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Changes in the milk serum proteome after thermal and non-thermal treatment

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ABSTRACT

Milk serum contains many immune-active proteins that are sensitive to heat treatment. This study compared the effects of thermal (63 °C, 30 min; 72 °C, 15 s; 85 °C, 5 min) and non-thermal (ultraviolet-C, UV-C; thermo-ultrasonication, TUS) treatments on bovine milk serum proteins by using label-free LC-MS/MS-based proteomics. UV-C (4500 J/L) and TUS (60 W, 6 min) treatments achieved a 5log microbial reduction as determined by plate counting. Proteomics showed that e.g., complement proteins, xanthine dehydrogenase/oxidase, and fatty acid-binding protein decreased significantly ($p<0.05$, $|\text{fold change}|>1$) after thermal treatments, and almost no lactoferrin, immunoglobulin, and lactoperoxidase was retained after heating at 85 °C for 5 min, whereas these proteins were mostly retained after non-thermal treatments. Most of these heat-sensitive proteins were located in membrane and extracellular regions and were involved in cellular and metabolic processes, response to stimulus, binding, immune process and catalytic functions. Finally, part of the proteomics results were verified by ELISA. This study thus provided insights for the development of optimized thermal and novel non-thermal treatments for dairy processing.

Industrial relevance: As alternatives to thermal processing technique, UV-C and ultrasonication showed a great potential in the processing of milk. This study not only showed that UV-C and ultrasonication were able to largely reduce the microbial load of raw milk, but also better retained the immune-related milk serum proteins than thermal processing, especially for the UV-C treatment. ELISA assays also demonstrated that the LC-MS/MS based proteomics technology used in this study was a robust method for quantifying damage to the milk serum proteome upon processing. Taken together, this study provided insights for development of optimized thermal and novel non-thermal techniques for dairy processing.

1. Introduction

Milk, a highly nutritious and readily digestible food, is one of the major sources of protein and micronutrients in the human diet all over the world (Boland & Singh, 2020; van Lieshout, Lambers, Bragt, & Hettinga, 2019). Besides, milk could also provide the neonate with many other essential compounds such as protective components, hormones, and growth factors (Braun-Fahrländer & Von Mutius, 2011). Among these components, the milk serum proteins are especially nutritionally important and provide a wide range of biological functions, such as antibacterial and immunomodulatory activity, which contributes to the development of the immune system in neonates (Hettinga et al., 2011; Lu et al., 2018). Lactoferrin, an iron-binding glycoprotein in milk serum, may exert a series of physiologic effects in the intestine. Many clinical studies have demonstrated that a number of potentially favorable biological effects were associated with lactoferrin in infants and children (Manzoni, 2019). CD14 is a 53- to 55-kDa glycosylphosphatidylinositol-anchored protein, and acts as a cellular receptor for bacterial

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lipopolysaccharide (LPS) (Paape, Lilius, Wiitanen, Kontio, & Miller, 1996). Accumulated studies have revealed that CD14 is able to modulate cellular and humoral immune responses by interacting with both T and B lymphocytes, playing a crucial role in protecting the host from an LPS challenge and Gram-negative bacteria-induced infections (Arias et al., 2000).

However, the milk serum proteins are sensitive to industrial dairy processing such as homogenization, pasteurization, sterilization and drying (Zhang et al., 2016), which are commonly used to make sure microbial safety and extend the shelf-life of the milk products. Traditionally, thermal treatments are widely used to achieve these goals with satisfactory results; however, quite a few studies also showed that thermal processing could cause heat damage and chemical modifications to the heat-sensitive proteins, such as glycation, oxidization, denaturation, and aggregation (Liu, Zhang, Han, Zhang, & Zhou, 2020). These processing-induced modifications may damage the bioavailability and functionality of milk proteins (van Lieshout et al., 2019). Studies also showed that denaturation of milk proteins in general, and the retention of immuno-active proteins in particular, are closely related to the thermal processing intensity and low-heat intensity processing would have a better protective effect on those bio-active proteins (Brick et al., 2017; Escuder-Vieco et al., 2018). Different thermal pasteurizations were also shown to have highly variable effects on milk serum proteins, which may be due to the highly variable heat loads of these different heat treatments. For example, Escuder-Vieco, Espinosa-Martos, Rodríguez, Fernández, and Pallás-Alonso (2018) reported that high temperature short time (HTST) treatments had higher retention of IgG in relation to holder pasteurization. However, a systematic comparison among different pasteurizations on milk serum proteins remains to be investigated.

Besides thermal treatments, which always lead to a certain loss of immune-active proteins, a number of other studies focused on non-thermal processing treatments as alternative. Among all nonthermal processing techniques, ultra-sonication (Shanmugam, Chandrapala, & Ashokkumar, 2012), high pressure processing (Pitino et al., 2019), and ultraviolet radiation treatment (Buhler et al., 2019) are gaining increased attention in recent years. Of these, especially the effect of ultrasonication and UV-C on bovine milk proteins has hardly been reported. Ultrasonic pasteurization (20–100 kHz) is an emerging technology for food preservation via producing inertial cavitation, which forms microscopic bubbles that rapidly collapse, producing shockwaves and localized heating, and disrupts cellular membranes leading to cell lysis (Czank, Simmer, & Hartmann, 2010). According to these authors, the combination of ultrasound and heating (thermo-ultrasonication) is an emerging food preservation technique that may retain higher quantities of bioactive components. Ultraviolet-C (UV-C), with wavelength between 200 and 280 nm, has a strong microbicidal efficacy, and is able to destroy bacteria, moulds, viruses, yeasts, protozoa, and algae (Bintsis, Litopoulou-Tzanetaki, & Robinson, 2000). UV-C irradiation kills these microorganisms by causing photoproducts of DNA bases which makes the DNA strands unable to replicate, thus leading to cell death (Christen, Lai, Hartmann, Hartmann, & Geddes, 2013). These studies were focusing on microbial inactivation, shelf-life extension, and retention of bio-active components (Pitino et al., 2019). Even though the effect of processing on retention of major whey proteins, such as lactoferrin, immunoglobulin G, β-lg, and α-la, has been reported (Bogahawaththa, Chandrapala, & Vasiljevic, 2017), the effect of non-thermal treatments on retention of the immune-active proteins present at low concentration in milk serum has not been well characterized.

This study compared the effects of low-intensity (63 °C for 5 min and 72 °C for 15 s), and high-intensity (85 °C for 5 min) thermal pasteurization, and two non-thermal treatments (UV-C and ultrasonication) on the milk serum proteins. Measurement of native milk serum protein concentrations and SDS-PAGE were first performed to determine the overall changes to the milk serum proteins. Second, a label-free quantitative proteomic approach was used to characterize the detailed qualitative and quantitative changes of the milk serum protein profiles, and explore the potential functionalities of these significantly changed proteins. Finally, we verified the LC-MS/MS based proteomics by determining the IgG and lactoferrin content by enzyme-linked immunosorbent assay (ELISA), and lactoperoxidase activity by an enzyme activity assay.

