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Shotgun proteomics of homogenate milk reveals dynamic changes in protein abundances between colostrum, transitional, and mature milk of swine

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Abstract

Milk is an easily digestible source of nutrients and bioactive factors, its composition reflects the neonate's needs, and changes from colostrum to transitional and mature milk. Our objective was to measure milk fat, lactose, total carbohydrate, and protein content in parallel with global proteome of homogenate milk samples to characterize changes across the three phases of swine lactation. Milk samples were collected from multiparous sows ($n = 9$) on postnatal day 0 (D0; colostrum), 3 (D3; early transitional), 7 (D7; late transitional), and 14 (D14; mature). On D3, percent fat (16 ± 2.1) and lactose (3.8 ± 0.3) were higher ($P < 0.05$) than on D0 (10 ± 3.9 and 1.5 ± 0.3 , respectively). Levels of fat and lactose were not different between D3 and D14. Percent total protein decreased ($P < 0.05$) between D0 (11 ± 2.1) and D3 (5 ± 0.7), but there was no significant change in percent protein between D3 and D14. Total carbohydrates increased ($P < 0.05$) between D3 ($944 \pm 353 \mu\text{g/mL}$) and D14 ($1,150 \pm 462 \mu\text{g/mL}$). Quantitative proteomic analysis using liquid chromatography tandem mass spectrometry (LC-MS/MS) of homogenate D0, D3, and D14 milk samples ($n = 6$) identified 772 protein groups which corresponded to 501 individual protein-coding genes. A total of 207 high confidence proteins were detected in $n = 3$ sows/day. Of the high confidence proteins, 81 proteins were common among all 3 days of lactation. Among the proteins that decreased between the days (false discovery rate; FDR < 0.05) were multiple apolipoproteins and XDH which decreased between D0 to D3. Proteins that increased across the days (FDR < 0.05) were complement factors and 14-3-3 proteins (YWHAQ, YWHAE). Our data provide a good characterization of milk proteome changes that likely reflect mammary function as well as the neonate's phase-specific developmental needs. This data may be useful in developing approaches to enhance the health and welfare of swine.

Key words: mammary gland, milk, lactation, proteome, sow

Abbreviation

ACN	acetonitrile
AGC	automatic gain control
BSA	bovine serum albumin
DAVID	database for annotation, visualization, and integrated discovery
DTT	dithiothreitol
FDR	false discovery rate
HCD	higher energy collisional dissociation
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
LC MS/MS	liquid chromatography-tandem mass spectrometry
LD	cytosolic lipid droplets
LFQ	label-free quantitation
PBS	phosphate-buffered saline
PSM	peptide spectral match
UPLC MS/MS	ultra-performance liquid chromatography-tandem mass spectrometry

Introduction

Milk is the only source of nutrients and bioactive factors for the suckling neonate and there are marked changes in the composition of milk across the course of lactation. These changes reflect both the metabolic activity of the mammary gland and the species-specific developmental needs of neonates (Hassiotou et al., 2013; Farmer, 2019; Quesnel and Farmer, 2019). During gestation and around the time of parturition, the mammary gland undergoes structural and functional changes that prepare the organ for milk synthesis and secretion. The endocrine milieu of pregnancy initiates and drives structural changes in the gland and the progressive differentiation of milk-producing lactocytes. Lactocyte secretory differentiation encompasses the first stage of lactogenesis and results in colostrum accumulation in the gland.

This first stage of lactogenesis is characterized by low levels of milk protein and lactose synthesis and active transport of immunoglobulin G (IgG) molecules across lactocytes (Quesnel et al., 2012; Quesnel and Farmer, 2019).

Colostrum is available to neonates immediately after birth, and the high energy and IgG content of colostrum is particularly important in swine, as piglets have almost no body-fat reserves, no circulating antibodies, and lack a fully functional immune system (Le Dividich et al., 2005; Šamanc et al., 2013). Thus, neonatal pigs rely on colostrum intake for thermogenesis and the establishment of passive immunity. Colostrum is typically available to the piglet the first 24 h after birth, which is the window of time that neonates can absorb IgG (Hurley, 2015). The second phase of lactogenesis begins 24–36 h post parturition and is marked by secretory activation of lactocytes. Secretory activation is hormonally driven and initiated by the drop in progesterone and the concomitant rise of prolactin and glucocorticoids that occur around the time of farrowing (Stelwagen et al., 1999; Neville et al., 2001; Farmer, 2019; Quesnel and Farmer, 2019). These periparturient hormonal changes stimulate tight junction closure between lactocytes, cessation of paracellular transport, and the onset of copious milk synthesis (Capuco et al., 2003). The capacity for de novo fatty acid synthesis in lactocytes and increased capacity for lactose synthesis are

primary markers for secretory activation (Arthur et al., 1989; Neville et al., 2001), and thus characteristics of the onset of the transitional phase of milk production (Anderson et al., 2007).

The growth rate of piglets is the highest in the first week postnatal than at any other time after birth. Compared with 4 weeks old piglets, at 1 week of age, piglets exhibited enhanced protein synthetic capacity in skeletal muscle and other organs in response to feeding. This enhanced capacity for protein synthesis was marked by high ribosomal content and increased efficiency of protein translation (Davis et al., 2008). The high energy content of transitional milk, defined as 34 h to 9 days postpartum, is thus likely needed to support this period of accelerated growth. With the period of transitional milk production, there is a decline in protein synthetic capacity in conjunction with gradual changes in milk composition. The period of mature milk synthesis in swine is considered to begin around 10 days after parturition and continue until weaning (Theil et al., 2014). Compared with earlier stages, macronutrient concentrations in mature milk remain stable throughout lactation (Hurley, 2015).

The overall premise driving this study was the concept that milk composition evolved to reflect the suckling neonate's growth and developmental needs. Therefore, a comprehensive survey of milk proteome changes across the course of swine lactation that will likely help in understanding the early postnatal developmental needs of piglets, and enable research aimed at developing unique nutritional-management strategies that decrease the currently high rate of preweaning morbidity and mortality. Previous studies of swine milk proteome employed one- and two-dimensional polyacrylamide gel electrophoresis and multidimensional protein identification technology for shotgun analysis (Wu et al., 2010; Ogawa et al., 2014). From these studies, it was demonstrated that the proteomic profile of swine milk is unique (Ogawa et al., 2014; Wu, et al., 2018). However, the swine milk proteome remains understudied and lacks vital information to better understand the nutritional and developmental needs of neonates. The objective of this study was to characterize the changes in macronutrient composition and the proteome of homogenate milk samples between colostrum, transitional, and mature milk phases of lactation using LC-MS/MS.

