Changes in serum metabolites in response to ingested colostrum and milk in neonatal calves, measured by nuclear magnetic resonance-based metabolomics analysis

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ABSTRACT

Uptake of colostrum is of central importance for establishing a passive immunity transfer in neonatal calves. Studies of absorption and transmission of colostral immunoglobulins have been widely reported; however, changes in the serum in response to the absorption of colostral components in neonatal calves have not been completely characterized. Here, a nuclear magnetic resonance-based metabolomics approach was used to investigate the changes in metabolites in ingested colostrum, milk, and serum after neonatal calves were fed colostrum or milk. Twenty-seven neonatal male Holstein calves were assigned to 1 of the following groups: (1) calves not fed colostrum or milk and slaughtered approximately 2 h after birth (control group, $n = 6$), (2) calves fed colostrum at 1 to 2 h after birth and slaughtered 8 h after birth ($n = 6$), (3) calves fed 2 colostrum meals (at 1–2 and 10–12 h after birth) and slaughtered 24 h after birth ($n = 6$), (4) calves fed 3 colostrum meals (at 1–2, 10–12, and 22–24 h after birth) and slaughtered 36 h after birth ($n = 6$), or (5) calves fed 2 milk meals (1–2 and 10–12 h after birth) and slaughtered 24 h after birth ($n = 3$). Concentrations of valine, leucine, lactate, lysine, and isoleucine were higher and concentrations of lactose were lower in the groups fed colostrum and milk compared with groups not fed colostrum and milk, respectively. Metabolite changes between groups fed or not fed colostrum and milk were similar and may reflect the primary metabolic requirements of ingestion by the small intestine of neonatal calves. Concentrations of serum metabolites choline, valine, leucine, and glutamate were higher in the serum of calves that received colostrum compared with control calves. Furthermore, concentrations of serum phenylalanine, valine, and glutamate were significantly higher, whereas serum concentrations of citrate and very low density lipoproteins were lower in calves that received colostrum compared with calves

fed milk. Our results indicate that concentrations of leucine, valine, and glutamate, which were higher in the calves that ingested colostrum, may transfer into the bloodstream, and that these metabolites are associated with health benefits in the neonatal calves that received colostrum. These findings provide novel information to help us understand the mechanism by which colostrum components are metabolized and absorbed in the small intestine and then transferred into bloodstream of neonatal calves.

Key words: serum, colostrum, intestine, metabolomics

INTRODUCTION

Colostrum contains many bioactive and nutritional components and is a unique diet for neonates. Most importantly, colostral immunoglobulins play a central role in neonatal survival, as transmission into the bloodstream establishes a passive immunity transfer within the first 24 h of life (Quigley, 2004; Osaka et al., 2014). For this reason, many studies have investigated colostral and blood immunoglobulins and the relationship between them (Stott et al., 1979a,b; Osaka et al., 2014). In addition to establishing a passive immunity transfer, colostrum intake plays a role in stimulating intestinal mucosal growth and function as well as in enhancing the absorptive capacity of the gastrointestinal tract (Hammon et al., 2013; Steinhoff-Wagner et al., 2014). As a result, studies have found that glucose and galactose originating from lactose and xylose are absorbed in the small intestine and transferred into the bloodstream (Girard, 1990; Scharrer and Grenacher, 2000; Steinhoff-Wagner et al., 2011a). However, increases in concentrations of several growth factors, such as IGF-I and IGF-II, and insulin in the blood of calves depended on the timing of first colostrum feeding (Hammon et al., 2000), whereas absorption of colostral growth factors was barely observed in the intestine (Hammon and Blum, 1997; Ontsouka et al., 2004a). These results suggest that colostrum feeding in neonates does not only provide abundant bioactive substances and nutrition, but also contributes to improving the intestinal function for the absorption of specific colostral components.

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Although colostrum digested in the small intestine of neonatal calves has received little attention, studies on changes in the serum of neonatal calves have been widely reported. In previous studies, increases in concentrations of several free EAA and decreases in glutamine concentrations were observed in serum after calves received colostrum (Hammon and Blum, 1999; Zanker et al., 2000). Additionally, concentrations of essential fatty acids, carotene, retinol, and α -tocopherol were significantly higher in serum of calves fed colostrum on d 1 postcalving compared with delayed colostrum-fed calves (Blum et al., 1997). These results provide useful information for understanding the effects of colostrum intake on blood components and the potential health benefits for neonatal calves. It is notable that colostrum is a significant source of metabolites, as several metabolites can be used as potential bioactive compounds (Sundekilde et al., 2013). However, changes in blood metabolites related to colostrum intake have not been fully elucidated in neonatal ruminants.