2. Material and methods

2.1. Milk sampling and treatments

Fresh raw bovine milk was collected from CARUS farm (Animal Sciences Department, Wageningen University Campus) and kept in the refrigerator at 4 °C before treatments. Milk samples were separated into six parts and one part was raw milk as control (R), the other five parts were treated with thermal and non-thermal processing. In each processing, three replicates were used. Thermal processing included low-intensity pasteurizations at 63 °C for 30 min (abbreviated as "63") in a water bath and at 72 °C for 15 s (abbreviated as "72") with an in-house made high temperature short time (HTST) pasteurization system, and high-intensity pasteurization at 85 °C for 5 min (abbreviated as "85") in a water bath. Theses thermal treatments were all monitored by a digital thermometer. The non-thermal processing was conducted by an in-house made UV-C system based on published literature (Christen et al., 2013) and a ultrasonicator (Branson Digital Sonifier® 450) equipped with a Branson Sonifier Sound Enclosure and microtip probe (length of 60 mm and diameter of 10 mm). In detail, for the UV-C treatment, 200-mL milk was transferred into a sterilized beaker (250 mL) and was irradiated by a UV-C lamp (UV-C radiation of 1.1 W, Philips) under magnetic stirring. For thermo-ultrasonication (TUS), 70-mL milk samples were treated in batch mode in sterilized 100-mL glass beakers with 20–25 mm distance from the microtip to the bottom. The glass beaker containing the sample was surrounded by circulating water of 40 °C to keep the sample temperature constant. The ultrasonicator was operated in pulse-pause mode, where the pulse length was set at 59.9 s of continuous pulse followed by 30 s of pause to avoid large temperature fluctuations. The temperature was checked, and confirmed to remain below 60 °C during the whole ultrasonication process. The UV-C and thermo-ultrasonication dosage were determined by a series of preliminary experiments, where different UV-C and ultrasonication dosages were applied to the milk samples to achieve a 10^5-CFU/mL reduction in native bacteria in milk. According to the results of these pre-experiments, a 4500J/L UV-C dosage and 60 W ultrasonication for 6 min (at 40 °C) were able to achieve a 10^5 reduction of total bacterial count in milk by plate counting method (details shown in Fig. S2), which is generally considered sufficient to achieve microbial safety according to literatures (Christen et al., 2013; Gunter-Ward et al., 2018; Wang, Fritsch, & Moraru, 2019).

2.2. Ultracentrifugation and BCA

To obtain the native milk serum proteins that were not denatured/aggregated during processing, the pH of milk samples was adjusted to 4.6 with 1 mol/L HCl to precipitate the casein and denatured milk serum proteins (Law & Leaver, 2000). After that, milk samples were transferred into ultracentrifuge tubes and centrifuged at 100,000 ×*g* for 90 min at 25 °C (Optima L-80, Beckman Coulter, USA). The obtained milk serum samples were collected and the total protein concentrations were determined by the BCA assay kit (Thermo Fisher Scientific, USA). These milk serum samples were stored at −20 °C before further analysis.

2.3. SDS-PAGE

Milk serum samples were diluted 10-fold with distilled water. Then, 10-μL diluted milk serum was mixed with 10-μL 2× concentrated loading buffer and 2-μL 10× concentrated sample reducing agent

(Thermo Fisher Scientific, Massachusetts, USA) and heated at 70 °C for 10 min. Of these samples, 10 μL was loaded onto a 12% Bis-Tris gel and run in MOPS buffer at 120 V for ~60 min. A PageRuler Prestained Protein Ladder (Catalog No. 26616, 10–140 kDa, Thermo Scientific) was used as protein marker. The gel was stained with Coomassie Brilliant Blue R-250 for 1 h and then destained with washing buffer (10% ethanol and 7.5% acetic acid in ultrapure water) overnight while mildly shaking.

2.4. Filter aided sample preparation (FASP) for LC-MS/MS

The sample preparation for LC-MS/MS was conducted according to published methods with some modifications (Hettinga et al., 2011; Wiśniewski, Zougman, Nagaraj, & Mann, 2009). Briefly, milk serum samples were diluted in 100 mmol/L Tris (pH 8.0) in low binding tubes (Eppendorf, Hamburg, Germany) to a protein concentration of ~1 μg/μL. Into these samples, 10% of their volume in DTT (dithiothreitol; 150 mmol/L) was added and the samples were incubated at 45 °C for 30 min. After that, 44 μL reduced milk serum (~40 μg protein) was added into 136 μL 8 mol/L urea in 100 mmol/L Tris/HCl (pH 8.0), after which another 20 μL 200 mmol/L Acrylamide in water was added and samples were incubated for 10 min at room temperature. Then, 100 μL alkylated sample was transferred onto a Pall 3 K omega filter (10–20 kDa cut off) and centrifuged at 16,900 ×*g* for 30 min. After centrifugation, 110 μL 50 mmol/L NH_4HCO_3 was added to the filter and centrifuged again. The filter was then moved to a new 2-mL low-binding tube, after which 100 μL trypsin in NH_4HCO_3 solution (5 ng/μL) was added. Samples were incubated overnight while mildly shaking. After digestion, the tubes were centrifuged at 16,900 ×*g* for 30 min and 100 μL 1 mL/L HCOOH in water was added on the top of the filter, followed by another centrifugation. The obtained peptide fractions were stored at −20 °C before injection into the LC-MS/MS system.

2.5. LC-MS/MS analysis and protein quantification

Of the peptide samples, 1.5–3.4 μL were injected directly onto a 0.10 * 250 mm ReproSil-Pur 120 C18-AQ 1.9 μm beads analytical column (prepared in house) at a pressure of 800 bar. Peptides were eluted with an acetonitrile gradient at a flow of 0.5 μL/min, using gradient elution from 9% to 34% acetonitrile in water with 0.1 *v*/v % formic acid in 50 min. An electrospray potential of 3.5 kV was applied directly to the eluent via a stainless steel needle fitted into the waste line of the micro cross that was connected between the pump and the analytical column. Full scan positive mode FTMS spectra were measured between *m/z* 380 and 1400 on a Q Exactive HF-X (Thermo Electron, San Jose, CA, USA). Higher-energy collisional dissociation (HCD)-fragmented MS/MS scans of the twenty most abundant 2–5 + −charged peaks in the Fourier-transform mass spectrometer (FTMS) scan were recorded in data-dependent mode. The obtained raw MS files were used to search against the uniprot-Bos taurus protein database (Uniprot UP000009136) and analyzed using MaxQuant (1.6.3.4). The parameters were set as follows: protein modifications, propionamide (C) (fixed), oxidation (M) (variable), enzyme specificity: trypsin, maximum 2 missed cleavages, first search 20 ppm peptide tolerance, main search 4.5 ppm tolerance, MS/MS fragment match tolerance of 20 ppm. Proteins identified by minimally 2 peptides of which at least 1 unique and 1 unmodified were considered as reliable and used for further analysis.

