient in infant formula. Lactoperoxidase (LPO) is one of the most prominent enzymes in bovine milk, which exerts its function as a natural antibacterial agent, and studies have shown that lactoperoxidase would lower disease activity of asthmatic patients and reduce damaging effects of hydrogen peroxide (Al Obaidi, 2007). Besides these decreased proteins, the abundance of a few proteins in heated milk showed a relative increase compared to the raw milk, such as caseins (including α_{s1} casein, α_{s2} casein, β-casein and κ-casein), nucleobindin, apolipoprotein and other unidentified proteins. In this study, one main reason for the increased abundance of caseins in the heated milk samples is that the differences in abundance of the proteins is determined relatively, as the same total amount of protein was used for the analysis. The decreased abundance of milk serum proteins would thus make the abundance of caseins and other heat-stable proteins increase. During the heating process, heat-stable caseins can dissociate from casein micelles or can aggregate with whey proteins or other components in milk (Corredig & Dalgleish, 1999; Yang et al., 2018), and such heat-induced protein cross-linking in milk is mainly due to the formation of intermolecular disulfide bonds milk (Manzo et al., 2015).

3.3. *Retention of immunoglobulins, lactoferrin and xanthine oxidase (XO)*

Immune-active proteins play an important role in bacteriostasis, antivirus, immunomodulatory activities, such as immunoglobulins, lactoferrin, lactoperoxidase, and xanthine oxidase (Spitsberg, 2005; Xiong et al., 2020). Fig. 4 shows the retention of immunoglobulins and of xanthine oxidase activity in milk after different industrial treatments. As shown in Fig. 4a, the content of both immunoglobulins and lactoferrin decreased after thermal treatments, especially after severe treatments (E, U, and S). It could be found that about 50% of lactoferrin and 60% of IgG survived pasteurization (both L and H), while only 35% IgA and 30% IgM survived after these treatments. Another study also suggested that HTST would retain more immunoglobulins, growth factors, and hormones in donor human milk (Escuder-Vieco et al., 2018) compared with holder pasteurization, although that could not be completely confirmed in our results. This is potentially induced by the variation in milk species (human vs. bovine) or different detection methods. Immunoglobulins and lactoferrin were almost completely diminished after E, U and S treatments. Previous research has shown that IgG could be detected in pasteurized milk, but not in UHT processed

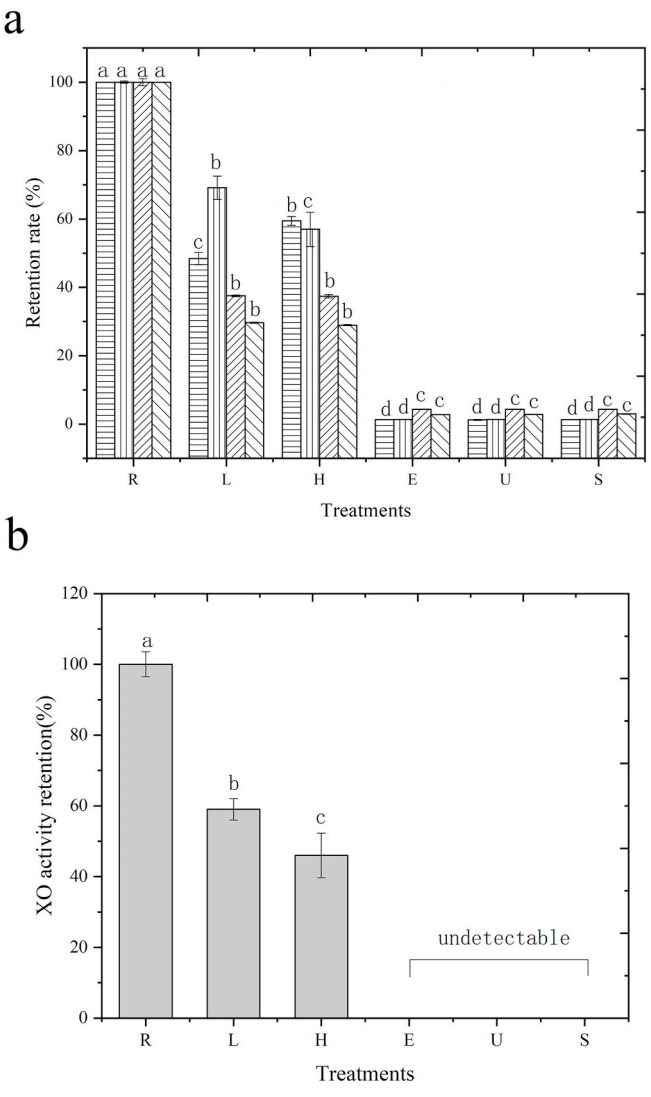

Fig. 4. Retention of lactoferrin, immunoglobulins (a) and xanthine oxidase (b) in milk after different industrial processes (▭ represents lactoferrin; ▥ represents IgG; ▨ represents IgA; ▧ represents IgM). Different letters indicate statistically significant difference among different heat treatments ($p < 0.05$) based on ANOVA.