Metabolomics, an omics field in systems biology, is widely used to investigate small metabolites within biological samples (Putri et al., 2013). As nuclear magnetic resonance (NMR) spectroscopy is a powerful method that provides direct relationships between metabolite contents and resonances, NMR approaches have been widely used to investigate the changes in metabolites of milk or serum, as well as correlations between milk and serum metabolites in dairy animals in recent years (Sundekilde et al., 2011; Klein et al., 2012; Palma et al., 2016). The results of such studies indicate that NMR-based metabolomics techniques provide a powerful tool for exploration of the effects of colostrum intake on serum metabolites in neonatal calves.

The objective of our study was to use an NMR approach to investigate the changes in serum metabolites in response to the absorption of colostrum or milk in the small intestine of neonatal calves, and to explore the absorption of metabolites into the bloodstream. We hypothesized that colostrum could be absorbed in the gastrointestinal tract and that a wide range of small molecules would transfer into the bloodstream of the calves fed colostrum. We expected our findings would provide novel information for a better understanding of the changes in serum metabolites corresponding to colostrum uptake and digestion in the gastrointestinal tract.

MATERIALS AND METHODS

Animals and Experimental Procedures

Twenty-seven neonatal male Chinese Holstein calves from the Chuzhou dairy farm (Chuzhou, China), with

birth weight of 40 ± 2 kg ($\pm$ SD), were used in this study (Supplemental Figure S1; <https://doi.org/10.3168/jds.2017-14287>). Animal care and use procedures were approved by the Animal Care Advisory Committee of the Anhui Academy of Agricultural Sciences. After birth, calves were separated from their mothers to prevent them from directly suckling colostrum. The first 2 milking colostrum samples were taken from healthy, multiparous Holstein dairy cows and pooled, and bulk milk from a healthy herd of experimental dairy farm cows was collected, placed in plastic bottles, and then stored at -20°C . Before use, the colostrum (not ingested colostrum) or milk (not ingested milk) was incubated in a water bath at 40°C and provided at approximately 8.0% of BW for each calf via a stomach tube, as described in previous studies (Chigerwe et al., 2012; Conneely et al., 2014). Six calves were not fed colostrum or milk and were slaughtered approximately 2 h after birth. These 6 calves constituted the control (Ct) group. Six calves received 1 colostrum meal at 1 to 2 h after birth and were slaughtered approximately 8 h after birth (CI group). Six calves received 2 colostrum meals at 1 to 2 and 10 to 12 h after birth and were slaughtered approximately 24 h after birth (CII group). Six calves received 3 colostrum meals at 1 to 2, 10 to 12, and 22 to 24 h postpartum and were slaughtered approximately 36 h after birth (CIII group). Three calves were fed milk instead of colostrum after birth, with 2 milk meals at 1 to 2 and 10 to 12 h after birth; milk-fed calves were slaughtered approximately 24 h after birth (M group). Samples of colostrum and milk were frozen and transferred to the laboratory.

Blood samples were collected from the jugular vein of individual calf in all groups before the calves were slaughtered. Samples were stored overnight at room temperature and centrifuged at $3,000 \times g$ for 15 min at 4°C . Supernatant was collected and aliquoted into 1.5-mL tubes, then frozen at -20°C . After the blood samples were collected, the calves were slaughtered. Mid-duodenum, -jejunum, and -ileum segments were separated within 30 min after opening the abdominal cavity (Liang et al., 2014). Ingested colostrum or milk was collected from mid-jejunum segments, frozen in liquid nitrogen, and then stored at -80°C until analysis.

NMR Spectroscopy Analysis

Colostrum and milk samples were thawed at room temperature and centrifuged at $4,000 \times g$ for 30 min at 4°C . The fat layer was removed and skim milk was collected. A total of 500 μL of skim milk from each sample was mixed with 1.0 mL of methanol and 500 μL of double-distilled H_2O , followed by periodical vortexing. The mixture was centrifuged at $12,000 \times g$ for 10

min at 4°C. The supernatant was collected and centrifuged to dry with a Speed Vac Concentrator (SPD121P, Thermo Savant, Waltham, MA). Dried samples were dissolved with 600 μ L of solution (0.1 M $\text{NaH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ and 0.1% NaN_3 in deuterium oxide, pH 7.4), followed by periodical vortexing. To analyze serum samples, 200 μ L of thawed serum was mixed with 400 μ L of solution (0.045 M $\text{NaH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ and 0.1% NaN_3 in deuterium oxide, pH 7.4). The mixtures were centrifuged at $16,000 \times g$ for 10 min at 4°C, and 550 μ L of supernatant from each sample was collected and placed into a 5-mm NMR tube. Two replicates of each sample were prepared for NMR spectroscopy.