2.6. ELISA verification

To verify the data of the LC-MS/MS, the concentration of lactoferrin (LTF) and IgG in the samples after the different treatments were measured by ELISA according to Liu et al. (2020). Milk serum samples were diluted 2000 and 5000 times with MilliQ water for LTF and IgG, respectively, according to the provided protocols (Cat. No. E11–126 and E11–118, Bethyl Laboratories, USA). After coating, incubation, and washing, 100-μL TMB was added into 96-well plate and incubated in the dark for 30 min. The reaction was stopped with 100-μL 0.18 M H_2SO_4 and the plate was read at 450 nm with a plate reader (xMark ™, Bio-Rad Laboratories, USA). The standard curve of this assay was fitted with a 4-parameter curve fitting equation that was used to calculate the LTF and IgG concentrations.

2.7. Lactoperoxidase (LPO) activity

The LPO activity was measured using the IDF method (Marks, Grandison, & Lewis, 2001) with minor modifications. Briefly, the rate of oxidation of 2,2'azinobis (3-ethyl-benzothiazoline-6-sulphonic acid, ABTS) was measured spectrophotometrically at 412 nm; 1 mmol/L ABTS (diammonium salt; Sigma) and 0.3 mmol/L H_2O_2 solutions were prepared freshly in 0.1 mmol/L PBS at pH 6.7 and stored at 22 °C. Then, 0.1-mL milk serum sample (4 °C) was mixed with 2 mL of ABTS solution and left for 5 min at 22 °C to warm up the samples and 1 mL H_2O_2 was added and mixed quickly to start the reaction. Absorbance at 412 nm was recorded by a Cary 60 UV–Vis Spectrophotometer (Agilent, CA, USA) every 10 s during a total time of 2 min. The linear slope of the absorbance against time in the first 60 s was then calculated as the LPO activity. Each sample was measured in three replicates.

2.8. Data analysis and visualization

For proteomics, Perseus software (version 1.6.2.3) was used to analyze the iBAQ (Intensity based absolute quantitation) data. Student's *t*-test was performed in Perseus to determine significant differences in proteins after the different treatments. Differences with a p value <0.05 were considered statistically significant. The obtained p-values were adjusted with Permutation-based FDR (false discovery rate) correction. Only those proteins identified in at least two of three replicates were used in a Venn diagram and subsequent data analysis. Cluster and PCA analysis were performed after $\log_{(2)}$ transformation, data filtering and imputation (with Perseus; imputation based on a normal distribution with a down shift of 1.8 and width of 0.3). ANOVA (SPSS 18.0) was used for the ELISA and LPO data analysis, and a p value<0.05 was considered a significant difference by the Duncan test. GO (Gene Ontology) enrichment analysis of milk serum proteins was performed based on biological processes, cellular components, and molecular functions, using the online tool Panther (http://www.pantherdb.org/). The result of the cluster analysis was visualized by Java TBtools software (Chen et al., 2020) and principal components analysis (PCA) was performed with the SIMCA 14.1 software (Umetrics, Umeå, Sweden).

3. Results and discussion

3.1. Native milk serum protein concentration and SDS-PAGE

Fig. 1a shows the total native milk serum protein concentration after different treatments, from which it can be found that the milk serum protein concentration in 85 °C-treated samples decreased significantly ($p<0.05$). Xiong, Li, Boeren, Vervoort, and Hettinga (2020) also found that milk heated at 75 °C for 30 min would cause major differences to the milk serum protein levels, which significantly affected the bacteriostatic activity. Further, Fig. 1b and c display the milk serum protein patterns by SDS-PAGE under reducing and non-reducing conditions, respectively. The protein patterns under reducing conditions mostly included α-lactalbumin (α-la), β-lactoglobulin (β-lg), LTF, bovine serum albumin (BSA), IgG-heavy chain (IgG-HC) and lactoperoxide (LPO), while under non-reducing SDS-PAGE, some aggregates could be observed (shown in Fig. 1c). Thiol groups of cysteine residues of milk serum proteins may get exposed during protein unfolding, and can subsequently trigger thiol-disulfide exchange reactions leading to the formation of protein aggregates (Manzo, Nicolai, & Pizzano, 2015). These aggregates would disappear under reducing conditions (Anema, 2020; Manzo et al., 2015). After severe heating treatment (85 °C for 5

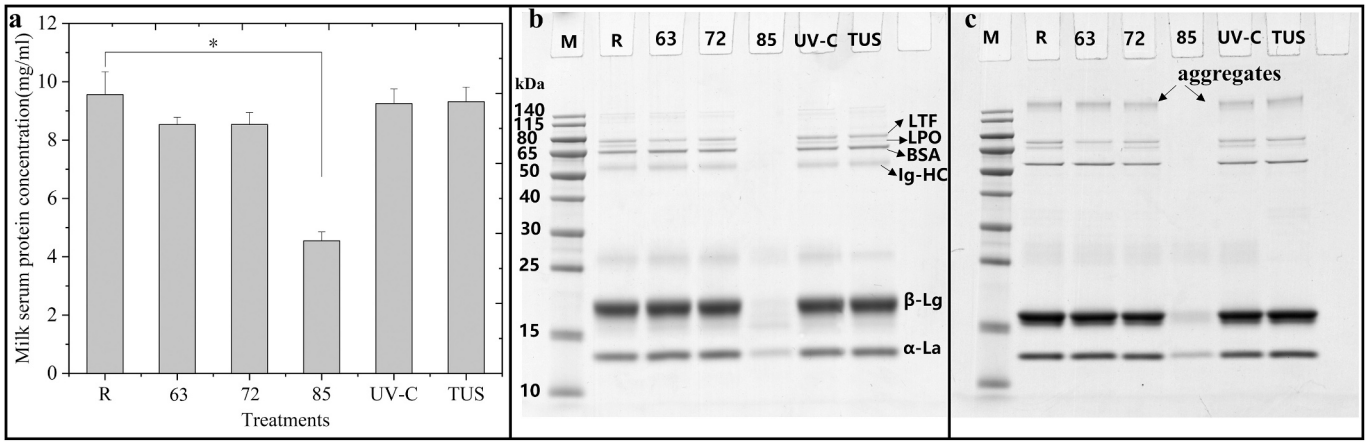

Fig. 1. Native milk serum protein concentration by BCA method (a); SDS-PAGE of milk serum proteins under reducing (b) and non-reducing (c) conditions. * indicates a significant difference ($p<0.05$). M: protein marker; R: raw milk; 63: heated at 63 °C for 30 min; 72: heated at 72 °C for 15 s; 85: heated at 85 °C for 5 min; UV-C: ultraviolet-C treatment; TUS: thermal ultrasonication.

min), the larger aggregates would be removed during the pH adjustment and ultracentrifugation as applied in the sample preparation, so no obvious aggregates would be expected in the 85 °C-treated group. Almost all the bands representing major milk serum proteins were reduced or completely disappeared after the 85 °C treatment, such as α-la, β-lg, LTF, LPO, BSA, and IgG-HC, which supports the BCA results. On the other hand, the low pasteurizations and non-thermal treatments (UV-C and TUS) had little effect on the protein patterns compared to the raw milk. During the heating, these heat-sensitive milk serum proteins would denature and aggregate with casein micelles or milk fat globules which were removed by the acidification and ultracentrifugation (Zhang et al., 2016). Overall, low-intensity pasteurizations (both at 63 °C and 72 °C) and non-thermal treatments (UV-C and TUS) have almost no influence on the milk serum proteins based on BCA and SDS-PAGE results.