Materials and Methods**Experimental animal design**

All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (Purdue IACUC Protocol #1605001416 and NC AT 18-007.0-A) prior to the start of the experiments. Milk samples used for this study were collected from nine multiparous sows at the Purdue University Animal Research and Education Center (ASREC) Swine Unit. Standard farrowing protocols for the ASREC Swine facility were followed, with sows were allowed to farrow naturally, after being moved to maternity crates on day 112 of pregnancy. On postnatal day one, all litters were standardized to 12–14 piglets.

Milk collection

Colostrum samples were collected from sows ($n = 9$) 1 h after delivery of the first piglet on day 0 (D0). Early transitional milk was collected on day 3 (D3) and late transitional milk was collected on day 7 (D7). Milk samples collected on day 14 (D14) represented mature milk. During colostrum collection, piglets remained with the dam, and sample collection was done during

active labor when natural levels of oxytocin are high. On days 3, 7, and 14, at 0600, piglets were removed from the sow for 1 h before milk collection. To stimulate milk letdown, just prior to collection, sows were injected with 1 mL oxytocin (VetOne; Boise, ID; 20 USP/mL) intravulvar using a 20-gauge × 1.5-inch needle. If milk was not let down in response to exogenous oxytocin, one or two piglets were returned to sow to further stimulate milk letdown. Teats were cleaned with 70% ethanol and wiped clean with sterile gauze then sprayed with water to remove excess ethanol left behind and wiped clean again with sterile gauze. All sows were hand milked, and samples were collected into sterile 50 mL polypropylene conical tubes. Milk was collected from at least two posterior and anterior teats. For colostrum and milk samples, at least 45 mL of milk was collected. Milk and colostrum were collected evenly from thoracic, abdominal, and inguinal mammary glands. Following collection, homogenous milk samples were aliquoted equally into 2 mL conical tubes, flash frozen in liquid nitrogen, and stored at -80°C until further analysis.

Protein content analysis

Total protein content of colostrum and milk samples was measured using the Coomassie Plus (Bradford) Protein Assay (Pierce Coomassie Plus Protein Assay cat #23236, Thermo Fisher Scientific) following the manufacturer's protocol. Whole milk samples were diluted 1:50 in sterile 1X phosphate-buffered saline (PBS). Absorbance was read on a Sparks 10M spectrophotometer (TECAN) at 595 nm. Protein content was determined using the standard curve generated using cubic polynomial curves with online software (<https://www.elisaanalysis.com/>).

Total carbohydrate analysis

Total carbohydrate concentration in milk samples was measured using the Total Carbohydrate Assay Kit (Total Carbohydrate Assay Kit Quantification cat #155891, Abcam) following the manufacturer's protocol. Whole milk samples were diluted at 1:500 in deionized water and 30 μL of each sample was transferred in duplicate into a 96-well plate. Absorbance was read on a microplate reader (Molecular Devices, SpectraMax Spectrofluorometers) at 490 nm.

Proteomic analysis

Proteomic analysis was conducted in the Purdue Proteomics Facility at the Bindley Bioscience Center, Purdue University on whole milk samples collected from six of the nine sows. Selection of the sows for proteomic analysis was random, and limited to D0, D3, and D14 samples for analysis. The D7 time point that was omitted at this time point also represents transitional milk. Briefly, samples were thawed, homogenized, and 150 μL were lysed on a Barocycler using 90 cycles of 20 s at 33 kPsi, and 10 s at 1 ATM, at 4°C (NEP2320, Pressure Biosciences Inc.). Following lysis, 3 volumes of 8 M urea were added, and centrifuged at 17,200 g for 10 min at 4°C . The supernatant was collected, and protein concentration was measured using bicinchoninic acid assay (BCA; Pierce Chemical Co.). The supernatant containing 50 μg of protein (equivalent volume) was mixed with 4 volumes of cold (-20°C) acetone and incubated overnight at -20°C . Samples were centrifuged at 17,200 rcf for 10 min at 4°C , the supernatant was discarded, and the protein pellets were briefly dried in a vacuum centrifuge. Protein was reduced by adding 10 μL of 10 mM dithiothreitol (DTT) and 8 M urea to the samples and incubated at 37°C for 1 h. Then, 10 μL of alkylation mixture (195 μL acetonitrile [ACN], 1 μL triethylphosphine, 4 μL

iodoethanol) was added, and samples were incubated at 37°C for 1 h. Samples were then digested with LysC/Trypsin (Promega) at a ratio of 1:25 using a Barocycler, with 60 cycles of 50 s at 20 kPsi and 10 s at 1 ATM, at 50°C . Peptides were desalted with C18 MicroSpin Columns (The Nest Group, Inc.).

Shotgun label-free liquid chromatography tandem mass spectrometry (LC MS/MS) was used for proteome analysis. Peptides were analyzed in a Dionex UltiMate 3000 RSLC nano System (Thermo Fisher Scientific, Odense, Denmark) coupled online to an Orbitrap Fusion Lumos Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), as described previously (Mohallem and Aryal, 2020; Zembroski et al., 2021). Reverse phase peptide separation, using a trap column (300 μm ID × 5 mm) packed with 5 μm 100 Å PepMap C18 medium coupled to a 50-cm long × 75 μm inner diameter analytical column packed with 2 μm 100 Å PepMap C18 silica (Thermo Fisher Scientific). The column temperature was maintained at 50°C . The sample was loaded to the trap column in a loading buffer (3% acetonitrile, 0.1% formic acid) at a flow rate of 5 $\mu\text{L}/\text{min}$ for 5 min and eluted from the analytical column at a flow rate of 200 nL/min using a 120-min LC gradient as follows: linear gradient of 6.5% to 27% of solvent B (80% acetonitrile, 19.9% water, and 0.1% formic acid) in 82 min, 27%–40% of solvent B in next 8 min, 40%–100% of solvent B in 7 min at which point the gradient was held at 100% of solvent B for 7 min before reverting to 2% of solvent B and held at 2% for 15 min for column equilibration. The column was washed and equilibrated using three 30-min LC gradients before injecting the next sample. All the data were acquired in the Orbitrap mass analyzer using an higher energy collisional dissociation (HCD) fragmentation scheme. The MS scan range was from 350 to 1,600 m/z at a resolution of 120,000. The automatic gain control target was set at 4×10^5 , maximum injection time (50 ms), dynamic exclusion 30 s, and intensity threshold 5.0×10^4 . MS data were acquired in data dependent mode with a cycle time of 5 s/scan. MS/MS data were collected at a resolution of 15,000. LC-MS/MS data were queried for protein identification and label-free relative quantitation using MaxQuant software (version 1.6.3.3) against *Sus scrofa* sequence database downloaded from UniProt (www.uniprot.org), using the following parameters: precursor mass tolerance of 10 ppm; enzyme specificity of trypsin/Lys-C allowing up to 2 missed cleavages; oxidation of methionine (M) and Acetyl N-term as a variable modification and iodoethanol (C) as a fixed modification. False discovery rate (FDR) of peptide spectral match and protein identification was set to 0.01. Results were filtered to retain only proteins with label-free quantitation (LFQ) > 0 and MS/MS (spectral counts) ≥ 2 for further analysis. The LC-MS/MS raw data files are available in the Massive data repository (massive.ucsd.edu) under ID MSV000087094.