The NMR scans were performed at a temperature of 298 K, with a proton frequency of 599.83 MHz, using an Agilent DD2600 MHz spectrometer (Agilent Technologies, Santa Clara, CA). The one-dimensional nuclear Overhauser enhancement spectroscopy preset sequence for water resonance was selected. A 90° pulse length with a relaxation delay of 10 μ s and an acquisition time of 1.36 s were applied, and a total of 32,000 data points were collected, with 64 scans and a spectral width of 20 ppm. Prior to applying the Fourier transformation, free-induction decays of the collected ^1H -NMR spectra were extended using a line-broadening factor of 1.0 Hz to improve the signal-to-noise ratio. The NMR spectra were manually phase- and baseline-corrected, and all were referenced to sodium trimethylsilyl-[2,2,3,3- $^2\text{H}_4$]-1-propionate (Merck, Darmstadt Germany) and evaluated for the individual metabolite signals. For the signals of the milk samples, the spectra were segmented into integral regions of chemical shifts (δ) 9.5 to 0.6 with a width of 0.002 ppm for each segment, and water resonance (δ 5.20–4.72 ppm) and buffer resonance (δ 3.327–3.40 ppm) regions were removed. For the signals of the serum samples, the spectra were segmented into integral regions δ 9.5 to 0.5 with a width of 0.002 ppm for each segment, and water resonance (δ 5.20–4.20 ppm) and urea resonance (δ 6.20–5.50 ppm) regions were removed.

Multivariate Statistical Analysis

The intensity of each peak signal in the NMR spectra was proportional to the concentration in the samples. The NMR data were normalized and transferred to the SIMCA-P⁺13.0 software package (Umetrics AB, Umeå, Sweden) for multivariate statistical analysis. To investigate the changes in metabolites of colostrum and milk, colostrum metabolites were compared with the ingested colostrum metabolites from the CI, CII, and CIII groups; milk metabolites were compared with the ingested milk from M group. In the serum analysis,

metabolites from Ct group were compared with CI, CII, and CIII, as well as M groups, respectively. In addition, metabolites of ingested colostrum or milk and serum were also compared between CII and M groups. Principal component analysis (PCA) was applied to test clustering profiles and trends among the studied samples. Supervised partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA) procedures were performed on the unit variance scaling. Three parameters, including R^2X and R^2Y (to assess the model quality) and Q^2 (to measure the model predictive ability), were used to evaluate the quality and confidence of the OPLS-DA models, calculated by cross-validation procedures. Of them, R^2X and R^2Y were used to assess the model quality, and Q^2 was used to measure the model predictive ability.

Score plots of the OPLS-DA model were used to visualize the separation between the studied groups, and coefficient loading plots were used to examine the spectral variables contributing to sample differentiation on the score plot. The colors of spectral signals projected onto the coefficient plots are proportional to the intensity of metabolite signals that are used to distinguish between 2 studied groups, in which red coloring indicates a high correlation and blue coloring indicates no correlation. Correlation coefficients were calculated to evaluate potential associations between metabolites in the experimental groups on the basis of the Pearson product-moment regression coefficient. Relative concentrations of metabolites were subjected to the nonparametric Mann–Whitney U test used to assess statistically significant differences between the 2 groups. Differential metabolites between the 2 groups were assigned by comparison with signals of chemical shifts in the Human Metabolome Database (www.hmdb.ca).

RESULTS

Differences in Metabolites in Calves Fed or Not Fed Colostrum and Milk

Representative ^1H -NMR spectra (δ 0.5–5.4 and δ 5.4–9.5) of groups not fed colostrum and milk and fed colostrum and milk are shown in Supplemental Figure S2 (<https://doi.org/10.3168/jds.2017-14287>). Several signals from lactose and other metabolites, such as carnitine, creatinine, and citrate, were detected. A PCA approach was used to present the clustering patterns of samples based on the mean-centered NMR spectra data. The PCA score plots from groups not fed colostrum and milk and fed colostrum and milk are shown

in Supplemental Figure S3 (<https://doi.org/10.3168/jds.2017-14287>). Metabolite profiles between calves not fed colostrum and intestinal colostrum of CI, CII, and CIII groups as well as between calves not fed milk and fed milk were clearly different. The ellipse in the score plots defines the Hotelling's T^2 confidence region and provides a 95% confidence interval for the observed samples.