3.2. Identification of milk serum proteins

In the present study, 165 milk serum proteins were identified in total. After data filtering based on detection in at least two out of three replicates, 102 milk serum proteins were used for the subsequent quantitative data analysis. Fig. 2 showed the UpSet plot of the numbers of

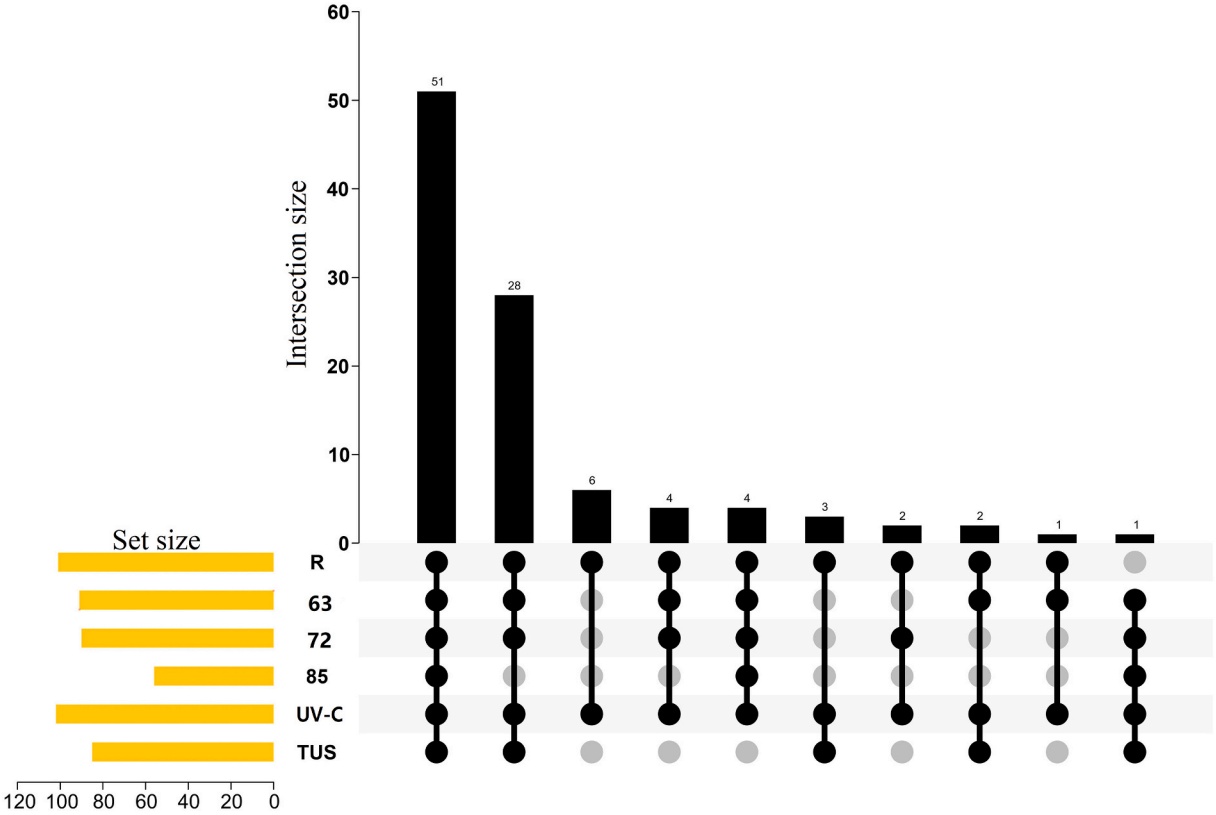

Fig. 2. UpSet plot of identified milk serum proteins in different groups. R: raw milk; 63: heated at 63 °C for 30 min; 72: heated at 72 °C for 15 s; 85: heated at 85 °C for 5 min; UV-C: ultraviolet-C treatment; TUS: thermal ultrasonication. The yellow bar represents the number of identified proteins in each sample group; the black bar represents the number of proteins present in multiple samples groups simultaneously; the dots represent the applied treatments associated with each black bar.

identified protein, which showed that 101, 91, 90, 56, 102 and 85 proteins were identified and quantified in the R, 63 °C, 72 °C, 85 °C, UV-C, and TUS milk serum samples, respectively. The number of identified milk serum proteins decreased under thermal treatments and TUS. Among these 102 proteins, 51 proteins were present in all the groups, 79 proteins were present in R, 63 °C, 72 °C, UV-C, and TUS groups, which suggested that 28 proteins were lost in 85 °C treated group. In addition, 88 proteins were both present in 63 °C and 73 °C treated groups, and 101 proteins were present in R and UV-C treated group, indicating that these groups may have similar protein patterns. The loss of identified proteins can mostly be attributed to heat-induced denaturation and aggregation, as also reported by Brick et al. (2017), who reported that the number of identified milk serum proteins would decrease with increasing heating intensity. Yang et al. (2018) investigated the changes in bovine milk fat globule membrane (MFGM) proteins caused by heat procedures and found that the number of absent proteins in the MFGM fraction was increased from pasteurized milk to ultrahigh-temperature milk. These findings were very similar to the results here, indicating that both milk protein types show a similar response to heating.

3.3. Quantitative and cluster analysis of milk serum proteome

A PCA biplot, which combines a common PCA score plot with a plot of the PCA loadings, is shown in Fig. 3a. Each grey point represents an identified protein, distinguishing the different treatments. According to this loadings plot, the serum proteins in the direction of PC1 may be the markers discriminating the 85 °C samples from the other groups. Fig. 3a also shows that the first two PCs explained 71% of the total variance of the original data set, which may be due to a strongly correlated response among all proteins towards the applied heat treatment. Whereas the 85 °C-treated milk serum was separated in the direction of PC1, TUS treated milk serum was separated in the direction of PC2. The other groups of treated milk serum could not be clearly separated from the raw milk, which indicates that they have a relatively similar protein composition. However, once the 85 °C and TUS-treated milks were taken out of the analysis, the milk serum protein profiles of the raw, 62 °C, 73 °C, and UV-C treated samples could be differentiated from each other (shown in Fig. S3), indicating that differences still exist among these samples. PC1 contained most of the highly variable proteins, such as vitamin D-binding protein, complement C7, lactoferrin, xanthine and dehydrogenase/oxidase, polymeric immunoglobulin receptor, and folate receptor alpha, whilst PC2 only contained α_{s1}-casein, SCGB2A2, and nucleobindin-1.

