THE BOVINE MILK PROTEOME: WHAT'S IN IT AND HOW CAN IT BE MANIPULATED?

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INTRODUCTION

Thousands of proteins have been identified in bovine milk to date, and the dairy industry has an opportunity to benefit from identification and characterization of this proteome on both the cow and consumer side. Pre-weaned calves are responsive to the quality of colostrum and mature milk, which could be in part due to the protein profile and could impact their immune system development and growth. Consumer health is also impacted by the protein profile of bovine milk, with some proteins in milk being heat resistant and persisting as intact bioactive compounds even in pasteurized milk products sold at the supermarket. Beyond the interest in the enhancement of bioactive proteins within milk, the possible use of proteins within milk as biomarkers for cow productivity or health is also a possibility. Diet and management-related treatments appear to affect some of the proteins present in milk, providing a mechanism to naturally shift the milk proteome. This lecture will review the overall makeup of the proteome, some examples of known bioactive proteins, and what is currently known about management and nutritional factors that can influence the proteome.

WHAT IS THE MILK PROTEOME?

Simply put, the milk proteome encompasses all proteins present in milk. However, these proteins are diverse in their origin, function, and proportions, and this simplistic definition juxtaposes the complexity of the protein profile highlighted through characterization of the proteome. In terms of the basic protein profile, approximately 80% of milk proteins are caseins. Caseins, along with the two major whey proteins, α-lactalbumin and β-lactoglobulin, are considered to be the high abundance milk proteins. These are synthesized in the mammary gland and are expelled from the secretory cells by simple exocytosis (Mercier and Gaye, 1980). The term low abundance milk proteins encompasses all other whey proteins. It is this low abundance protein fraction that has garnered the most attention over the past several years, with high throughput techniques such as mass spectrometry (MS) being included in the laboratory workflow as a method to identify thousands of milk proteins present in a milk sample. Separating these different milk proteins through phasic separation is perhaps the simplest and most crude method for categorizing milk proteins. Centrifugation of a milk sample can accomplish this basic separation such as described by Nissen et al. (2013), yielding three different categories of proteins: 1) milk fat globule membrane (MFGM)-associated proteins, 2) skim milk-associated proteins, and 3) cell pellet-associated proteins. As reported by Nissen et al. (2013), there is some overlap in the proteins identified across

the different fractions; however, these fractions also each contain their own unique protein profiles and can, in a sense, each be considered as their own unique proteome.

Low abundance proteins have been identified within the milk fat globule membrane (MFGM) and are secreted by the mammary secretory cells in association with MFGM secretion (Reinhardt and Lippolis, 2008; Pisanu et al., 2011). Endogenous losses likely also contribute a large portion of the milk low abundance protein profile through sloughing of secretory cells and through secretion of cytosolic crescents, which are portions of the secretory cell cytosol that become entrained within the MFGM prior to its secretion (Heid and Keenan 2005). The average number of cytosolic crescents entrained within the MFGM ranges across species (Huston and Patton, 1990). Proteins associated with the MFGM include a large proportion of membrane-associated proteins, with research reporting a wide variation in this proportion. For example, Yang et al. (2015) reported that approximately 35% of the proteins identified in the MFGM were membrane-associated proteins, while Reinhardt and Lippolis (2008) reported that approximately 70% of identified proteins being membrane-associated in their trial. In terms of functionality, a high proportion of proteins isolated with the MFGM are associated with binding function (Yang et al., 2015, identified 49% of the MFGM-associated proteins being involved with binding).