The supervised OPLS-DA approach was used to evaluate the different metabolites in the small intestine in groups not fed and fed colostrum and milk. The OPLS-DA score plots between the studied groups were well separated without any overlap. Data originated from 1 predictive component and 2 orthogonal components, in which Q^2 values were at least 0.832, indicating that the models were effective for predictive reliability. Differences in metabolites between colostrum and milk and samples from CI, CII, CIII, and M groups identified using OPLS-DA score plots and corresponding coefficient loading plots are shown in Figure 1. The differential metabolites based on the signals of chemical shift are listed in Table 1 and Supplemental Table S1 (<https://doi.org/10.3168/jds.2017-14287>).

Based on the results of the NMR analysis, we found lower concentrations of lactose and citrate and higher concentrations of valine, leucine, isoleucine, and creatine in colostrum compared with milk. Compared with groups not fed colostrum, concentrations of several metabolites, such as lactate, succinate, and glutamate, were significantly higher in the CI, CII, and CIII groups, whereas concentrations of lactose and uridine diphosphate were significantly lower. Compared with groups not fed milk, concentrations of several metabolites, such as inosine, succinate, and hypoxanthine, were higher whereas concentrations of lactose and citrate were lower in calves in the M group. Although many differences were observed between the metabolites in milk and colostrum, changes in metabolites from colostrum to ingested colostrum and from milk to ingested milk were unexpectedly very similar after calves were fed colostrum and milk. However, concentrations of 5 metabolites were different in the ingested colostrum and milk approximately 24 h after birth; of them, inosine, uridine diphosphate, hypoxanthine, and 2 unknown metabolites were higher in the ingested milk.

Differences Between Serum Metabolites in Calves Not Fed or Fed Colostrum and Fed Milk

Representative NMR spectra (δ 0.5–5.4 and δ 5.4–9.0) of the serum from calves in the Ct group, CI, CII, and CIII groups, and calves in the M group are shown in Supplemental Figure S4 (<https://doi.org/10.3168/jds.2017-14287>). The NMR spectra data were subjected

to PCA analysis to provide the clustering patterns of the studied samples. The PCA score plots are shown in Supplemental Figure S5 (<https://doi.org/10.3168/jds.2017-14287>), which clearly shows that the metabolite profiles of serum of Ct calves and CI, CII, and CIII calves were different. Similarly, metabolite profiles of serum in calves from CII and M groups were clearly different.

The supervised OPLS-DA approach was also used to investigate the differential metabolites in serum between the different treatment groups. Differences in serum metabolites of calves from the control and experimental groups obtained using OPLS-DA score plots and corresponding coefficient loading plots are shown in Figure 2. Models of 1 predictive component and 2 orthogonal components were present, in which Q^2 values were at least 0.817, indicating robust predictive reliability. The identified serum metabolites in the different treatment groups are listed in Table 2 and Supplemental Table S2 (<https://doi.org/10.3168/jds.2017-14287>).

According to the results of OPLS-DA analysis, concentrations of metabolites of glutamate, glycerophosphocholine/phosphocholine, lipid, choline, and tyrosine were significantly higher in serum of calves from the CI, CII, and CIII groups, whereas concentrations of glycerol and an unknown metabolite were lower compared with the Ct group. In addition, concentrations of valine, leucine, and histidine were higher, whereas concentrations of creatinine, citrate, and glutamine were lower in serum of calves in the CII and CIII groups compared with calves in the Ct group. However, in the M group, serum concentrations of pyruvate and histidine were higher whereas concentrations of glycerol, allantoin, guanine, and citrate were lower compared with calves in the Ct group. We also found that changes in serum metabolites in calves fed colostrum (CII group) and milk (M group) had a similar pattern, and only 7 serum metabolites were significantly different. Of these, concentrations of valine, glutamate, and creatine were higher, whereas concentrations of citrate and *N*-acetyl glycoprotein signals were lower in calves fed colostrum (CII group) compared with the calves fed milk.