The quantified proteins were further analyzed by hierarchical clustering, as shown in Fig. 3b, suggesting that these treatment groups were classified into four major clusters. Milk serum proteins from the two types of low pasteurized milks (both 63 °C and 72 °C) could not be clearly distinguished and formed one cluster; milk serum from UV-C and raw milk also showed a similar proteomic pattern, and formed another cluster. The TUS and 85 °C treated milk serums formed another two clusters separately, according to their specific proteomic patterns. This clustering pattern was consistent with the PCA results.

It could be found that TUS has an obvious effect on the milk serum proteins even though it is used as a non-thermal treatment. The changes of milk serum proteins under TUS can potentially be attributed to two aspects. Firstly, the temperature of milk during ultrasonication would increase to nearly 60 °C (as shown in Fig. S1), even though it was initially heated at only 40 °C, which is inevitable for thermo-ultrasonication (Czank et al., 2010). Secondly, the physical forces of acoustic cavitation and shear force may also affect the structure of the milk proteins. According to some previous studies, globular whey proteins may be denatured under the influence of the high shear occurring during ultrasonication (Shanmugam et al., 2012). The bubble surfaces generated during the ultrasound cavitation can readily damage proteins which are located at the bubble interface and facilitated denaturation (Thomas & Geer, 2011); moreover, the hydrophobic character at the bubble interface would facilitate these partially denatured protein monomers to interact with each other and form aggregates (Sluzky, Klibanov, & Langer, 1992). These denatured whey protein fractions may finally form insoluble aggregates with other whey proteins, casein micelles and/or milk fat globules through thiol-disulphide exchange reactions, after which they would have been removed during acidification and centrifugation. Shanmugam et al. (2012) also found that the major whey protein fractions showed a decrease when the ultrasound treatment is over 30 min due to the formation of soluble whey–whey/whey–casein aggregates. According to Czank et al. (2010), a typically homogenization of the milk fat globules would be observed after 2 min of ultrasonication, where they would become smaller, causing an obvious homogenization effect on the milk fat. Native fat membrane will then be disintegrated, forming smaller milk fat globules with modified membranes and these membranes would be prone to be covered with the hydrophobic part of caseins or denatured whey protein particles (Bermúdez-Aguirre, Mawson, & Barbosa-Cánovas, 2008). This attached milk protein would be removed in the subsequent sample preparation, in addition to the protein already bound by the disulphide bridges.

The quantitative differences in overlapping proteins between groups were analyzed using two-sample *t*-tests (Fig. 4). Significantly different proteins were filtered based on the criteria of "Log_2 (Fold Change) >1.0 and $p < 0.05$". Compared with raw milk, a number of 24, 29, 78 and 41 proteins were down-regulated in the 63, 72, 85, and TUS treated groups, respectively; whereas no proteins were significantly decreased in the UV-C treated group. A considerable decrease of protein abundance in the 85-treated group was observed, as shown in the supplementary data. Based on a GO analysis of the significantly different proteins, we found that most were involved with immunity, enzyme, binding and transport activities. Overall, both low and high intensity pasteurizations and TUS treatment induced damage on milk serum proteins, including protein abundance and types while UV-C is better to keep them intact.

Heat-labile milk serum proteins, containing many valuable constituents, would denature and aggregate during heating processing, leading to a loss of biological functionality. Among these valuable constitutes, lactoferrin, lactoperoxidase and immunoglobulins are the main immune-active proteins (Madureira, Pereira, Gomes, Pintado, & Xavier Malcata, 2007). In addition, other low abundant proteins, such as complement components and CD14 may also involve with prevention of allergy and asthma (Bieli et al., 2007). Some of these proteins may withstand the gastric digestion, and may directly exert physiologic functions in the intestine (Jasion & Burnett, 2015), such as lactoferrin. It was reported that bovine lactoferrin was able to help prevent necrotizing enterocolitis (Manzoni et al., 2014) in very-low-birth-weight neonates through randomized clinical trials. Lactoperoxidase (LPO) and xanthine dehydrogenase/oxidase (XDH/XO) are important natural enzymes in milk, which are secreted from the mammary gland andreported to exert antimicrobial properties and thereby provide innate immunity protection (Kussendrager & van Hooijdonk, 2000; Silanikove, Shapiro, Shamay, & Leitner, 2005).

3.4. GO enrichment analysis of absent and significantly reduced proteins

Some heat-sensitive proteins would suffer heat damage and get lost/decrease in concentration during the treatments, which could not be detected or showed a decreased abundance in LC-MS/MS based proteome. Functional analysis of the absent and significantly decreased proteins from heated milk samples compared with raw milk samples is shown in Fig. 5. Compared with the raw milk, UV-C treatment did not reduce the number of identified proteins or their abundance, so the comparison between these samples is not shown. Generally speaking, the number of absent proteins related to each GO category (Fig. 5a–c) increased from 63 °C and 72 °C treatment to TUS and 85 °C treated milks. Most of the absent proteins were originally located in membrane and extracellular regions and were involved in cellular and metabolic processes, response to stimulus, binding, immune process and catalytic