Proteomic analysis identified >4,000 peptides which mapped to unique UniProt/Swiss Prot I.D.s in the *S. scrofa* database. The UniProt I.D.s were converted into Ensembl I.D.s using Database for Annotation, Visualization, and Integrated Discovery (DAVID; Huang et al., 2009) and Ensembl BioMart (Howe et al., 2021) software. Duplicate Ensembl IDs were removed by selecting the reads with the highest intensity across all timepoints. This resulted in 501 unique Ensembl IDs corresponding to proteins that were used for subsequent analysis. A protein was considered present on a day when it was detected in at least three samples within that day, resulting in identification of high confidence proteins. MetaboAnalyst 4.0 (Xia et al., 2009), which was written in R, was used for data visualization and statistical analysis of high confidence protein abundance across days. Tests used included principal component analysis (PCA), hierarchical cluster analysis, ANOVA for time-series,

and paired t-test analysis. In MetaboAnalyst, features with missing values were replaced with 1/5 of minimum intensity across all timepoints and proteins. Data were normalized using logarithmic transformation. Proteins were identified as differentially abundant between days using an adjusted *P*-value cutoff of (0.05). To identify protein common and unique between days, Ensembl IDs were uploaded into Venny 2.1 (Oliveros, 2007–2015). In order to retain robustness of data during functional annotation analysis, *S. scrofa* Ensembl IDs were converted into human Ensembl IDs before uploading into DAVID (Huang et al., 2009). GeneCards (Stelzer et al., 2016) and Uniprot databases were used to determine protein functions.

Statistical analysis of macronutrient composition

GraphPad Prism statistical software version 5.0 (GraphPad Prism 5, 2007) was used for quantitative comparisons. Two-way ANOVA followed by post hoc Tukey test was used to determine the effect of day and sow on protein, fat, carbohydrate, and lactose content of milk. *P* < 0.05 was considered significant and were reported as mean ± standard deviation (SD).

Results

Composition of colostrum and milk

Total carbohydrate increased (*P* < 0.05) between D3 (944 ± 353 µg/mL) and D14 (1,150 ± 462 µg/mL), but there was no difference in total carbohydrate content between D0 (1,160 ± 687 µg/mL) and D7 (1,070 ± 234 µg/mL; Figure 1A). Mean protein percent decreased (*P* < 0.05) between D0 (11 ± 0.63) and D3 (5 ± 0.59) and between D3 (5 ± 0.59) to D7 (4.1 ± 0.28; Figure 1B). There was no difference in protein percent between D7 (4.1 ± 0.28) and D14 (4 ± 0.25). There was no significant effect of sow on milk composition.

Global milk proteome

MaxQuant software (version 1.6.3.3) mapped data to 772 unique Uniprot/Swiss Prot I.D.s in the *S. scrofa* database (Supplementary Table S1), which corresponded to 501 unique Ensembl gene IDs (Supplementary Table S2). Correlation analysis of label free quantification of proteins between samples indicated good relationships and repeatability between biological replicates within a day, with mean ± SD of the correlation coefficient (*r*) for D0, D3, and D14 being 0.96 ± 0.01, 0.92 ± 0.04, and 0.97 ± 0.005, respectively (Supplementary Table S3). PCA (Figure 2A) and hierarchical cluster analysis showed distinct clustering of

samples by day (Figure 2B). The dendrogram associated with the heat map from hierarchical cluster analysis showed a primary grouping of D0 from D3 and D14 and a secondary branch that divided D3 from D14 samples (Figure 2B). There were 207 unique proteins (Supplementary Table S4) found in at least three samples within a day. The dynamic range of abundances of the identified proteins was up to four orders of magnitude, indicating the capturing of both major and minor milk proteins. The most abundant proteins across the three days were alpha-casein, beta-casein, kappa casein, and progesterone-associated endometrial protein (PAEP), which is a homolog of beta-lactoglobulin (BLG; Reinhardt and Lippolis, 2008). Also, among the most abundant proteins, were albumin, three iron transport proteins (HP, LTF, and TF), three milk fat globule membrane proteins (MFGE8, XDH, and BTN1A1), and multiple immunoglobulin proteins.

High confidence proteins were sorted by day to distinguish common proteins and those identified only in one milk group across lactation using the Venny 2.1 tool. There were 81 proteins common across D0, D3, and D14 (Figure 2C; Supplementary Table S5) and 37 proteins were only identified in D0, 23 in D3, and 13 in D14 (Figure 3). Proteins only identified in D0 samples included three apolipoproteins (APOE, APOA1, and APOA4), two complement proteins (C8B and CFD), two protease inhibitors (ITIH2 and TIMP2), extracellular matrix associate proteins (ECM1, FGB, and COL1A1), an amino acid transporter (SLC6A14), lipopolysaccharide binding protein (LBP), and long-chain fatty acid CoA ligase 3 (ACSL3), which activates long-chain fatty acids to target for synthesis and beta-oxidation.

The proteins identified only in D3 milk samples were heterogeneous nuclear ribonucleoprotein (HNRNPA2B1), that fat transporter CD36, and mannan-binding lectin serine protease 2 (MASP2), which activates the complement system. Also found unique to D3 samples were proliferating cell nuclear antigen (PCNA) and cell division control protein 42 (CDC42). Proteins unique to mature milk samples included lumican (LUM) which binds collagen and glycosaminoglycans in extracellular matrix, and the actin binding protein profilin (PFN1). The blood coagulation factor XI (F11), mucin, and PDIA3, which modulates protein folding. Two ribosomal proteins (RPS25 and RPL18) and two 14-3-3 proteins (YWHAE and YWHAZ) were also unique to D14 samples. The 14-3-3 are phospho-serine/threonine binding proteins that function in metabolism, protein trafficking, signal transduction, apoptosis, and cell cycle regulation.