Relationships Between Metabolites in Ingested Colostrum and Serum Metabolites After Ingestion of Colostrum

Concentrations of leucine, valine, isoleucine, glutamate, lysine, tyrosine, lactate, and succinate were higher in the ingested colostrum collected from the gastrointestinal tract than in colostrum that was not ingested, whereas concentrations of lactose, choline, and uridine diphosphate were lower in the ingested co-

Corresponding coefficient loading plots

Figure 1. The score plots and corresponding coefficient loading plots of orthogonal partial least squares discriminant analysis derived from nuclear magnetic resonance spectra of the colostrum compared with the ingested colostrum collected from the jejunum of the calves fed colostrum approximately 8 h (CI group; A), 24 h (CII group; B), and 36 h (CIII group; C) after birth, respectively; milk compared with the ingested milk collected from the jejunum of the calves fed milk approximately 24 h after birth (M group; D); and ingested colostrum from CII group compared with the ingested milk from M group (E). Changes in metabolites between the 2 studied groups are labeled in the corresponding coefficient loading plots, respectively. UDP = uridine diphosphate; NAG = *N*-acetyl glycoprotein signals; OAG = *O*-acetyl glycoprotein signals; GPC = glycerophosphocholine; PC = phosphocholine; U1 and U2 = unknown metabolites. Color version available online.

Table 1. Differential metabolites from colostrum, milk, and their ingested products in the gastrointestinal tract of the calves

Metabolite	Chemical shift	Correlation coefficients ¹						Metabolic pathway
		Colostrum-CI	Colostrum-CII	Colostrum-CIII	Milk-Colostrum	CII-M	Milk-M	
Tyrosine	7.20, 6.91	0.769	0.95	0.858	—	—	0.978	Amino acid
Leucine	0.96, 0.97	0.787	0.993	0.9	0.984	—	0.996	Amino acid
Lysine	1.74, 1.92, 3.02	0.817	0.983	0.896	—	—	0.997	Amino acid
Glutamine	2.45	0.854	0.88	0.852	—	—	0.996	Amino acid
Valine	1.05, 1.00	0.87	0.926	0.88	0.98	—	0.962	Amino acid
Isoleucine	1.02, 0.94	0.872	0.961	0.906	0.985	—	0.995	Amino acid
Creatine	3.04, 3.93	0.879	0.925	—	0.993	—	0.912	Amino acid
Phenylalanine	7.33, 7.38, 7.43	0.882	0.986	0.924	—	—	0.996	Amino acid
Alanine	1.48	0.895	0.928	0.899	—	—	0.967	Amino acid
Glutamate	2.35	0.904	0.898	0.927	—	—	0.995	Amino acid
Histidine	7.10, 7.85	—	—	—	—	—	0.987	Amino acid
O-Acetyl glycoprotein signals	2.13	0.864	0.968	0.824	0.989	—	0.998	Carbohydrate
Lactose	5.24, 4.67, 4.46	−0.987	−0.993	−0.996	−0.993	—	−0.999	Carbohydrate
N-Acetyl glycoprotein signals	2.04	—	−0.956	0.878	0.997	—	—	Carbohydrate
Galactose	5.27, 4.64, 4.68	0.937	—	—	—	—	—	Carbohydrate
Lactate	1.33, 4.12	0.981	0.89	0.968	—	—	0.945	Energy
Succinate	2.41	0.889	0.921	0.911	—	—	0.992	Energy
Citrate	2.52, 2.68	—	—	—	−0.996	—	−0.998	Energy
Phosphocholine	3.22	—	0.974	—	0.996	—	0.841	Lipid
Glycerophosphocholine	3.22	—	0.974	—	0.996	—	0.841	Lipid
Choline	3.2	—	−0.952	−0.963	—	—	—	Lipid
Hypoxanthine	8.2	—	—	—	—	0.792	0.897	Nucleotide
Inosine	8.35, 6.10	—	—	—	—	0.84	0.964	Nucleotide
Uridine diphosphate	7.96, 5.97, 5.98	−0.986	−0.982	−0.966	0.994	0.869	0.968	Nucleotide
Uridine	7.88, 5.89, 5.90	—	—	—	0.995	—	—	Nucleotide
Unknown 1	6.3	—	—	—	—	0.985	0.992	—
Unknown 2	6.49	—	—	—	—	0.902	0.836	—

¹CI, CII, and CIII = ingested colostrum collected from the jejunum of the calves fed colostrum approximately 8, 24, and 36 h after birth, respectively; M shows ingested milk collected from the jejunum of the calves fed milk approximately 24 h after birth.

lostrum. Accordingly, concentrations of choline, lipid, glycerophosphocholine/phosphocholine, tyrosine, and glutamate were higher in the serum of the calves in CI, CII, and CIII than in Ct calves. On the basis of changes in metabolites in the ingested colostrum and serum, several metabolites from the ingested colostrum, such as tyrosine and glutamate, were clearly positively associated with increased concentrations of these metabolites in serum.

