Heat treatment of bovine colostrum: Effects on colostrum metabolome and serum metabolome of calves

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ABSTRACT

Bovine colostrum is important for neonates' health due to its nutritive and non-nutritive components. Heat treatment of colostrum is a well-established management tool, but it may influence colostrum components and affect the health status of calves. In our previous studies, we had shown that colostrum proteome and serum proteome of calves was altered by heat treatment to different degrees. Our objectives in this study were to investigate the effects of heat treatment on colostrum metabolome and the effect of feeding heat treated colostrum on the serum metabolome of newborn calves. Further, the changes in serum metabolome from before to after colostrum feeding were characterized. Newborn Holstein female calves (n=10) were randomized within pairs and fed heat-treated (n=5; 60°C, 60 min) or raw (n=5) colostrum at 8.5% of birth BW by esophageal feeder within 1 h of birth. After a single colostrum feeding, calves were not fed until after the 8 h time point when milk was offered free-choice. Blood samples were taken immediately prior to feeding (0 h) and 8 h after feeding. The colostrum and serum metabolome were first analyzed using reverse-phase chromatography and tandem mass spectrometry (RPLC-MS), and serum metabolome was then further

analyzed using hydrophilic interaction chromatography and tandem mass spectrometry (HILIC-MS). In colostrum metabolome, 458 features were identified and 328 were annotated, and a trend of separation between raw and heated colostrum could be observed through multivariate analysis. In serum metabolome, 3360 features were identified and 1439 were annotated, but no trend of separation was observed between the two groups of calves fed raw colostrum vs. heat-treated colostrum. The serum metabolome presented substantial differences comparing before (0 h) and after colostrum feeding (8h), in partiluar a tripeptide, β-homovaline-β-homoalanine-β-homoleucine, and 1-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-1D-myo-inositol had higher concentrations after colostrum feeding than before, along with other metabolites that were not fully annotated. Based on a relatively small sample size, our findings point to the effect of heat treatment on the change of colostrum metabolome, but not on the change of serum metabolome of calves fed raw colostrum vs. heat-treated colostrum. Further studies using larger sample size and complementary analytical techniques are warranted to further explore potential heat treatment induced alterations in colostrum metabolome.

INTRODUCTION

Colostrum, the first milk that neonates receive after birth, is rich in nutrients and non-nutritive biologically active factors (Hammon et al., 2013). Colostral nutrients including lactate, amino acids (especially alanine), and glycerol, and these nutrients can be used as substrates in neonates for gluconeogenesis within a short time (8 hour) after birth (Girard et al., 1992). The non-nutritive factors of the colostrum include potentially bioactive compounds, such as immunoglobulins, hormones and growth factors, which improve the growth, function, and absorptive capacity of the neonatal gastrointestinal tract (Hammon et al., 2013, Fischer et al., 2018), and can affect the serum metabolites of the bovine neonate (Zhao et al., 2018). Colostrum feeding thus not only fulfills nutritional requirements, but also improves growth and health in neonatal mammals (Blum, 2006, Guilloteau et al., 2009). Early intake of adequate amounts of high-quality

colostrum is critical for the health and growth of the bovine neonate (Godden et al., 2019), as a biological mechanism in early postnatal life when enhanced metabolic plasticity allows for an extended maternal care through colostrum and milk (Bartol et al., 2013). The concentrations of essential fatty acids, carotene, retinol, and α-tocopherol were significantly higher in serum of calves fed colostrum on day 1 post natum compared with delayed colostrum-fed calves (Blum et al., 1997). In terms of feeding management, heat treatment of colostrum is used to decrease bacterial contamination, extend storage post-harvest, and control infectious agents that could be transmitted to the neonate (Fischer et al., 2018, Godden et al., 2019). However, heat treatment might impair the function of heat-labile bioactive compounds, such as immunoglobulins, whey proteins and enzymes, and cholesterol, as suggested for both bovine and human colostrum (Johnson et al., 2007, Sousa et al., 2014, Parrón et al., 2016).