There were 96 proteins shared between D0 and D3 (Table 1), with 19 increased and 37 decreased in abundance between D0 and D3 (FDR < 0.05). Proteins that decreased in abundance between

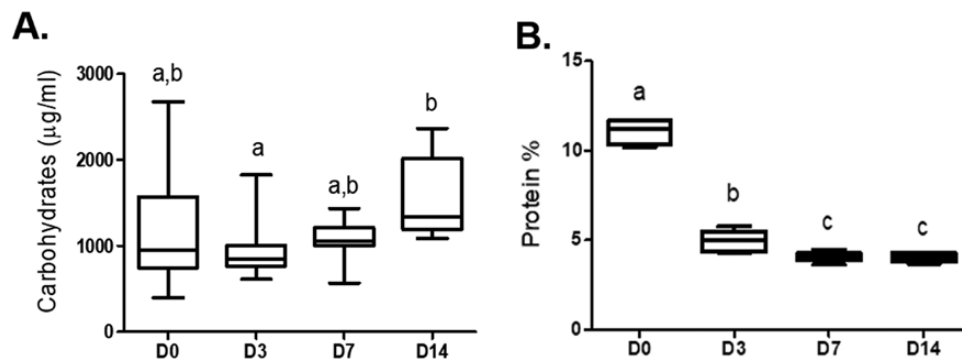


Figure 1. Compositional analysis of milk collected from sows at the time of farrowing (day 0, D0, colostrum), and on day 3 (D3, early transitional milk), day 7 (D7, late transitional milk), and day 14 (D14 mature milk). Percent fat (A), percent lactose (B), total carbohydrate concentration (C), and protein percent (D). Milk samples were collected from *n* = 9 sows. ANOVA followed by post hoc Tukey test, different letters, indicate a difference at *P* < 0.05.

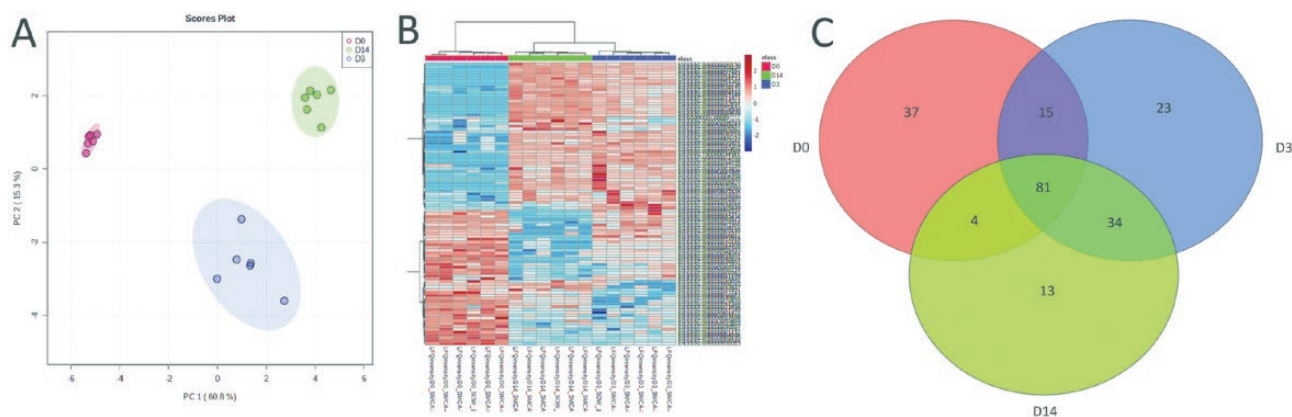


Figure 2. PCA (A) and heat map and dendrogram of hierarchical cluster analysis (B) of proteins isolated from milk samples collected from sows ($n = 6$) on day 0 (D0, red), day 3 (D3, blue), and day 14 (D14, green) of lactation. PCA shows distinct clusters of samples by day, whereas heat maps illustrated 125 most differentiating proteins that are greater (red) and lesser (blue) in abundance relative to D0 (red), D3 (blue), and D14 (green). Venn diagram of the distribution of the 207 high confidence proteins (detected in at least three samples per day between day 0 (D0, red), day 3 (D3, blue), and day 14 (D14, green)).

Unique D0	Unique D3	Unique D14	Common	D0	D3	<0.05	Common	D3	D14	<0.05
APOE	FCGR2B	YWHAZ	HP	31.88	29.44		NUCB1	32.97	33.93	*
APOA1	CLDN3	RAB1A	XDH	30.22	28.96	*	WAP	27.71	28.61	
PAPLN	PCNA	MUC1	IGKV1-12	29.13	26.73	*	SOD1	27.56	27.25	
CSTL	HSPA6	PFN1	DAG1	28.22	26.21	*	LMNA	27.45	26.67	
FGB	HNRNPA2B1	LOC110257903	AOAH	27.28	25.10	*	ANPEP	27.45	29.35	*
CPM	TRN	PDIA3	LPL	27.00	30.08		MYH9	27.42	28.47	*
SLC34A2	TINAGL1	RPS25	SERPINF1	26.97	24.37	*	DDX5	26.51	26.13	
APOH	TCN1	LUM	CFB	26.94	26.02	*	IGH	26.42	26.25	
SERPINC1	CD36	YWHAE	CDH1	26.45	24.94	*	CRP	26.31	24.11	
LPO	ACTA1	SELENBP1	B4GALT1	26.33	25.57		FABP3	26.10	25.94	
AZGP1	S100A1	PRDX5	CFI	25.59	25.16		CD14	25.60	25.19	
SOD3	PLBD2	RPL18	AGT	25.30	25.06		ALDOA	25.25	25.45	
IGHE	unknown	F11	SDCBP	25.26	26.89		LOC100739163	25.06	25.82	*
ACSL3	ACSL6		NPC2	24.38	25.81	*	FSTL1	25.00	25.78	*
QSOX1	H1-4		HEXA	22.68	22.54		KRT18	24.99	25.34	
MCFD2	ORF1						SMPDL3B	24.91	23.73	*
ITIH2	CDC42						LOC100153783	24.79	24.27	
ATP6AP1	MASP2		Common	D0	D14	<0.05	RPL37a	24.47	23.24	
OS9	NAGA		NPG4	25.56	23.43	*	FOLR1	24.33	22.99	
APOA4	CXCL2		COL4A2	24.61	25.08		ENO1	24.30	24.57	
CLEC3B	HSP90B1		CHID1	23.62	25.11	*	HBA	24.24	22.99	*
COL1A1	YBX1						MYL12B	24.08	25.82	*
MUC15	VDAC2						YWHAQ	24.00	23.14	
ECM1							HBB	23.87	23.48	*
LBP							DSC2	23.78	24.03	
C8B							FLNB	23.71	24.55	
SLC6A14							SLA-1	23.69	23.91	
IGFBP5							GAPDH	23.67	25.19	*
CFD							RPS18	23.66	24.78	*
C1QA							GRK1	23.16	23.48	
C7							UGDH	22.67	23.84	
PPIB							HNRNPH1	22.49	22.55	
TIMP-2										
ANXA2										