DISCUSSION

Several previous studies indicated that bovine colostrum is a significant source of metabolites, with higher concentrations of AA, carbohydrates, lipids, and vitamins than milk (Gopal and Gill, 2000; Kamada et al., 2007; McGrath et al., 2016). In accordance with these results, we found that concentrations of valine, leucine, creatine, and uridine were higher, whereas concentrations of lactose were lower in colostrum as compared with milk, determined by the NMR-based metabolomics in the current study. Similarly, in sows, concentrations of several metabolites, such as citric acid, glycerophosphocholine, creatine, and choline were higher in colostrum than in milk, whereas concentrations of betaine, phosphocholine, and taurine were lower in colostrum than in milk based on NMR approaches (Curtasu et al., 2016). Studies of human breastmilk metabolites found that concentrations of alanine, glutamate, valine, and choline were higher in colostrum than in milk (Spelvacek et al., 2015; Wu et al., 2016). Taken together, these studies suggest that colostrum is rich in some metabolites that may contribute to meeting the specific requirements of newborns compared with milk.

In neonatal calves, colostrum ingested in the gastrointestinal tract is used to meet the absorption requirements of the small intestine. We found that several metabolites associated with carbohydrates, AA, lipids, and nucleotides were altered in the ingested colostrum compared with colostrum not ingested. For example, concentrations of lactose were lower in the ingested colostrum because the lactase activity in the small intestine increased after neonates were fed with colostrum, which served as a primary determinant of lactose digestion (Burrin et al., 1994; Kelly and Coutts, 2000; Ontsouka et al., 2004b). As a result, lactose was digested as glucose and galactose, and these were absorbed in the small intestine to meet the energy demands of neonates after birth (Girard, 1990; Steinhoff-Wagner et al., 2014). In accordance with results from previous studies, we found significantly lower lactose concentrations in the ingested colostrum from CI, CII, and CIII groups

compared with colostrum not ingested. Similarly, we also found that lactose concentrations in the ingested milk from calves in the M group were lower than in milk not ingested. In addition, lactose concentrations were not different between the ingested colostrum from calves in the CII group and the ingested milk from calves in the M group, although lactose concentrations were significantly lower in colostrum than in milk. Interestingly, lactose in human breastmilk has been considered an inducer of innate immunity (Cederlund et al., 2013). According to the changes in AA, we found higher concentrations of valine, leucine, glutamate, glutamine, and lysine in the ingested colostrum from calves in the CI, CII, and CIII groups compared with colostrum not ingested. A previous study found that concentrations of total EAA were relatively low in colostrum, and several AA, such as threonine, arginine, and glutamine, were not detected in colostrum (Zanker et al., 2000). Subsequently, a study of colostrum passing through the stomach and gut of piglets indicated that caseins and β -LG are likely to be digested when they reach the small intestine, based on proteomics analysis (Danielsen et al., 2011). Based on our results and the discussion of previous studies, we concluded that several types of colostrum components were digested in the gastrointestinal tract, resulting in higher concentrations of succinate, valine, leucine, alanine, and lysine in the ingested colostrum. This result may reflect the primary ingested requirements of colostrum by the neonatal intestine that contributed to providing a suitable metabolite profile for the absorption by the small intestine of neonates; however, these aspects need further investigation.

As a result of feeding colostrum, glucose concentrations were significantly higher in the serum of calves. Our results were similar to the results of a previous study that found that serum glucose concentrations were increased in calves that received colostrum (Steinhoff-Wagner et al., 2011b). For neonatal calves, lactose intake is not sufficient to meet glucose demands (Girard, 1990), and endogenous glucose production and gluconeogenesis have been observed in labeled-glucose studies (Steinhoff-Wagner et al., 2011b). In contrast, glycerol concentrations were lower in the serum of calves fed colostrum. Previous studies suggested that glycerol is an important gluconeogenic substrate that is taken up by the liver and converted into glucose (Phillips et al., 2002; Steinhoff-Wagner et al., 2011b). Similarly, we found that glycerol concentrations were lower in the serum of calves fed milk compared with control calves. Unexpectedly, glucose and glycerol concentrations were not different in the serum of calves from CII and M groups; thus, we believe that serum glycerol could en-