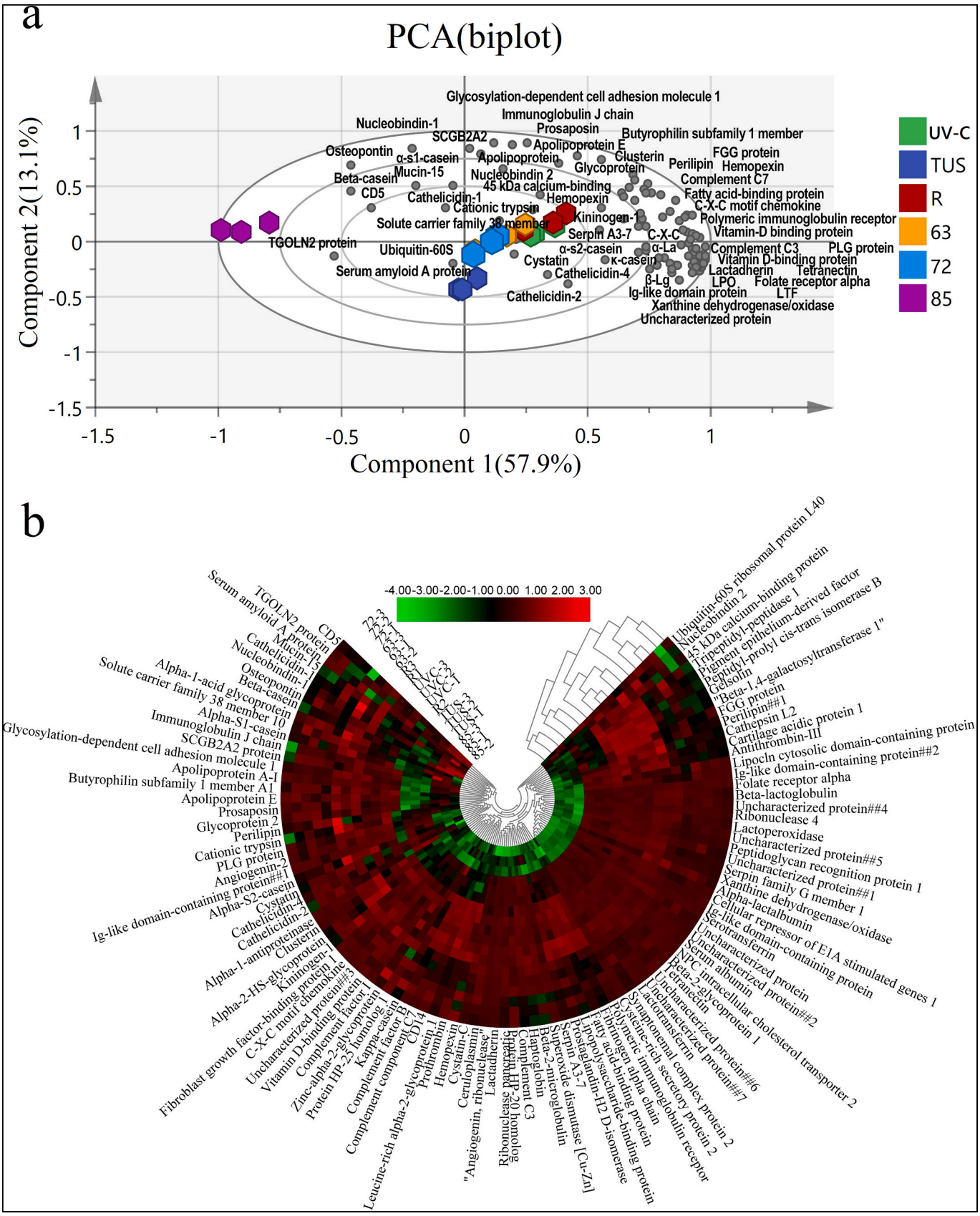

Fig. 3. PCA-biplot (a) and hierarchical clustering (b) of quantified milk serum proteins in different groups. Each grey dot and colored hexagon (Fig. 3a) represent a quantified protein and treatment for milk; the bar color represents a logarithmic scale from −4.0 to 3.0. R: raw milk; 63: heated at 63 °C for 30 min; 72: heated at 72 °C for 15 s; 85: heated at 85 °C for 5 min; UV-C: ultraviolet-C treatment; TUS: thermal ultrasonication.

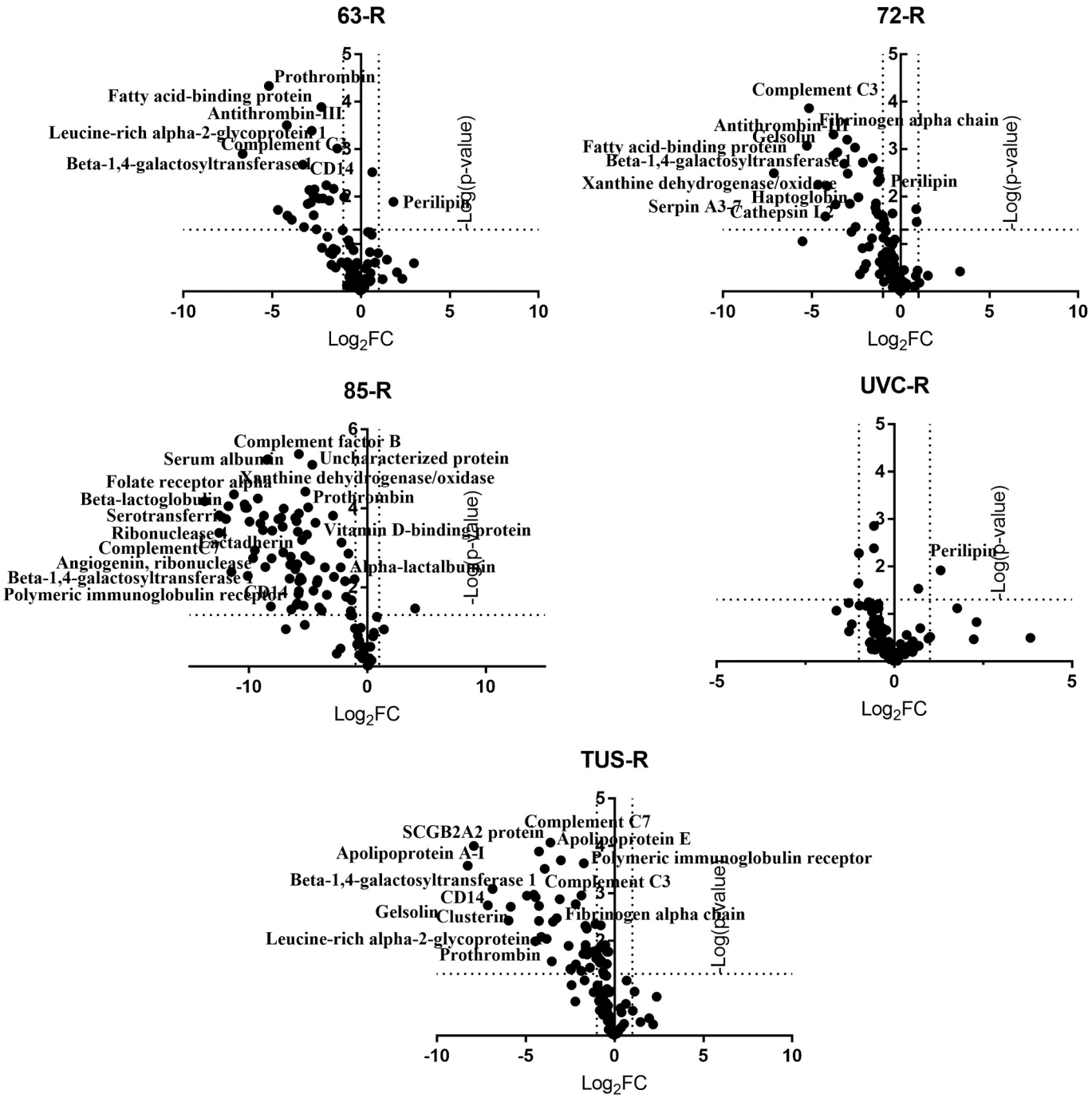

Fig. 4. Volcano plots of milk serum proteins in different groups vs raw milk. a, represents the 63 °C- 30 min vs raw milk (63 vs R); b, represents the 72 °C- 15 s vs raw milk (72 vs R); c, represents the 85 °C- 5 min vs raw milk (85 vs R); d, represents the UV-C vs raw milk (UV-C vs R); e, represents the TUS vs raw milk (TUS vs R).

functions. Fig. 5(d–f) demonstrated the GO enrichment analysis of the milk serum proteins which decreased significantly in abundance compared with the raw milk. In each GO category, the number of decreased proteins increased in the order of UV-C < 63 °C < 72 °C < TUS < 85 °C. The GO functions of proteins that decreased in abundance were more than that of the absent proteins and these proteins were mostly located in the extracellular region or cell parts and were involved in cellular process, biological regulation, response to stimulus, metabolic process and immune system process.