Figure 3. Proteins unique to day 0 (D0), day 3 (D3), and day 14 (D14), and common between D0 and D3, D3 and D14, and D0 and D14. Values are mean log base 2 label free quantification (LFQ) of protein by day. Asterisk indicates differences between days (FDR < 0.05). Color-code shading indicate relative abundance of proteins to each other, with most abundant shaded deepest red and least abundant deepest blue.

day 0 and day 3 were xanthine dehydrogenase (XDH) and two iron-binding transport proteins transferrin (TF), lactotransferrin (LTF). Among the 15 proteins found in D0 and D3 samples, but not

on D14, were three proteins that regulate lipid transport. These were lipoprotein lipase (LPL), the cholesterol transporter NPC2, and XDH. E-cadherin (CDH1) and beta-dystroglycan (DAG1) that

mediate cell-cell and cell-extracellular matrix adhesion were also found only in colostrum and transitional milk samples. Also, among these proteins were angiotensin (AGT), beta-1,4-galactosyltransferase (B4GALT1), which is a component of lactose synthase and haptoglobin (HP), which binds free hemoglobin, has antibacterial activity, and plays a role in modulating many aspects of the acute phase response. Of the 85 proteins shared between D0 and D14 (Table 1), 23 proteins increased and 29 proteins decreased in abundance (FDR < 0.05; Table 1). Proteins that increased in abundance were PAEP. Proteins that decreased in abundance were two iron binding proteins (LTF and TF) and complement C3 (C3).

There were 115 proteins shared between D3 and D14 (Table 1), with 24 of these proteins increased and 16 decreased in abundance (FDR < 0.05). Proteins that increased in abundance were immunoglobulin heavy constant gamma (IGHG), J-chain, nucleobindin 1 (NUCB1), and casein alpha S1 (CSN1S1), which play an important role in the capacity of milk to transport calcium phosphate. Proteins that decreased in abundance from transitional milk (D3) to mature milk (D14) included immunoglobulin kappa constant (IGKC), and lactalbumin alpha (LALBA). Amongst D3 and D14, there were 34 shared proteins that were not found in D0 samples. Proteins included heterogeneous nuclear ribonucleoprotein H1 (HNRNPH1), ribosomal protein S18 (RPS18), UDP-glucose 6-dehydrogenase (UGDH), whey acidic protein (WAP), the glycolytic protein enolase (ENO1), and the 14-3-3 protein YWHAQ. Also, among the proteins found in transitional and mature milk were two components of hemoglobin (HBA and HBB), myosin chain proteins (MYL12B and MYH9), a keratin (KRT18), and CD14, which is a coreceptor for bacterial lipopolysaccharide expressed by macrophages.

Nucleobindin 1 (NUCB1), which functions in calcium transport and the folate receptor (FOLR) were found to of relatively high abundance in D3 and D14 samples.

Functional annotation analysis of the 81 proteins present across the 3 days sampled (days 0, 3, and 14), categorized 80% as secreted proteins (Table 2; Supplementary Table S6) that were differentially expressed between days of lactation (Figure 4). Among these were the proteins most abundant in milk across all 3 days such as multiple caseins (CSN1S1, CSN2, and CSN3), lactoferrin (LTF), and alpha-lactalbumin (LALBA). About 14 of the 81 proteins were categorized as negative regulators of endopeptidase activity, to include 4 serpins, 3 complement factors, and 3 inter-alpha-trypsin inhibitor heavy chain proteins (ITIH3, ITIH1, and ITIH4). Also, among the 81 proteins found on all 3 days were multiple immunoglobulin gamma heavy chains, the polymeric immunoglobulin receptor (PIGR), and the J-chain protein component of the antibodies IgM and IgA. The J-chain is required for secretion and facilitates binding to PIGR, whereas PIGR functions in the transcytosis of immunoglobulins across epithelial cells.

Discussion

Shotgun proteomic analysis of homogenate sow milk samples by LC-MS/MS enabled detection of 207 unique high confidence proteins across colostrum, transitional, and mature milk lactation phases. Most of the previous proteome studies, fractionated milk samples and examined proteins associated with milk fat globule membranes or whey-skim fractions Reinhardt et al., 2008; Zhang et al., 2013; Yang et al., 2016; Hernández-Castellano et al., 2018; Fahey et al., 2020). Although

Table 1. Number (No.) and percent (%) of proteins differentially abundant between days of lactation

Day	No. shared ¹	Total no. of proteins shared between the day		
		No. differentially express (%)	No. increased (%)	No. decreased (%)
D0-D3	96	56 (58)	19 (34)	37 (66)
D0-D14	85	52 (61)	23 (44)	29 (56)
D3-D14	115	40 (35)	24 (60)	16 (40)

¹Protein was defined as present on each day when detected by LC MS/MS in at least three samples per day in sow (n = 6), of the 501 detected, there were 207 proteins that met this criteria. Of the 207 proteins, 81 were commonly shared.