Metabolomics is a high-throughput technique that can identify, quantify, and characterize hundreds to thousands of low-abundant metabolites from biological samples using targeted or global analytical approaches (Ryan and Robards, 2006). Based on blood samples of calves, metabolomics has identified the plasma biomarkers of immune response (Gray et al., 2015), and screened the absorption and transmission of colostral components to serum within 8 to 36 h after birth (Zhao et al., 2018). Hence, a metabolomics-based overview of metabolite profiles could help us understand the effect of heat treatment on the colostrum metabolome, and the effect of feeding heat-treated colostrum on the serum metabolome of calves. Among metabolomics analyses, LC-MS has been widely used as a targeted measurement due to its high sensitivity and wide range of metabolite coverage (Kuehnbaum and Britz-McKibbin, 2013). Aiming to obtained a larger set of metabolites (non- and moderately polar compounds) in whole-body metabolome, untargeted metabolomics can be performed using reverse-phase liquid chromatography (RPLC, mainly C18-bonded silica columns) (Want et al., 2010, Dunn et al., 2011), and using hydrophilic interaction liquid chromatography (HILIC) that offers a complementary selectivity to RPLC (Ilves et al., 2012).

Our previous work showed that heat treatment (60°C, 60 min) altered the proteome profile of low-abundant proteins in colostrum and reduced insulin and immunoglobulin concentrations (Mann et al., 2020a). Further we showed that heat treatment also altered the serum profile of low-abundant proteins, but not immunoglobulins (IgA and IgG) in calves fed with heat-treated colostrum (Mann et al., 2020b). The effect of heat treatment on the colostrum metabolome, and the effect of heat-treated colostrum on the serum metabolome of calves have not yet been reported. We hypothesized that the colostrum metabolome can be altered by heat treatment due to the degradation of heat-labile molecules. If such changes affect biologically active colostrum components, the calves' serum metabolome can also be altered after feeding the heat-treated colostrum. Further, we hypothesized that the serum metabolome undergoes substantial changes within 8 hours on day 1 after birth, due to the adaptation to postnatal life and/or due to colostrum feeding. Therefore, based on the metabolome revealed by RPLC in colostrum, and the combination of RPLC and HILIC in serum of calves at the time points of 0h and 8h after feeding, the objectives of this study were: *i*) to evaluate the effect of heat treatment on colostrum metabolome, *ii*) to evaluate the effect of heat treatment on calf serum metabolome, and *iii*) to characterize the difference in the serum metabolome between 0 h and 8 h after colostrum feeding.

MATERIALS AND METHODS

Cows and Colostrum Samples

All animal procedures were reviewed and approved by the Cornell University Institutional Animal Care and Use Committee (protocol no. 2018-0021). The study was performed between July and August 2018 on a commercial dairy farm in New York State after obtaining written consent from the owner. Holstein cows were housed indoors year-round in free-stalls and moved to the calving pen following a just-in-time approach.

Colostrum from all animals with at least 28 d of dry period length and that were clinically healthy immediately postpartum were eligible for enrollment, as described in

Mann et al. (2020a). In brief, colostrum of individual cows was harvested into sanitized buckets, and gently mixed with a whisk before taking an aliquot to test Brix% on a digital refractometer (Palm Abbe, Misco, Cleveland, OH). Colostrum ≥ 22% Brix and ≥ 8 L total volume was eligible to be used in the study. Colostrum (n=5) was whisked to mix thoroughly while avoiding foam production, and then filled into 2 separate 4 L disposable bags (Perfect Udder, Dairy Tech, Inc., Windsor, CO). Raw colostrum bags were placed on ice for 30 min and then stored in a refrigerator at 4°C for up to 24 h. The paired aliquot of each colostrum batch was heat treated using a commercial pasteurizer (Dairy Tech, Inc., Windsor, CO) at approx. 60°C which lasted approx. 25 min. After cooling down to approx. 43°C, the bags were removed and immediately placed on ice for 30 min to rapidly cool before storage at 4°C for up to 24 h.

Calves and Colostrum Feeding

Female Holstein calves born with a birth weight of 34.0 to 47.0 kg, absence of birth defects, and having been delivered without assistance. Calves were removed from dams within 10 min of birth and not allowed to suckle. Calves were enrolled to be fed raw (**R;** n=5) or heat-treated (**H;** n=5) colostrum following a randomized block design with 2 calves per block according to the time of birth on the same day. Raw or heat-treated colostrum as prepared above was adjusted to 8.5% of the calf's birth BW (Conneely et al., 2014). None of the calves were fed their own dam's colostrum. Colostrum was administered to calves within 1 h of birth using an esophageal feeder (Dairy Tech., Inc.) according to manufacturer instructions (www.dairytechinc.com) and consistent with farm protocols. Both treatments (Raw and Heated) of a single batch were administered to each pair of newborn calves on average within 2 h, and within a maximum of 4 h from each other. Bags of refrigerated raw or heat-treated colostrum were then placed in a 43°C water bath (MilkWorks, Dairy Tech., Inc.) for 20 min to warm to feeding temperature.

