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## Colostrum and milk protein rankings and ratios of importance to neonatal calf health using a proteomics approach

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### ABSTRACT

Administration of colostrum to the newborn calf before gut closure is pivotal to its health, because of the transfer of passive immunity. Traditionally, passive immunity has been attributed to the transfer of immunoglobulins although it is increasingly clear that multiple other factors contribute, including innate immune proteins, developmental factors, immunomodulatory factors, and the presence of cellular immunity. The objective of this study was to produce a comprehensive comparison of the bovine colostrum proteome and the milk proteome by applying 2-dimensional liquid chromatography-tandem mass spectrometry. Further, the objectives were to rank proteins mutually and generate protein ratios from the spectral counts of the 2 proteomes and ELISA to gain insight into which proteins could be of most relevance to neonatal calf health. To obtain an in-depth picture of the bovine colostrum and milk proteome, we compared the contents of different fractions from bovine colostrum and milk from our 2 previous studies. A total of 140 colostrum fluid-phase proteins and 103 milk fluid-phase proteins were detected. In the cellular fraction, 324 and 310 proteins were detected in colostrum and milk, respectively. In total, 514 proteins were detected, of which 162 were in the fluid phase. Of these, 50 proteins were exclusively seen in colostrum, 13 were exclusively seen in milk, and 99 were common to colostrum and milk. Ranking of proteins mutually and calculating protein ratios based on spectral counts and ELISA resulted in new information on how proteins were associated with the fluid or cellular fraction of the samples. Interestingly, despite lower counts/concentrations than the classical

proteins such as immunoglobulins,  $\beta$ -lactoglobulin, and lactotransferrin, several proteins appeared in higher or similar colostrum:milk spectral count ratios as these. Using this approach indicated, for example, that osteopontin, haptoglobin, milk amyloid A, and gelsolin may be interesting molecules to study in detail in their relation to calf health. Although the sensitivity, identification, and ranking of proteins varied between the 2 methods, and proteome analysis clearly suffers from low sensitivity, we believe that this idea and approach of generating ratios and ranking proteins can contribute new information and perspectives on how to prioritize the importance of multiple proteins, beyond immunoglobulins, in relation to neonatal calf health.

**Key words:** *Bos taurus*, colostrum proteome, milk proteome, transfer of passive immunity

### INTRODUCTION

The period just after birth is critical in neonatal life. The neonate calf has an immature immune system and is exposed to many environmental challenges. The gut epithelium loses the ability to take up massive loads of proteins from the colostrum approximately 24 h postpartum, in a process referred to as gut closure (Stott et al., 1979; Bush and Staley, 1980); thus, early administration of colostrum to the newborn calf, before gut closure, is pivotal to health and survival and has traditionally been attributed to the transfer of passive immunity (TPI) via immunoglobulins (Weaver et al., 2000). Proteins other than immunoglobulins that are involved in the humoral immune system could play a role in the neonate. This could include, for example, complement factors, antimicrobial peptides, and immunomodulatory factors. Further, developmental factors transferred from colostrum are believed to play important roles both locally in the calf's gastrointestinal (GI) tract and systemically in the calf (Stelwagen et

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al., 2009; Wheeler et al., 2012). The biological mechanism of TPI ensures that these components are transferred to the neonatal calf. At gut closure, the role of colostrum shifts from being immunological to primarily nutritional and growth, which prevents further absorption of intact immunoglobulins, proteins, and peptides from colostrum or milk.

Indeed, cow milk also contains immunologically important proteins, although these are in relatively low amounts and are thus believed to have little biological significance compared with that of colostrum (Stelwagen et al., 2009; Nissen et al., 2013). The proposed primary immunological function of these proteins is to protect the dam's udder from developing mastitis caused by infection with extra-mammary pathogenic bacteria (Rainard and Riollet, 2006). A second immunological function is to provide local immunity to the calf's GI tract, which readily may be infected with environmental pathogens.