3.5. Retention of IgG, lactoferrin by ELISA and LPO activity

Fig. 6a shows the retention of lactoferrin, IgG contents and LPO activity after different treatments. The lactoferrin content decreased significant after different treatments in comparison to the raw milk. It seems that the UV-C and TUS treatments both were better at retaining lactoferrin (80% retention) in comparison with the thermal pasteurizations. Furthermore, lactoferrin concentrations in milk serum after 72 °C-treatment showed a higher retention (70% retention) than the holder pasteurization (63 °C) did (50% retention). IgG showed a difference in retention relative to lactoferrin. UV-C treatment almost did not decrease the concentration of IgG ($p^{\wedge}0.05$) compared with the raw milk. The 63 °C and TUS treatments both significantly decreased the IgG concentration by ~10% while HTST decreased the IgG concentration by nearly 20%. Both IgG and lactoferrin could hardly be determined in 85 °C -treated group, due to a much lower concentration than in the

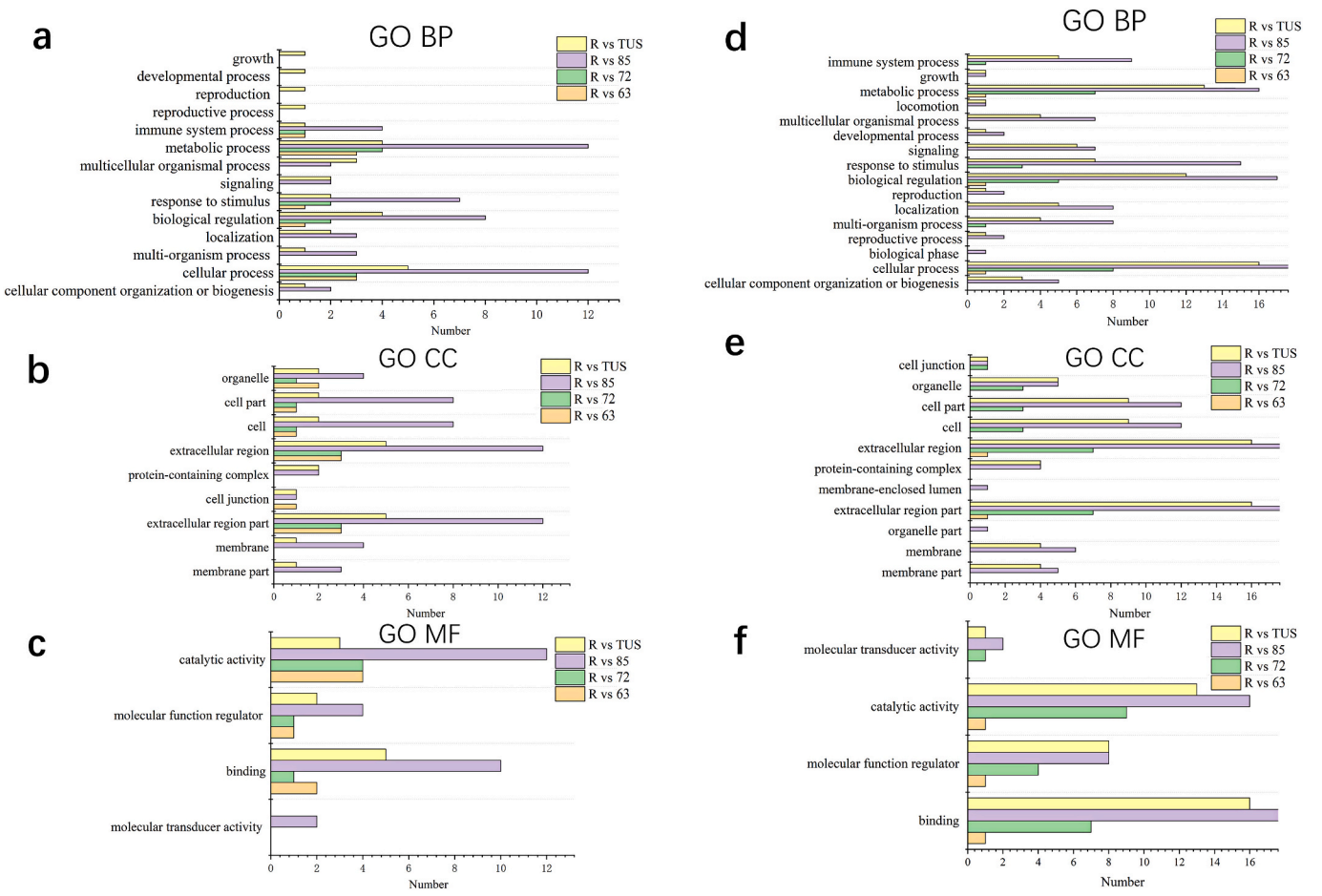

Fig. 5. Gene ontology (GO) of absent (a–c) and significantly decreased (d–f) proteins compared with the raw milk serum. GOBP, GO biological process; b, GOCC, GO cellular component; c, GOMF, molecular function. R: raw milk; 63: heated at 63 °C for 30 min; 72: heated at 72 °C for 15 s; 85: heated at 85 °C for 5 min; UV-C: ultraviolet-C treatment; TUS: thermal ultrasonication.

other samples. Our findings slightly differ from the reports of Escuder-Vieco, Espinosa-Martos, Rodríguez, Fernández, and Pallás-Alonso (2018), who found a higher human milk IgG retention after HTST treatment. This was potentially induced by the variation in milk species (human vs. bovine) or different detection methods. In summary, we showed that IgG in bovine milk had a better retention in both thermal and non-thermal treatments than lactoferrin. 72 °C treatment had a better retention of lactoferrin, whereas the 63 °C treatment showed a better retention of IgG. UV-C and TUS were shown to realize a better retention of both these immune-related proteins compared to any thermal treatment.