or canned milks (Kummer, 1992). Chen and Chang (1998) also reported that IgG was susceptible to denaturation when treated at temperatures >75 °C. They found almost no IgG when milk was heated at 95 °C for 15 s, which is in agreement with our results. Fig. 4b demonstrates the activity retention of xanthine oxidase in milk samples. Compared to the raw milk, the activity of XO is decreased to 58% and 46% after L and H treatments; moreover, no active XO was detected in the E, U and S milks. XO is the second most abundant protein (12%–13%) in the milk fat globule membrane (MFGM), and serves as an important bactericidal agent in milk. Overall, the concentration of these important immune-active proteins all decreased, or lost their activity, after severe heat treatments (E, U and S).

3.4. ELISA verification for LC-MS/MS by lactoferrin content

LC-MS/MS based proteomics was used to quantify the native proteins in milk serum; however, the ability of proteomics to determine the variation in proteins, as induced by heat processing, has not be explicitly confirmed. To verify the LC-MS/MS results, the concentration of lactoferrin and IgG was determined by ELISA and compared with that in LC-MS/MS. Fig. 5 showed the lactoferrin and IgG contents determined by LC-MS/MS (iBAQ, intensity based absolute quantification) and ELISA respectively, which showed a similar trend for both methods. In addition, both methods showed that lactoferrin has a higher retention in HTST treatment compared to holder pasteurization. Recently, Chen et al. (2019) also found a good correlation between determining the β-LG and κ-casein in goat milk both by ELISA and label-free proteomics. In general, the consistent variation trends of lactoferrin determined by ELISA and LC-MS/MS showed that the LC-MS/MS based proteomics applied in this study is robust.

3.5. Characterization on glycation degree of whey proteins during industrial process

The Maillard reaction modifies the side chain of the protein; the ε-amino groups of lysine (Lys) residues represent the primary target for glycation by reducing sugars such as glucose and lactose to form the Amadori product lactulosyllysine, which may subsequently undergo a series of further reactions leading to a variety of modified structures, which may eventually influence the functionality or digestibility (van Lieshout, Lambers, Bragt, & Hettinga, 2019; Wada & Lönnerdal, 2014). To investigate the degree of protein glycation during the heat processing, the whey protein fractions in the milk were further analyzed using UPLC-MS. Fig. 6 displays the changes in mass distribution patterns of α-LA (Fig. 6A) and β-LG (Fig. 6B), respectively. Based on the relative intensity of the signal in the MS spectra, α-LA and β-LG in the raw milk did not show any glycation with lactose. In addition, α-LA and β-LG were also not glycated during pasteurization (L and H). However, treatments with higher heating intensity did lead to glycation of these two proteins. As shown in Fig. 6A, α-LA was glycated by 1 or 2 lactose units during E, U or S treatments. For β-LG, both genetic variants A and B, with a mass of 18,275 and 18,360 kDa, respectively, were detected as shown in Fig. 6B. Similarly, β-LG was also not modified by lactose during either type of pasteurization. It was observed that β-LG was also glycated after thermal treatments with high intensity. In general, the whole glycation of whey protein in milk was not very extensive during any of the different thermal treatments, and only slight glycation (by 1 or 2 lactose units per protein molecule) happened during thermal treatments under the highest heating intensities. It has been reported that protein glycation can be affected by several factors (Chen et al., 2018; Semagoto et al., 2014), of which high temperature was one of the most important factors facilitating glycation (Wada & Lönnerdal, 2014). Overall, no obvious glycation of whey protein appeared during pasteurizations and only slight glycation of whey proteins occurred during severe thermal treatments such as ESL, UHT and spray-drying.

4. Conclusion

Aiming to elucidate the changes of milk serum proteins after industrial thermal processing, several common thermal treatments were simulated in this study, including holder pasteurization, HTST, ESL, UHT, and spray-drying. The proteome of milk serum after these treatments were examined by label-free proteomics. In total, 433 proteins were identified in the samples, of which 54 proteins were quantified in all the samples. Notably, a number of immune-active proteins decreased in abundance after severe thermal treatments, such as lactoferrin, complement C3, and lactoperoxidase. Almost no active immunoglobulins or xanthine oxidase could be detected after the severe heat treatments (UHT, ESL or spray-drying) while 30–65% of immunoglobulins were retained after pasteurization. To verify the results of proteomics, ELISA for lactoferrin and IgG was used. In addition, lactose glycosylation of whey proteins was observed after UHT, ESL and spray-drying treatments. Even though the conditions of heat treatments used in this study may not be representative of actual industrial processing, the findings here were still able to be used to update the current knowledge into the changes of milk proteins after thermal treatments. Types of heat treatment should be carefully selected for achieving better nutritional potential of milk-based products.

Declarations of interest

None.