The contribution of colostrum to the neonatal calf's health via cellular immunity has been the subject of research (Le, 1996). The cells present in colostrum consist mainly of leukocytes and mammary epithelial cells. Besides providing protection to the mammary gland itself, the leukocytes serve a dual role of contributing with proteins to the fluid phase (Stelwagen et al., 2009) and also directly contributing to the neonatal calf's health locally by translocating to the mucosa in the GI tract of the calf or systemically by translocating from the GI tract to the calf's blood circulation (Le, 1996).

Several researchers have investigated the changes in proteins during transition from colostrum to milk. Yamada et al. (2002) used 2-dimensional electrophoresis followed by microsequencing and MS and identified 29 proteins. Reinhardt and Lippolis (2008) explored the changes in the proteome of milk fat globule membranes by shotgun proteomics and *isobaric tags for relative and absolute quantitation* (iTRAQ; >138 proteins detected). Senda et al. (2011) used an approach similar to Yamada et al. (2002) and detected 25 proteins, and Le et al. (2011) applied ion-exchange separation combined with MS to provide semiquantitative spectral counting-based analysis of 293 proteins. More recently, Zhang et al. (2015) used shotgun proteomics and a dimethyl labeling approach to quantify the changes between colostrum and milk (212 proteins detected).

Previously, we published 2 comprehensive proteome-based studies of the of the proteins present in bovine colostrum sampled from the first milking after calving and in milk sampled on d 10 (Nissen et al., 2012, 2013). These results allow us to investigate whether biologically active proteins other than immunoglobulins were exclusive to colostrum or present in higher concentrations in colostrum than in milk. The aims of

the present study were, first, to compare proteins in colostrum and milk from healthy animals, by mapping the unique complementary proteins as well as common proteins in colostrum and milk. Proteins were grouped according to their relative presence in either fluid phase or cellular fraction, or both. The second aim was to conduct a semiquantitative spectral count (SC) analysis to rank proteins and estimate protein abundance in the 2 samples. Finally, we calculated protein ratios between colostrum and milk from the SC as well as from preselected proteins analyzed by ELISA to investigate whether this novel approach could be used to generate new knowledge and perspectives on how to prioritize the importance of multiple proteins in relation to neonatal calf health beyond the immunoglobulins.

## MATERIALS AND METHODS

This investigation was based on the results previously reported for proteins identified in colostrum (Nissen et al., 2012) and milk (Nissen et al., 2013). Both publications contain a thorough description of the procedures of fractionation and analysis; therefore, only a brief summary and an illustration of the fractionation and sample processing is presented here (Figure 1).

### Animal Health and Milk Sampling

Composite colostrum and milk were collected on d 1 and 10 postpartum, respectively, from 4 healthy Danish Holstein-Friesian cows in second lactation. The cows had been dried off 5 to 7 wk before calving. Each cow was milked twice a day at 0700 and 1600 h. Udder health and body temperature were recorded in the period from parturition (d 1, colostrum sampling; cut-off value as healthy <39.1°C) to d 10 (milk sampling; cut-off value healthy <37.9°C). Udder inflammation based on foremilk strippings was scored using a semiquantitative cow-side test, California Mastitis Test (CMT; Kruse, Marslev, Denmark; Pyörälä, 2003). The milk was collected immediately after calving and was accepted if CMT score was <3. On d 10 (milk sampling), milk was accepted if the CMT score was <2. Bacteriological analyses were done on foremilk samples according to National Mastitis Council guidelines on the first 6 milkings and on d 10 and were negative for major bacterial pathogens (National Mastitis Council, 1999). Further, milk samples were collected at the first 6 milkings postpartum and on d 10 and analyzed for SCC in a diagnostic dairy laboratory (Eurofins Steins Laboratory, Holstebro, Denmark). All collected milk samples for 2-dimensional liquid chromatography-tandem mass spectrometry (2D-LC-MS/MS) analysis had SCC  $<2.9 \times 10^6/\text{mL} \pm 8.0$  (colostrum, d 1) and SCC  $<9.4$

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