Table 2. Categories enriched with the 81 proteins found common across colostrum (D0), transitional (D3), and mature (D14) milk samples

Term	%	P-Value	Genes
Secreted	80	1.55E-43	PIGR, ORM1, SERPINA1, MSTN, PZP, CSN3, CSN2, CHRDL2, HAPLN3, CLU, JCHAIN, C4A, C8G, CSN1S1, PGLYRP1, FGA, AHSG, BTN1A1, RNASET2, FGG, SERPINF2, PCOLCE, F2, SERPING1, LALBA, MFGE8, LTF, ITIH4, ITIH3, CFH, CD5L, PLG, A1BG, ITIH1, C3, VTN, HPX, C5, TTR, IGKC, LCN15, C9, A2M, GC, GSN, FN1, APOC3, CP, TF, LRG1, MGP, ALB, PAEP, FMOD, SCRG1, C1QC
Negative regulation of endopeptidase activity	20	1.60E-15	ITIH4, ITIH3, SERPINA1, SERPIND1, AHSG, SERPINF2, PZP, ITIH1, C3, C4A, VTN, C5, SERPING1, A2M
Acute-phase response	10	1.03E-08	ITIH4, ORM1, SERPINA1, AHSG, SERPINF2, FN1, F2
Innate immunity	15	1.60E-08	C3, C4A, FGA, C5, C8G, CFH, C9, SERPING1, PGLYRP1, CLU, C1QC
Antigen binding	4.3	5.00E-02	IGKC, IGHA1, JCHAIN
Fibrinolysis	8.6	1.81E-08	FGA, SERPINF2, FGG, SERPING1, PLG, F2
Extracellular matrix organization	8.6	1.18E-03	FGA, VTN, TTR, FGG, FN1, THBS1
Lysosome	5.7	5.00E-02	RNASET2, IDS, AGA, LGMN

NAME	Log base 2 LFQ			FDR <0.05			NAME	Log base 2 LFQ			FDR <0.05		
	D0	D3	D14	0 v 3	0 v 14	3 v 14		D0	D3	D14	0 v 3	0 v 14	3 v 14
PAEP	37.80	37.73	38.09	*	*	*	C4A	28.58	27.83	28.01	*	*	*
CSN1S1	36.54	36.73	37.18	*	*	*	FGG	28.54	28.12	24.74	*	*	*
CSN2	35.81	34.66	35.76	*	*	*	FN1	28.39	30.54	30.98	*	*	*
CSN3	35.55	36.22	36.62	*	*	*	PZP	28.18	29.08	28.96	*	*	*
Ig lambda chain C	35.34	33.48	33.44	*	*	*	SERPINA1	28.09	28.55	29.62	*	*	*
ALB	35.29	32.91	32.76	*	*	*	LOC106504545	28.09	29.64	29.44	*	*	*
IGKC	34.59	32.43	30.47	*	*	*	PLIN2	27.80	25.76	26.04	*	*	*
IGHG	34.56	36.10	36.44	*	*	*	AHSG	27.75	27.40	26.86	*	*	*
CSN1S2	34.52	32.25	30.91	*	*	*	GSN	27.71	26.40	27.19	*	*	*
IGHA1	33.89	34.53	34.63	*	*	*	ITIH3	27.67	24.75	24.06	*	*	*
MFGE8	33.30	31.16	30.03	*	*	*	TP23	27.55	30.35	31.29	*	*	*
LALBA	32.77	32.80	32.40	*	*	*	CHRD2	27.54	29.31	29.27	*	*	*
MSTN	32.17	31.32	31.53	*	*	*	AGA	27.32	25.90	25.42	*	*	*
MGP	31.64	29.65	29.59	*	*	*	LOC100156325	27.28	30.24	29.30	*	*	*
C3	31.56	30.18	29.58	*	*	*	SIRPG	27.15	27.16	27.77	*	*	*
LOC100302368	31.42	29.29	28.70	*	*	*	PCOLCE	27.09	26.87	27.14	*	*	*
CFH	31.06	31.07	31.32	*	*	*	HSPD1	27.02	25.61	26.33	*	*	*
PIGR	30.91	30.35	30.01	*	*	*	ACTG1	26.94	27.13	25.63	*	*	*
LTF	30.72	27.52	28.30	*	*	*	SERPINF2	26.56	26.77	26.69	*	*	*
THBS1	30.68	32.26	33.22	*	*	*	VTN	26.40	25.43	26.58	*	*	*
PGLYRP1	30.65	32.35	31.05	*	*	*	RNASET2	26.27	26.87	27.41	*	*	*
TF	30.51	28.97	28.68	*	*	*	PLG	26.24	23.81	23.53	*	*	*
HAPLN3	30.38	28.02	26.10	*	*	*	ATP6AP2	26.18	24.26	24.68	*	*	*
ITIH4	30.28	29.99	27.78	*	*	*	C5	26.15	28.28	29.20	*	*	*
LOC100153899	30.27	29.62	29.16	*	*	*	LCN15	26.15	29.82	27.18	*	*	*
A2M	30.11	29.87	30.11	*	*	*	DBI	25.91	25.12	23.73	*	*	*
BTN1A1	29.77	27.63	26.97	*	*	*	LOC100520329	25.71	25.31	25.44	*	*	*
ZNF70	29.55	29.02	29.33	*	*	*	F2	25.44	25.54	25.68	*	*	*
JCHAIN	29.50	28.83	28.82	*	*	*	C9	25.25	25.18	24.87	*	*	*
CLU	29.45	30.26	30.09	*	*	*	SERPING1	25.24	27.56	26.68	*	*	*
TTR	29.30	28.56	29.15	*	*	*	SERPIND1	25.01	27.04	25.71	*	*	*
GC	29.29	27.33	26.79	*	*	*	APOC3	24.76	27.18	26.49	*	*	*
FGA	29.15	31.39	30.36	*	*	*	CD5L	24.57	24.53	24.57	*	*	*
A1BG	29.08	28.32	27.95	*	*	*	LRG1	24.55	25.74	25.36	*	*	*
Ig-like	28.98	28.78	28.93	*	*	*	ITIH1	24.35	24.21	23.54	*	*	*
SERPINA3-2	28.95	29.61	29.71	*	*	*	LGMN	24.31	26.19	26.19	*	*	*
CP	28.89	28.53	28.73	*	*	*	C1QC	24.18	24.60	23.72	*	*	*
FMOD	28.78	27.96	27.97	*	*	*	GANAB	24.16	25.86	24.99	*	*	*
SCRG1	28.75	28.24	28.25	*	*	*	C8G	22.91	27.26	24.72	*	*	*
HPX	28.63	28.57	28.17	*	*	*	IDS	22.77	23.75	23.17	*	*	*
ORM1	28.62	31.10	28.30	*	*	*							

Figure 4. The 81 proteins common among all three sample days (detected in at least 3 sows on each day; D0, D3, and D14). Values are mean log base 2 of label free quantification (LFQ) of. Color is indicative of relative scale of abundance, deepest red (38.1) and deepest blue (22.7). Asterisk indicates differences between days (FDR < 0.05).

these approaches enabled an understanding of component milk proteomes, they precluded analysis of the relative abundance of proteins to each other and thus relative abundance available to neonates. We found that both factors changed dynamically

between the 3 days analyzed. Moreover, the unique proteome profiles of colostrum, transitional, and mature sow milk reflected the gland's metabolic activity and potentially the developmental needs of the neonate. This is discussed below

along with comparisons to previous studies of bovine, swine, and human milk changes across days of lactation.