CRediT authorship contribution statement

Yaowei Liu: Conceptualization, Methodology, Methodology, Writing - original draft. **Wenjin Zhang:** Data curation, Investigation. **Lina Zhang:** Visualization, Software. **Kasper Hettinga:** Software, Validation, Writing - review & editing. **Peng Zhou:** Supervision, Writing - review & editing, Funding Acquisition.

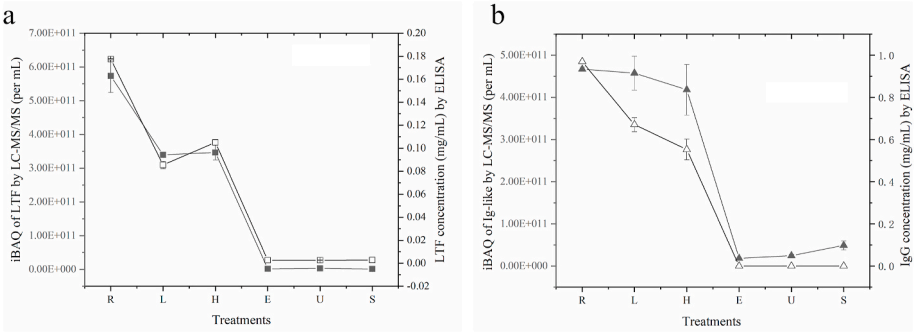

Fig. 5. Verification for LC-MS/MS results by comparing lactoferrin (a) and IgG (b) concentration by ELISA (–■– represents lactoferrin intensity by LC-MS/MS; –□– represents lactoferrin concentration by ELISA; –▲– represents IgG-like protein intensity by LC-MS/MS; –△– represents IgG concentration by ELISA).

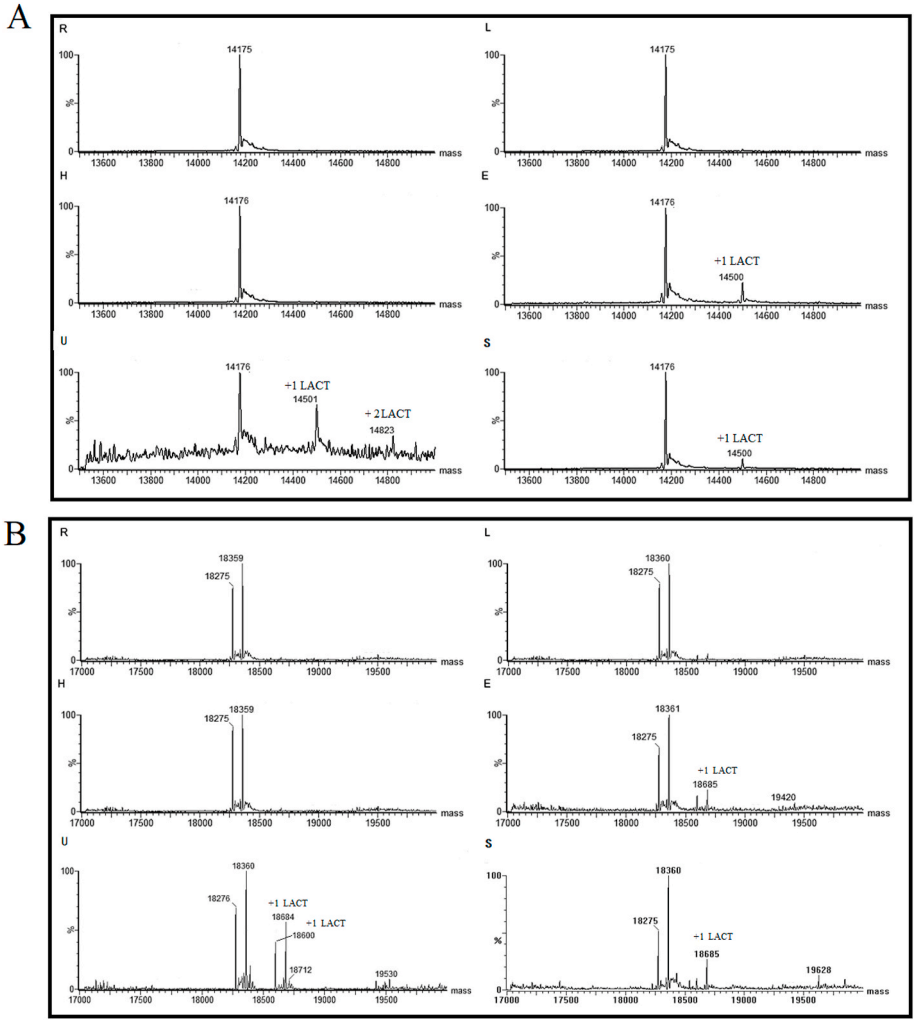

Fig. 6. Characterization on glycation degree of α-LA and β-LG in different milk samples by UPLC-MS.

Declaration of competing interest

The authors declared no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.110101.

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