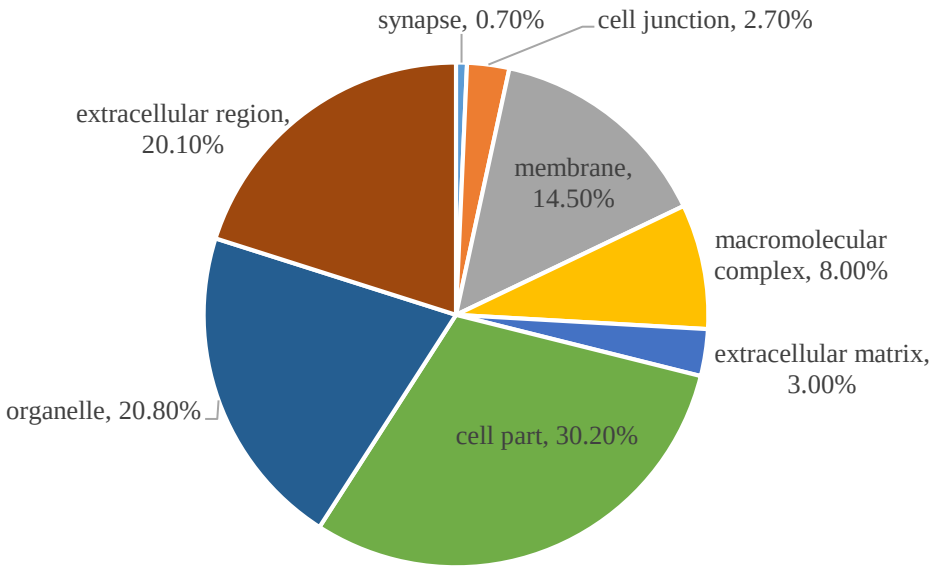

Figure 1. Cellular component analysis of low abundance proteins identified in the skim milk fraction by Tacoma et al. (2016).

While the skim milk fraction includes the high abundance proteins as well as many low abundance proteins, not all skim milk-associated proteins seem to be mammary cell-derived, some having a high concentration in blood (example, serum albumin). Evidence of protein secretion into the alveolar lumen primarily via transcellular routes (similar to casein secretion) but also paracellular routes (through ‘leaky’ tight junctions) exists (Peaker and Taylor, 1975). Gene ontology analysis of the skim milk

fraction helps to highlight the diversity of cell component localizations of these low abundance proteins within the cell. Using a data set listing skim milk-associated low abundance proteins published by Tacoma et al. (2016), we can more closely examine the cell component analysis of this proteome. PANTHER classification analysis (Mi et al., 2017) of this data set classified the majority of proteins as associated with the cell (Figure 1). This cellular component breakdown is similar to that identified through MFGM gene ontology classification (see Reinhardt et al., 2013, for example).

D'Alessandro et al. (2011) suggested that the majority of identified low abundance proteins appear to be involved in 1) host defense and immunity, 2) structure, 3) lipid transport, or 4) cellular growth and differentiation. These categories all do appear to be repeatedly represented across the different published data sets; however, some variation in their dominance is evident. Using again the Tacoma et al. (2016) data set, examination of the biological process classification of these proteins identified a lesser proportion of immune-associated proteins in skim milk from cows that were nearing mid-lactation (Figure 2). Conversely, the number of proteins with known immune system function are higher in colostrum. To visualize this, Figure 3 depicts the same biological process analysis using proteins identified in the skim milk fraction of colostrum by Tacoma et al. (2017b).

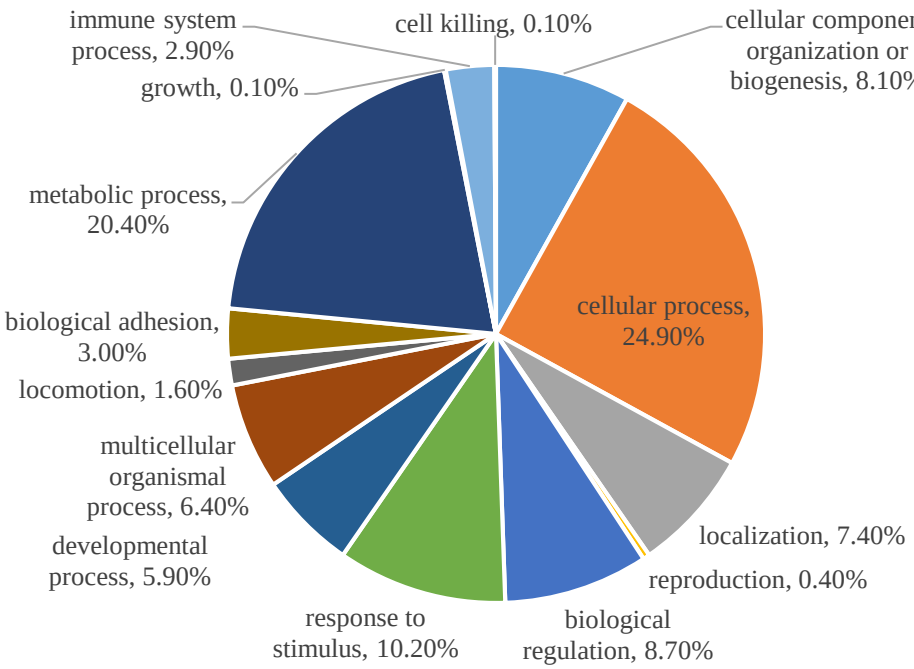

Figure 2. Biological analysis of low abundance proteins identified in the skim milk fraction by Tacoma et al. (2016). These milk samples were collected from Holstein cows nearing mid-lactation.