A study of human and macaque milk that aimed to comprehensively capture proteomes also used homogenate samples (Beck et al., 2015). Prior to LC-MS/MS, milk proteins were separated by gel electrophoresis and 1,606 proteins were identified across three mature human milk samples and 518 proteins across seven macaque monkey milk samples. Our analysis mapped LC-MS/MS data to 772 proteins corresponding to 501 non-redundant protein-coding genes, and thus, at least relative to macaque milk, appeared to be as robust without pre-fractionation. Moreover, most of the proteins identified in sow milk samples overlapped with those reported to be found in cow and human milk (Berg et al., 2002; Zhang et al., 2013; Hernández-Castellano et al., 2018; Fahey et al., 2020). Analysis of homogenate milk samples also proved to capture as many proteins that spanned a similar range in abundance as studies that fractionated milk to analyze whey or milk fat globule membrane proteins using LC-MS/MS. A study that compared milk fat globule membrane proteins between colostrum and mature milk samples of humans and cows identified a total of 411 proteins (Yang et al., 2016). The stringency of selection of proteins was not mentioned, but the study highlighted 263 proteins, with approximately half of these differentially abundant between colostrum and mature human milk samples. Most of the proteins identified in this analysis were the same proteins found in homogenate sow milk samples. There were also similar trends in changes in abundance between days, including several immunoglobulin heavy chain proteins decreasing in abundance between colostrum and mature milk phases. However, there were also differences in directional changes of protein abundance between colostrum and mature milk of several fat globule membrane proteins reported for bovine and humans and what was found for homogenate sow milk samples.

The differences in directional changes in abundance of proteins between days may have been related to species-specific differences, analysis of differential fractions of milk, or different days analyzed. Another study that analyzed changes in protein abundance in milk fat globule membrane proteins of Holstein cows between colostrum and day 7 (mature milk) found of the 138 bovine milk fat globule membrane proteins, with 26 proteins increased and 19 proteins decreased in abundance between D0 and D7 (Reinhardt et al., 2008). Our analysis of sow milk found many of the same proteins, and similar to cows, there was a decrease in apolipoproteins and immunoglobulin heavy chain proteins found between colostrum and mature milk samples. However, unlike homogenate sow milk samples, analysis of bovine milk fat globule membrane proteins alone found an increased abundance of XDH and butyrophilin (BTN1A1) between colostrum and mature milk. The discrepancy in XDH may be a species difference, whereas the difference in BTN1A1 is likely due to analysis of milk fat globule membrane fraction versus whole milk. This speculation is supported by studies of changes in the bovine whey proteome between parturition and approximately day 7 of lactation (Lee et al., 2018), which found a decreased abundance in BTN1A1 and an increased abundance in XDH. Although there were some differences in the direction of change in abundance between colostrum and mature milk in cow whey samples and sow milk samples, the 85 whey proteins measured in bovine samples were mostly identified in homogenate sow milk samples. Studies of changes in human milk whey protein between transitional and mature milk found that the abundance of more than 400 proteins changed

between phases of lactation (Zhang et al., 2013). The more significant number of proteins in the human whey proteome study than homogenate sow milk proteins was partly due to the greater stringency imposed in our study. Despite differences in the number of proteins, similar directional changes in the abundance of proteins were found in swine and human milk between early and later lactation phases.

A study of changes in the proteome of the skim fraction of swine milk between day 0 and 21 of lactation (Ogawa et al., 2014), identified 113 in colostrum samples and 118 in mature milk, of which 50 were shared between the days. Similar to our findings, beta-lactoglobulin (PAEP in our study) was the most abundant protein in colostrum and mature milk samples. Also, among the most abundant proteins were alpha-lactalbumin (LALBA), multiple caseins, immunoglobulin chain proteins, MFG8, lactotransferrin (LTF), polymeric Ig receptor (PIGR), serotransferrin (TF), and serum albumin (SA).

Proteins found unique to colostrum included apolipoproteins, which function to carry lipids in plasma and ACSL3, which is required for phosphatidylcholine synthesis. The unique presence of these proteins in colostrum likely reflects the dependence on plasma lipids as sources for fat at this stage of lactation. Insulin-like binding protein-5 (IGFBP5) was also among the proteins uniquely found in colostrum. IGF-binding proteins prolong the half-life of insulin-like growth factor (IGF). Although not detected in LC-MS/MS analysis, IGF-I concentrations have been reported to be 10-fold higher in swine colostrum than mature milk. Feeding recombinant IGF-I to neonatal pigs increased intestinal development and affected gut function (Donovan et al., 2004). Thus, the presence of IGFBP5 may reflect the critical role that colostrum plays in initiating the final stages of gut development in the early postnatal period (Wang and Xu, 1996).

Milk serves as a vehicle for passive transfer of immunity from the sow to the neonates, and the high protein content of colostrum reflects the highly concentrated content of immunoglobulins. Immunoglobulins are actively transported from maternal circulation into milk (Hine et al., 2019). Transport of IgA and IgM is mediated through the polymeric immunoglobulin receptor (PIGR; Rojas and Apodaca, 2002), and its abundance was relatively constant across the days sampled. This finding indicates that the transfer of IgA and IgM from circulation across mammary epithelial cells into the milk for delivery to neonates changed little between the days. The Fc fragment of IgG receptor IIb (FCGR2B), which specifically transports IgG across epithelial membranes (Van de Perre, 2003), was very high in transitional milk samples, but not detected in D0 and D14 samples. Thus, the changes immunoglobulin proteins between colostrum, transitional, and mature milk may reflect the secretory activation of the gland, as well as changes in transporter expression (Wall et al., 2015).

Upon secretory activation, the synthesis and secretion of milk proteins are increased, and proteome changes that reflected this change were marked by the increased abundance of PAEP, caseins, whey acid protein (WAP) alpha-lactalbumin (LALBA) between colostrum and mature milk. LALBA is the regulatory component of lactose synthase, and the increase in lactose content between day 0 and 3 was also indicative of secretory activation. Lactose is a major osmoregulator (Hurley, 2015). Increased lactose content increases the water content of milk. Increased water content resulted in the overall dilution of all proteins in milk and was evident in the decrease in percent protein found between day 0 and 3.

