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# Proteomic study on the stability of proteins in bovine, camel, and caprine milk sera after processing

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ABSTRACT

Milk proteins have been shown to be very sensitive to processing. This study aims to investigate the changes of the bovine, camel, and caprine milk proteins after freezing, pasteurization (62 °C, 30 min), and spray drying by proteomic techniques, filter-aided sample preparation (FASP) and dimethyl labeling followed by liquid chromatography–tandem mass spectrometry (LC–MS/MS). A total of 129, 125, and 74 proteins were quantified in bovine, camel, and caprine milk sera, respectively. The milk serum protein content decreased significantly after freezing, pasteurization, or spray drying, which can be ascribed to the removal of protein aggregates by the pH adjustment and ultracentrifugation during sample preparation. Some of the immune-related proteins were heat-sensitive, such as lactoferrin (LTF), glycosylation-dependent cell adhesion molecule 1 (GLYCAM1), and lactadherin (MFGE8), with losses of approximately 25% to 85% after pasteurization and 85% to 95% after spray drying. α-Lactalbumin (LALBA), osteopontin (SPP1), and whey acidic protein (WAP) were relatively heat stable with losses of 10% to 50% after pasteurization and 25% to 85% after spray drying. The increase of some individual proteins in concentration after freezing may be caused by the proteins originating from damaged milk fat globules and somatic cells. GLYCAM1 decreased significantly after pasteurization in bovine and camel milk but this protein is relatively stable in caprine milk. In conclusion, milk proteins changed differently in concentration after different processing steps, as well as among different species.



## 1. Introduction

Milk proteins can be divided into three groups, lipid (milk fat globule membrane, MFGM), colloidal (caseins), and soluble (whey or serum proteins) proteins. MFGM proteins are the smallest in amount, accounting for only 1–2% of the total milk protein content (Lu et al., 2011). Caseins ($\alpha s_1$, $\alpha s_2$, β, and κ), on the other hand, are the major milk proteins in commercial dairy species, like bovine, camel, and caprine (Moatsou et al., 2008). Serum protein accounts for 20% in commercial dairy animals, whereas it accounts for 40–50% in human milk. Because of the high content of casein in animal milk relative to human milk, adding serum protein to infant formula is an important step to make it more comparable to breast milk (Grant et al., 2005).

Serum proteins, however, are highly sensitive to heat treatment such as pasteurization and spray drying (Nabhan, Girardet, Campagna, Gaillard, & Le Roux, 2004). Pasteurization is an essential step used by the dairy industry for eliminating pathogens from milk. Spray drying is also frequently used in dairy industry for producing many different powders, including infant formula. However, these two processes can modify the structure of milk proteins, and thereby lead to changes in protein functionality (Li et al., 2013). Several immune-active compounds in bovine, camel, caprine, and human milk were found to be reduced in amount after pasteurization (Elagamy, 2000; Ewaschuk et al., 2011; Laleye, Jobe, & Wasesa, 2008; Li-Chan, Kummer, Losso, Kitts, & Nakai, 1995). Intensive heat treatment was shown to affect both the functional properties (Bu, Luo, Zheng, & Zheng, 2009) and solubility of milk serum proteins (Miyamoto et al., 2010).

This loss of protein functionality can be due to two processes occurring during heating. First, proteins may be modified by glycation. Second, denaturation and subsequent aggregation of denatured proteins can occur, which may result in loss of functionality. The effect of these processes on protein functionality is not the same. Glycation has, for example, been shown to be important for allergenicity (Taheri-Kafrani et al., 2009). Denaturation and aggregation, on the other hand, have been shown to be important for immune functionality, for example of lactoferrin and immunoglobulins (Levieux, Levieux, El-Hatmi, & Rigaudière, 2006).

In addition to pasteurization and spray drying, freezing is a technique used to preserve and extend the shelf life of milk (Voutsinas, Katsiari, Pappas, & Mallatou, 1995). Although freezing is commercially applied only for the widespread and constant supply of unheated milk on a year-round basis (Sun, 2005), it is used in producing ice cream in the dairy industry, preserving donated milk in human milk banks

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(Ogundele, 2000), and for scientific research purposes. Researchers have investigated the stability of major proteins from bovine, camel, and caprine milk during heat treatment (Elagamy, 2000; Ewaschuk et al., 2011; Laleye et al., 2008; Li-Chan et al., 1995). However, less is known about the stability of the whole proteome of bovine, camel, and caprine milk after pasteurization and spray drying, and even less is known about the stability after freezing. In addition, the worldwide consumption of camel and caprine milk increases (El-Agamy, 2008; Zervas & Tsiplakou, 2013) and consequently there is a need to know to what extent freezing, pasteurization, and spray drying influence the protein composition of these types of milk.

Thus, the objective of this study is to investigate the sensitivity of soluble milk serum proteins in bovine, camel, and caprine milk to freezing, pasteurization, and spray drying. Because of our interest in immune-active proteins, we focused on quantitative differences in serum proteins, by applying shotgun proteomics techniques, filter aided sample preparation (FASP), and dimethyl labeling combined with liquid chromatography–tandem mass spectrometry (LC–MS/MS). We were able to measure the changes of soluble milk serum proteins among species during processing.