The cell pellet is formed from centrifugation of a milk sample, whereby the larger cellular debris is separated as the heavier phase of the three. Of the protein fractions directly compared by Nissen et al. (2013), it was the cell pellet fraction that yielded the

highest number of proteins, as well as the protein profile that had the least overlap with the non-fractionated milk, the cell and fat free fraction, or the whey fraction. In this cell pellet fraction, the exosomal proteome is also captured and methods to further isolate this specific group are published (Reinhardt et al., 2012). As expected, the exosome proteome contains a higher proportion of organelle-associated proteins compared the MFGM or skim milk proteomes, but also includes a higher number of proteins with molecular and binding functions compared to the MFGM and skim milk proteins (Reinhardt et al., 2012).

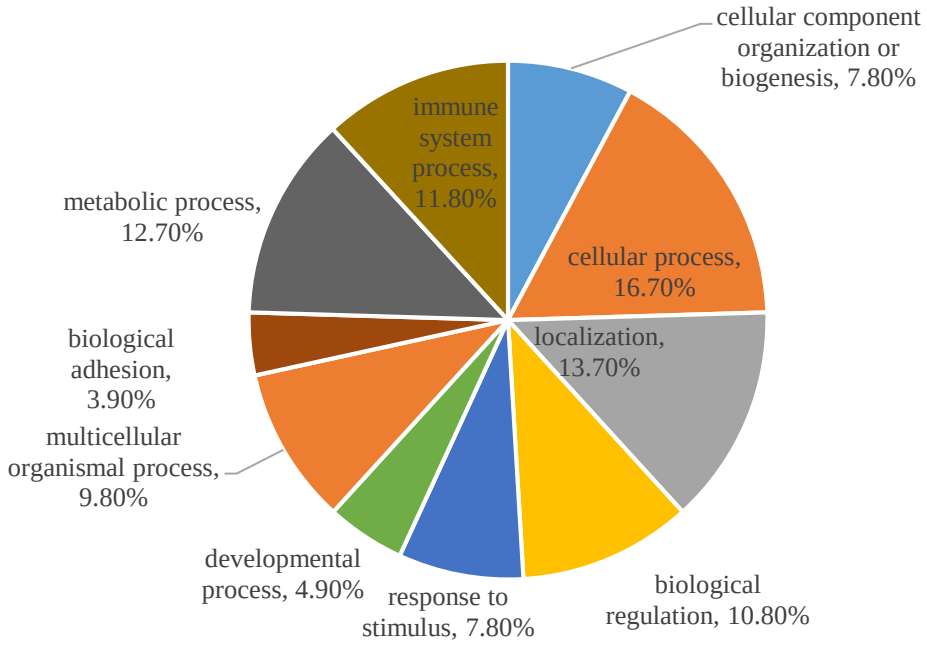

Figure 3. Biological analysis of low abundance proteins identified in the skim milk fraction by Tacoma et al. (2017b). These milk samples were collected from Holstein cows during the colostrum period.

KNOWN BIOACTIVES

While the gene ontology classifications of the milk proteome provide a sense of the likely biological targets, research focusing on the bioactivity of these proteins is underway. From this research, it is evident that the bioactivity of proteins and peptides is diverse in terms of target and potency. A comprehensive list of the bioactive proteins and peptides within milk has not been published for some time, but some examples of the diversity are well documented by Clare and Swaisgood (2000). Lactoferrin is a specific low abundance protein in milk that highlights the potential biological strength of these proteins. It stimulates intestinal epithelial cell proliferation and differentiation (Buccigrossi et al., 2007; Lönnerdal et al., 2011), intestinal epithelial cell sucrase and lactase activity (Buccigrossi et al., 2007) and antimicrobial activity (Lönnerdal, 2014). Additional low abundance proteins such as osteopontin, lysozyme, haptocorrin and lactoperoxidase all act on the milk-fed animal to stimulate biological activity (Lönnerdal, 2003; Lönnerdal, 2014).