Although casein and major whey proteins increased from colostrum to transitional and mature milk, there was a

decrease in the relative abundance of the major milk fat globule membrane protein MFGE8 in homogenate sow milk samples. The decrease in abundance reflects a decrease in per unit volume of milk and is likely indicative of an increase in the milk fat globule size from early to later phases of lactation. This supposition is supported by lipidome analysis of these same samples, which found a decrease in the proportion of plasma membrane lipids to triacylglycerol lipids (Suarez-Trujillo et al., 2021). The ratio of membrane lipids to triacylglycerols reflects the size of milk fat globule (Argov et al., 2008), with smaller globules having higher ratios (Wang et al., 2018).

Secretory activation is also marked by the closure of tight junctions and an increase in de novo fatty acid synthesis. The high level of the tight junction protein claudin 3 (CLDN3) in transitional milk samples may reflect the initiation of high levels of expression of this protein upon secretory activation. The decrease in abundance of apolipoproteins and LPL from colostrum to transitional and mature milk likely reflects the transition from the dependence of blood-born lipids as a source of milk fat to de novo fatty acid synthesis by lactocytes (Reinhardt et al., 2008; Zhang et al., 2013). Several proteins uniquely detected on D3, also likely reflect the very dynamic changes in the mammary gland around this time, including the detection of ACSL6 which encodes a long-chain acyl-CoA synthetase. Long-chain fatty acyl-CoAs suppress fatty acid synthesis, and so this enzyme may indicate the transition of dependence on preformed fatty acid to de novo synthesis of fatty acids in milk. Two proteins indicative of proliferation, PCNA and CDC42, were uniquely found on D3 and may reflect a burst in proliferative activity of mammary epithelial cells in the early postnatal period. The presence of heat shock proteins and the heterogeneous nuclear ribonucleoprotein on D3 likely reflected the increase in transcription, translation, and protein folding as secretory activation ensues.

Over the 3 days sampled (D0, D3, and D14), complement factor proteins fluctuated in abundance, with some increasing while others decreased across lactation. The complement system is a part of the host defense mechanism in the innate immune system, and responsible for destroying microorganisms and apoptotic cells (Yang et al., 2016). The proteins found that increased between D0–D3 and D0–D14 within the complement system were C5 and C7, which both play an essential role in the activation or act as constituents of the membrane attack complex (Zhang et al., 2013). Also found to increase in sow milk was CFI which plays an essential role in regulating the complement cascade (alternative pathway), and thus suggests an increase in the extent of the function of this pathway during lactation (Zhang et al., 2013). Our study also found other complement factors that decreased across the course of lactation, such as C3, which plays an essential role in regulating the immune response by controlling all complement pathways.

Three 14-3-3 proteins were detected in transitional and mature milk, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein theta (YWHAQ), zeta (YWHAZ), and t-epsilon (YWHAE). The family of 14-3-3 proteins is conserved regulatory molecules that bind various diverse signaling proteins, such as phosphoserine-containing proteins (Stelwagen et al., 1999). YWHAQ and YWHAE were relatively minor proteins (log base 2 LFQ ~24; Figure 3), with abundance approximately 0.00006 of the most abundant protein, PAEP. However, YWHAZ was a mid-range protein (log base 2 LFQ ~30; Figure 3), and was about 0.003 of the abundance of PAEP, suggesting its important as a milk protein. The 14-3-3 proteins may play a role in the regulation of mammary development and

milk protein synthesis (Thomas et al., 2011; Khudhair et al., 2015; Wang et al., 2018), and perhaps the increased abundance from not being detected in D0 to increasing from D3 to D14, similar to the increase in abundance of ribonucleoprotein H1 (HNRNPH1), ribosomal protein S18 (RPS18), UDP-glucose 6-dehydrogenase (UGDH) proteins, reflecting the increased metabolic output of the gland following secretory activation.

Proteins found in relatively high abundance across the three days of lactation were peptidase inhibitors, such as serpins and proteins that make up inter-alpha-trypsin inhibitors. Studies of human milk found antitrypsin and antichymotrypsin protein concentrations were relatively high and decreased throughout lactation (Chowanadisai and Lönnnerdal, 2002). In vitro studies indicated that antitrypsin may survive digestion and thus function to affect the survival and maintain bioactivity of other milk proteins. The levels of the inter-alpha-trypsin inhibitors and serpins remained relatively constant across sow lactation, suggesting an importance in their role in maintaining bioactivity of milk proteins for neonatal pigs. In contrast to these proteinase inhibitors, there were also several proteases that were present across all three days. Among them was legumain (LGMN), which may digest bacterial peptides, and iduronate 2-sulfatase (IDS), which degrades heparan sulfate, and the lysosomal protein aspartylglucosaminidase (AGA). AGA cleaves N-linked oligosaccharides of glycoproteins. Oligosaccharides in milk are not used as energy by neonates, but rather help to establish a healthy gut microbiome. Oligosaccharides are found free in milk or associated with glycoproteins and glycolipids, and thus AGA, may play a role in establishing the neonates' microbiome by cleaving oligosaccharides from glycoproteins.

Along with providing essential macronutrients, milk facilitates the adaptation of life outside of the womb, which includes continued neonatal growth and development coincident with new abiotic and biotic challenges. The composition and proteomic profile of swine milk changed dynamically with the stage of lactation. Compared to mature milk, colostrum had the highest concentration of protein and greater abundance of immunoglobulins, apolipoproteins, and IGFBP that reflect the need for passive immunity, energy for thermogenesis and maturation of the gastrointestinal tract needed immediately after birth. Transitional milk was marked by a relative increase in fat and lactose concentration. Although traditionally viewed as a dilution of colostrum, the unique proteome profile of transitional milk, suggests that it is more, and thus its composition may provide clues to the developmental needs of neonatal pigs in the first week after birth. Together, the knowledge of the relative abundance of milk proteins to each other and across days may help in development of innovative approaches that can decrease high mortality and morbidity rates in swine herds.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

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Author Contributions

Conceptualization was by T.M.C and C.V.B.; methodology—S.L., L.L., T.M.C, A.S-T, and C.V.B; LC-MS sample preparation—R.M. A.S-T, and U.K.A.; LC-MS data analysis—R.M. and U.K.A.; writing—original draft preparation—C.V.B, and T.M.C.; writing—review and editing—K.R.M., R.C.M., A.S.T, U.K.A., and T.M.C.; visualization—T.M.C. and R.C.M.; supervision—T.C.M.; project administration and funding acquisition—R.C.M.

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