Corresponding coefficient loading plots

Scores plots

Figure 2. The score plots and corresponding coefficient loading plots of orthogonal partial least squares discriminant analysis derived from nuclear magnetic resonance spectra of serum metabolites of control group (Ct group) compared with those of the calves fed milk approximately 8 h (C1 group; A), 24 h (C2 group; B), and 36 h (C3 group; C) after birth, respectively, and the calves fed milk approximately 24 h after birth (M group; D), and metabolites of C1 group compared with those of M group (E). Changes in metabolites between the 2 studied groups are labeled in the corresponding coefficient loading plots, respectively. GPC = glycerophosphocholine; L1 = low-density lipoprotein CH_2 - $(\text{CH}_2)_n$; L2 = very-low-density lipoprotein CH_2 - $(\text{CH}_2)_n$; L3 = low-density lipoprotein CH_2 - $(\text{CH}_2)_n$; L9 = lipid, $-\text{CH}=\text{CH}-$; NAG = *N*-acetyl glycoprotein signals; For = formate; PC = phosphocholine. Color version available online.

Table 2. Differential metabolites of serum derived from the calves not fed or fed colostrum and milk

Metabolite ¹	Chemical shift	Correlation coefficients ²					Metabolic pathway
		Ct-CI	Ct-CII	Ct-CIII	Ct-M	CII-M	
Glycerol	3.56, 3.68, 3.77	−0.892	−0.959	−0.948	−0.93	—	Lipid
Choline	3.2	0.643	0.84	0.926	0.953	—	Lipid
Lipid, -CH=CH-	5.27	0.675	0.876	0.802	0.829	—	Lipid
LDL, CH ₃ -(CH ₂) _n -	0.84	0.827	0.864	0.866	0.93	—	Lipid
Phosphorylcholine	3.22	0.872	0.747	0.837	0.922	—	Lipid
LDL, CH ₃ -(CH ₂) _n -	1.24	0.872	0.881	0.872	0.957	—	Lipid
Glycerophosphocholine	3.22	0.872	0.747	0.837	0.922	—	Lipid
Formate	8.45	—	0.699	—	0.732	—	Lipid
VLDL, CH ₃ -(CH ₂) _n -	0.92	—	—	—	—	0.919	Lipid
Tyrosine	6.88, 7.18	0.629	0.836	0.81	0.822	—	Amino acid
Glutamate	2.35	0.981	0.871	0.846	—	−0.772	Amino acid
Valine	0.98, 1.04	—	0.802	0.846	—	−0.756	Amino acid
Phenylalanine	7.33, 7.38, 7.43	—	0.842	—	—	−0.826	Amino acid
Histidine	7.06, 7.78	—	0.912	0.887	0.768	—	Amino acid
Betaine	3.26	—	0.777	−0.728	—	—	Amino acid
Glutamine	2.45	—	−0.801	−0.707	—	—	Amino acid
Leucine	0.95, 0.96	—	0.726	0.656	—	—	Amino acid
N-Acetyl glycoprotein signals	2.03	—	—	—	—	0.913	Amino acid
Citrate	2.52, 2.68	—	−0.833	−0.831	−0.747	0.954	Energy
Creatinine	3.04, 4.05	—	−0.844	−0.862	−0.801	—	Energy
Acetone	2.23	—	−0.655	—	−0.778	—	Energy
Pyruvate	2.36	—	—	—	0.82	—	Energy
Creatine	3.03, 3.92	—	—	—	—	−0.899	Energy
Allantoin	5.39	—	−0.852	−0.922	−0.806	—	Nucleotide
Guanine	7.67	—	−0.794	−0.826	−0.714	—	Nucleotide
Glucose	5.23	—	0.668	—	0.842	—	Carbohydrate
Unknown 3	4	−0.92	−0.977	−0.96	0.972	—	—

¹LDL = low-density lipoprotein; VLDL = very-low-density lipoprotein.

²Ct = serum derived from control calves; CI, CII, and CIII = serum derived from the calves fed colostrum approximately 8, 24, and 36 h after birth, respectively; M = serum derived from the calves fed milk approximately 24 h after birth.

ter the gluconeogenesis pathway to meet the energy requirements of neonatal calves.