Importantly, the bioactivity of bovine milk proteins affects not only the milk-fed calf, but also the human consumer due to cross-reactivity. Casein-derived peptides created from the digestion of cow-milk based infant formulas in the infant gut have functionality (Raikos and Dassios, 2014), while low abundance proteins present in these cow milk-based infant formulas also show human cross-bioactivity. For example, Lönnerdal et al. (2011) were able to enhance intestinal epithelial cell (Caco-2 cell) differentiation by 27% through the addition of 50 µg/mL of bovine lactoferrin to the media, while Buccigrossi et al. (2007) observed 20% higher Caco-2 cell lactase activity when treated with bovine lactoferrin compared to the human lactoferrin. In addition, Jiang and Lönnerdal (2013) have identified the regulation of 284 genes in human intestinal cells (HIEC cells) by bovine osteopontin, with the majority of affected genes being related to cell proliferation and immune function. Crucially, the presence and bioactivity of milk low abundance proteins even persists after pasteurization of milk regardless of species of origin (Maga et al., 2013; Sousa et al., 2014), providing the feasibility of large-scale commercialization to take advantage of these milk proteins in the human food market.

MANAGEMENT AND NUTRITIONAL MANIPULATION

The ability to change the milk protein profile has been validated in both our laboratory and by others. Some of the earliest research in this area demonstrated the potential for diet manipulation of the milk protein profile by reporting milk casein content. Ostersen et al. (1997) reported an increase in γ-casein and total whey protein in milk in cows with a higher calving body condition score (BCS), and early research by Christian et al. (1999a and b) reported shifts in the casein content of milk from cows given different pasture allowances or offered different base rations. While these two published works demonstrate the potential to alter the milk protein profile, they preceded MS technology, and hence could not give an indication of the dietary impacts on the greater milk proteome. Later research published by Danowski et al. (2013) uncovered some of the first evidence of diet-induced manipulation of low abundance proteins in cow's milk. In this study, milk lactoferrin concentrations were 52.6% lower (77.5 µg/mL less) from cows fed an energy restricted diet (49% of requirement) compared to milk samples from control cows, translating to 4.5 versus 1.9 g of lactoferrin secretion per day from control versus restricted cows, respectively, based on milk yield of the respective treatments. Focusing on the impact of energy balance, Lu et al. (2013) found that shifts in energy balance during the transition period affected the abundance of 10 milk proteins and numerous milk serum metabolites associated with energy utilization, including Acyl-CoA synthetase, NADH-cytochrome b5 reductase, β-hydroxybutyrate, carnitine, N-acetyl sugars, and acetoacetate. Research by Sun et al. (2015) reported the impact of low- and high- forage quality inclusion in the diet on the milk proteome. Along with a negative impact on milk production and milk efficiency, 8 milk proteins were affected by the diet, including creatine.

Manipulation of the diet beyond restriction is scarcer. Li et al. (2015) reported a shift in the abundance of zinc-α-2-glycoprotein (ZAG), a bioactive protein in milk, by manipulating the rumen degradation rate of the diet. These researchers used steam-flaked or finely ground corn, and either solvent-extracted soybean meal or heat-treated

soybean meal, to create diets that were considered to either undergo fast or slow rumen fermentation rates. Providing a diet that can be rapidly degraded was reported to increase the abundance of ZAG in milk. Manipulating the dietary RDP: RUP ratios does not appear to be the causative factor of such as shift, as Tacoma et al. (2017a) observed no difference in the 595 milk proteins identified by MS when cows were fed isoenergetic and isonitrogenous diets that included a 10% swing in the RDP: RUP ratio across the dietary treatments. While these results combined could indicate that the milk proteome is most reactive to energy balance or total intake, it is plausible that it could also be that other non-nitrogenous dietary components, such as the carbohydrate shifts used in Li et al. (2015), which may be the more dominant drivers of the milk proteome.

SUMMARY

The dairy industry could benefit from our knowledge of milk proteomics on several levels, including calf health, biomarker development, and commodity markets. The bioactivity of milk proteins, both at the calf and human level, appear to include a diverse array of functions; however, the variability of the protein profile and our understanding of how to manipulate it is less elucidated. Ongoing research to solidify our understanding of this unique aggregate is helping to tease out the possible drivers that impact the proteome, and will help in our exploitation of the healthfulness of milk or use of this fluid as a diagnostic tool.

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