## 2. Material and methods

### 2.1. Material

Camel milk sample was pooled milk obtained from 12 healthy camels (*Camelus dromedarius*) that were between 6 weeks and 12 months in lactation from Kamelenmelkerij Smits camel farm (Berlicum, The Netherlands). Bovine and caprine milk samples, were obtained from tank milk from several farms, and were provided by Lyempf company (Kampen, The Netherlands). Bovine samples were all collected from Holstein Friesian cows and goat samples were all collected from Saanen goats. All the spray dried milk powders were skim milk powders produced by Lyempf (Kampen, The Netherlands). The samples of each of the species, including unheated and processed samples, were collected from the same batch of milk. Samples were transported using an ice box to our lab within 2 h for further analysis.

### 2.2. Methods

#### 2.2.1. Pasteurization, freezing, and spray drying procedures

Milk samples of each of the species were divided into three portions (50 mL each). Fig. 1 shows the schematic workflow. In brief, one portion was used as control (unheated milk). The unheated milk was processed immediately for separating milk serum after we received it. The milk serum was stored at −20 °C for further analysis. The second portion was poured into glass tubes sealed with aluminium foil and heated for 30 min in a heating block at 62 °C–63 °C, which is a well-established condition for batch pasteurization (FAO&WHO, 2009). After heat treatment, the milk was immediately cooled to room temperature for centrifugation. The third portion was stored at −20 °C for three weeks. The frozen milk was thawed at room temperature before centrifugation. Spray dried milk powder was obtained by first pasteurizing and defatting at a maximum temperature of approximately 80 °C, and then evaporating at a maximum of approximately 90–95 °C. Lastly, samples were dried at a maximum of approximately 80 °C. Spray dried milk powder was dissolved in Milli-Q water, at a weight ratio of 1:10. To obtain a well-dissolved milk sample, Milli-Q water was slowly added to the milk powder under continuous manual stirring.

#### 2.2.2. Centrifugation

We prepared all samples in triplicates from centrifugation and did sample preparation and analysis separately for all samples. Samples were centrifuged at 1500 ×*g* for 10 min at 10 °C (with rotor 25.15, Avanti Centrifuge J-26 XP, Beckman Coulter, USA) to remove the fat.

#### 2.2.3. pH adjustment

After centrifugation, all skimmed milk samples were acidified by drop-wise addition of 1 M HCl under stirring, until a pH of 4.6 was reached. The samples were then kept at 4 °C for 30 min to equilibrate. When needed, pH was adjusted before the final pH reading. This pH adjustment was done to separate the denatured aggregated serum proteins from the native serum proteins during ultracentrifugation, as previously described by (Law & Leaver, 2000; Spiegel, 1999).

#### 2.2.4. Ultracentrifugation

The acidified skim milk was transferred to ultracentrifuge tubes followed by ultracentrifugation at 100,000 ×*g* for 90 min at 30 °C (Beckman L-60, rotor 70 Ti). After ultracentrifugation, samples were separated into three phases. The top layer was milk fat, the middle layer was milk serum, and the bottom layer (pellet) was casein. Milk serum was used for BCA assay and filter aided sample preparation (FASP) as described below.

#### 2.2.5. Bicinchoninic acid (BCA) assay

BCA Protein Assay (Thermo Scientific Pierce) was used to determine protein concentration, according to the manufacturer's instructions. Bovine serum albumin was used as the standard to make a calibration curve, covering the protein concentration from 0.02–2 μg/μL, and the milk serum protein concentration was determined and adjusted so that an equal amount of protein was used for proteomics sample preparation.

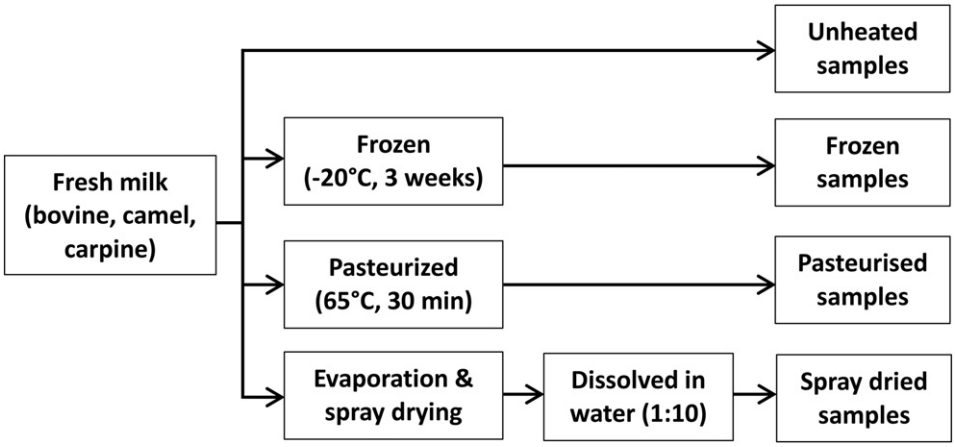


**Fig. 1.** Schematic workflow of the sample collection and preparation.