Moreover, several previous studies found that concentrations of total free EAA and several individual AA, such as lysine, methionine, threonine, and glutamate, increased in serum within 12 h after calves received colostrum (Hammon and Blum, 1999; Zanker et al., 2000). More recently, 2-dimensional GC-MS was used to investigate the changes in serum metabolites of neonatal calves in response to colostrum intake, and the results indicated that concentrations of several EAA, such as threonine, tryptophan, and methionine, were significantly higher in serum from calves fed colostrum and that these concentrations followed a time-dependent pattern (Miyazaki et al., 2017). In another study, compared with a baseline, postprandial plasma concentrations of isoleucine, lysine, tyrosine, and glutamine were significantly higher in calves fed a milk replacer twice daily using NMR spectroscopy (Ghaffari et al., 2017). In particular, previous studies have indicated that nearly all of the colostrum components were absorbed by the neonatal small intestine (Dividich et al., 2007; Lin et al., 2009). As a result, we found that several AA were

significantly higher in the serum of calves fed colostrum, whereas concentrations of glutamine were lower in the serum of calves fed colostrum than in control calves; this result was in agreement with the results of previous studies (Hammon and Blum, 1999; Zanker et al., 2000). However, serum concentrations of histidine and tyrosine were significantly higher in the calves fed milk than in control calves. More importantly, several metabolites have been implicated in multiple biological functions; for example, glutamine plays a crucial role in the metabolism of AA, carbohydrates, and lipids (Lobley et al., 2001; Roth et al., 2002). Glutamine could serve as the main fuel for newborn enterocytes and as a precursor for DNA synthesis to promote gut growth and development (Posho et al., 1994; Roth et al., 2002). Leucine, in particular, has been considered as a potent activator of glucose uptake and glycogen synthesis (Nishitani et al., 2002). Additionally, the branched-chain AA valine, leucine, and isoleucine have been implicated in promoting protein synthesis and transcription in liver and other tissues via mammalian target of rapamycin signaling pathways (D'Antona et al., 2010). Branched-chain AA have also been found to

be involved in regulating lymphocyte growth and proliferation and natural killer cell activity (Calder, 2006; Nakamura et al., 2007). Thus, we suggest that higher concentrations of metabolites in the ingested colostrum and serum of the calves fed colostrum could contribute to meeting the requirements of calves' growth and development, as well as immunity.

Furthermore, choline concentrations were lower in the ingested colostrum, whereas choline concentrations were higher in serum of calves fed colostrum. In breastfed infants at 12 to 180 d after birth, serum choline concentrations were positively correlated with choline, phosphocholine, glycerophosphocholine, and total choline contents of breastmilk (Ilcol et al., 2005). In general, phosphocholine, glycerophosphocholine, and phosphatidylcholine serve as a form of storage for choline (Hollenbeck, 2012). Importantly, choline is a precursor for the biosynthesis of phospholipids (phosphatidylcholine and sphingomyelin), which are essential structural components of all cellular membranes (Michel et al., 2006; Ridgway, 2013). Choline-deficient diets resulted in altered intestinal morphology and impaired intestinal lipid metabolism in lactating rats (da Silva et al., 2015). As evident based on these studies, choline may contribute to the structural integrity of intestinal membranes and lipid transport and metabolism. In addition, a previous study found higher phospholipid and cholesterol concentrations in serum of calves that received colostrum (Blum et al., 1997). Cholesterol and lipids were absorbed from the intestine and packaged into very low density lipoproteins for transport in the bloodstream (Scow et al., 1980; Beisiegel, 1998). We also found higher concentrations of lipids in serum of calves that received colostrum. Thus, we believe that changes in the ingested colostrum contributed to improving intestinal morphology for absorption efficiency of the small intestine, and that some of these metabolites may be transported into blood. However, the relationship between the colostrum components absorption by the small intestine and transmission into the bloodstream needs further investigation.

CONCLUSIONS

Ours is the first study to characterize the effects of colostrum and its digestion in the gastrointestinal tract on serum metabolites of neonatal calves using NMR approaches. The primary metabolite characterization of colostrum and milk ingested and not ingested revealed that concentrations of several metabolites, such as lysine, valine, leucine, lactate, and isoleucine were higher in the ingested colostrum and milk compared with colostrum and milk not ingested. In addition, the

metabolic profile of ingested colostrum was similar to that of ingested milk and was used to meet the requirements of neonates. Compared with concentrations of serum metabolites in control calves, concentrations of choline, tyrosine, and glutamate were higher in serum of calves that received colostrum. Concentrations of phenylalanine, valine, and glutamate were also significantly higher, whereas concentrations of citrate and very low density lipoprotein were lower in serum of calves that received colostrum compared with calves fed milk. These findings suggest that metabolites in ingested colostrum might facilitate the absorption of small molecule compounds by the small intestine of neonatal calves. Metabolomics-based NMR analysis of colostrum, ingested colostrum, and serum may provide novel information to help understand the mechanism of the absorption of colostrum components in the small intestine and contribute to understanding the serum metabolome profile of neonatal calves.

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