Lactoperoxidase (LPO) is a naturally-occurring antimicrobial enzyme in raw milk, which is active against both Gram-positive and Gram-negative microbes to varying extents (Siragusa & Johnson, 1989). Fig. 6a shows the LPO activity retention after the different treatments, which demonstrated that most treatments had no obvious effects on the activity of LPO, except the 85 °C-treated milk. After heating at 85 °C for 5 min, almost no LPO activity was detected. Marks et al. (2001) also pointed out that as the temperature increased, the LPO activity decreased rapidly until it could not be detected after heating at approximately 80 °C, which is in accordance with our findings here. Although LPO is sensitive to heating, we found that LPO retained its activity after the two types of low pasteurization. This also agrees with another study which reported that heat treatments below 74 °C would not decrease the LPO activity significantly (Marín, Sánchez, Pérez, Puyol, & Calvo, 2006). Therefore, it can be postulated that LPO remains active in holder pasteurized and HTST treated milks, and the two non-thermal treatments could also achieve an almost complete retention of the LPO activity.

In order to verify the LC-MS/MS results, the content of lactoferrin and IgG from ELISA, as shown in Fig. 6, was compared to the iBAQ intensities of the LC-MS/MS analysis, as shown in Fig. 6b and c. The variations in the lactoferrin and IgG concentrations in the different groups were very consistent between the ELISA and the LC-MS/MS results. Similarly, we also used the LPO activity to fit the quantified LPO abundance by LC-MS/MS and found that they showed a similar variation (Fig. 6d). In general, the results of LTF, IgG and LPO activity were thus consistent with the results of LC-MS/MS, indicating that the label-free proteomics used in this study is robust for protein quantification.

4. Conclusion

In summary, the profiles of the milk serum protein fractions from raw, thermal and non-thermal treated milks were, for the first time, mapped using a label-free proteomic approach. Both high intensity and low intensity heat treatments would lead to a decrease, or disappearance, of several immune-active milk serum proteins. Non-thermal treatments seem to better retain these milk serum proteins, especially for the UV-C treatment, whilst TUS would show a decreased protein abundance. These changes of the milk serum proteins were confirmed by determining lactoferrin and IgG by ELISA and LPO by enzyme activity assay. The proteomic data provided important insights into the changes in the milk serum proteins in response to the thermal and non-thermal treatments, establishing a basis for future studies on non-thermal

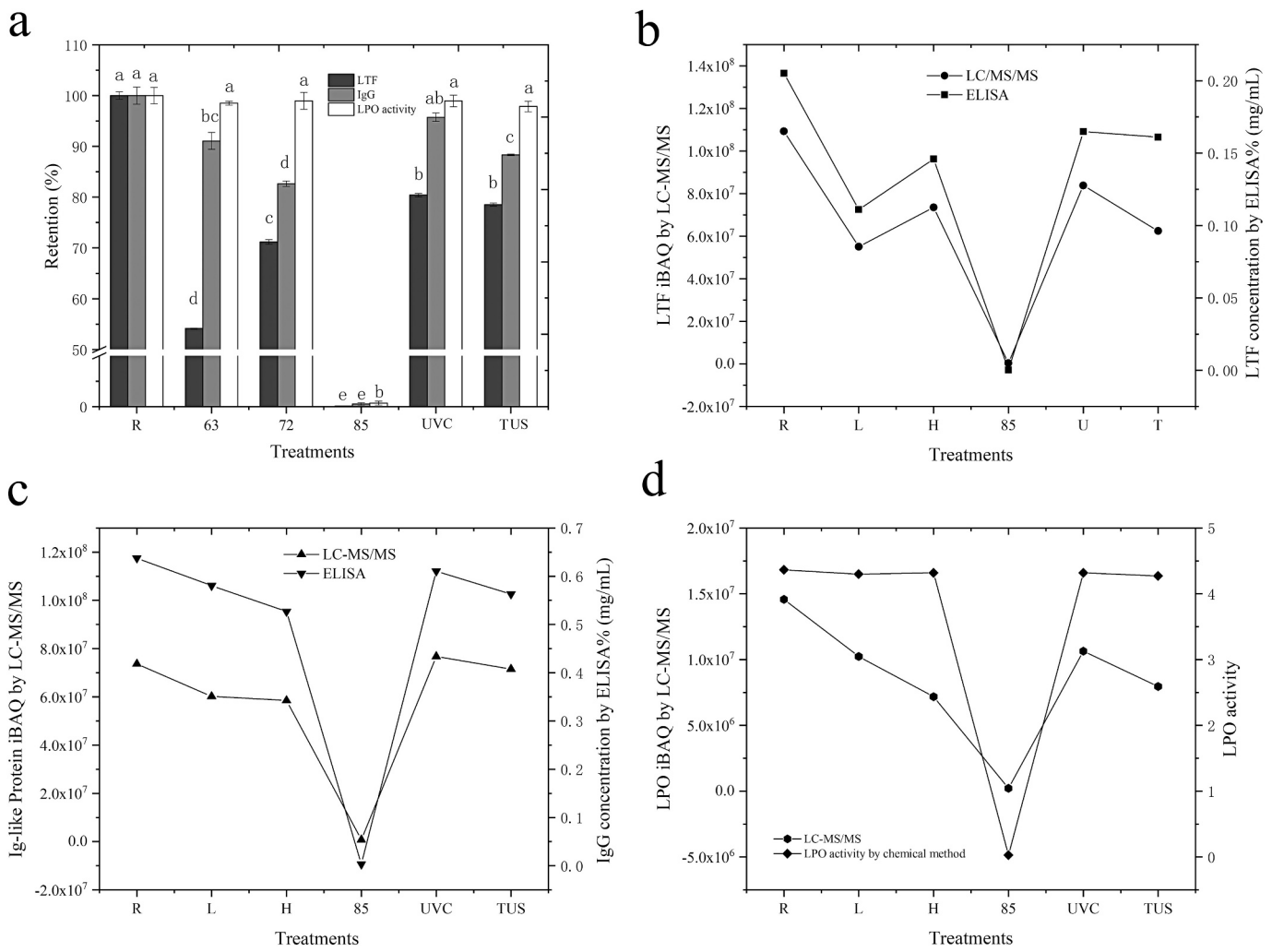

Fig. 6. Retention of lactoferrin, IgG and LPO activity in milk serum after different treatments by ELISA (a); different letters on the same annotation column indicate significant difference ($p<0.05$). Comparison of lactoferrin(b), IgG (c) content by ELISA, LPO (d) activity and LC-MS/MS (data were expressed by the average of three replicates). R: raw milk; 63: heated at 63 °C for 30 min; 72: heated at 72 °C for 15 s; 85: heated at 85 °C for 5 min; UV-C: ultraviolet-C treatment; TUS: thermal ultrasonication.

processing for dairy processing aimed at improved immune-active protein retention. Further studies, including sensory evaluations and digestibility after non-thermal treatments, are needed.

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Declaration of competing interest

KH is a general advisor on milk composition & milk processing to Tamarack Biotics and has performed analytical services. We declare no other conflicts of interests.

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