Blood Sampling

Blood samples were taken from the jugular vein of each calf immediately before colostrum feeding (0 h) and at 8 h after feeding. Blood was collected into evacuated 10

mL serum tubes (Monoject, Covidien, Dublin, Ireland) and was allowed to clot at room temperature for 10 min. Tubes were centrifuged for 20 min at 3,000 x g at 4°C within 30 min after collection. Harvested serum samples were snap frozen in liquid nitrogen, stored at -20°C for < 24 h, and then stored at -80°C until analysis.

Sample Preparation and Metabolomics Analysis

Plasma samples (100 µL) were removed from -80°C to 4°C, and added 300 µL cold methanol (4°C) incubated for 1 h for protein precipitation. Samples were then removed from 4°C to room temperature for 20 minutes. The supernatant was collected after centrifugation at 13,000 rpm for 10 min at 4 °C and evaporated to dryness. The dry extracts were reconstituted with 100 µL 60% acetonitrile (for RPLC) or 50% acetonitrile with 0.1% formic acid (for HILIC) before analysis. Metabolomics measurements were done using reverse-phase liquid chromatography (RPLC) in colostrum and in serum, and additionally by hydrophilic interaction liquid chromatography (HILIC) in serum. Chromatographic separation was performed on a Vanquish UHPLC system with a SeQuant ZIC pHILIC column (5µm, 2.1 x 150mm) coupled to a Q Exactive™ HF Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) for polar compounds, and a Vanquish UHPLC system with an Accucore Vanquish C18+ column (1.5µm, 2.1 mm id x 100mm) coupled to a Q Exactive™ HF Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) for nonpolar compounds. A quality control sample was prepared by pooling equal volumes of each sample. Three internal standards, sulfadimethoxine, 13C-pyruvic, and 13C-valine (CIL, MA, USA) were added to all samples to assess MS instrument reproducibility. The measurement conditions were as follow: column temperature 45°C in RPLC and 24°C in HILIC, flow rate 320 µL/min in RPLC and 250 µL/min in HILIC, and injection volume 2 µL.

Data processing and Statistical Analysis

All MS/MS samples were aligned against the pooled quality control reference run, and peak picking was performed on individual aligned runs to create an aggregate data set. Following peak picking, unique spectral features (retention time and m/z pairs) were

grouped based on adducts and isotopes, and individual features or metabolites were normalized to all features. Compounds with 25% coefficient of variance (CV) were retained for further analysis. Principal component analysis (PCA) was applied to the data to check a general trend in an unsupervised way. Partial least squares discriminate analysis (PLS-DA) was used to maximize the fitness of variables discriminating between the two groups in a supervised way. The PLS-DA model was tested by crossed-validation and the validated model was further considered in sparse PLS-DA (sPLS-DA). Based on the high number of features in the untargeted metabolome, sPLS-DA was chosen to select the most predictive or discriminative features in the data that help classify the samples (Lê Cao et al., 2011). A paired t-test with false discovery rate (FDR) correction was used to compare treatment effects. All multivariate analyses (PCA, PLS-DA, sPLS-DA) and univariate analyses (t-test) were performed using MetaboAnalyst 4.0 (Chong et al., 2019).

RESULTS & DISCUSSION

Metabolomics workflow in colostrum and serum

We used two complementary analytical methods of LC-MS: RPLC and HILIC (Figure 1). The RPLC method was first used for both colostrum and serum samples. Based on the data obtained via RPLC, we observed a trend for separation in the metabolome profiles between raw and heated colostrum (Figure 2), but not between the two groups of calves fed raw colostrum vs. heated colostrum (Supplementary Figure 1). To further explore any differences in the serum metabolome profiles between the two treatment groups, the HILIC method was further applied to the serum samples.

In the colostrum metabolome, 458 metabolites were detected after normalization and removing the background and false positives, and 400 metabolites were annotated based on our spectral databases (Figure 1). In the serum metabolome, 1879 and 1481 metabolites were detected after normalization and removing the background and false positives by RPLC and HILIC, respectively. Based on our spectral databases, 659 metabolites were annotated, and 822 got a formula prediction in the RPLC data;

whereas 780 metabolites were annotated, 920 got a formula prediction and 179 got a molecular weight in the HILIC data (Figure 1). Although untargeted metabolomics is known for a relatively larger metabolite coverage, the annotation of metabolites is often a challenging process (Cui et al., 2018). Therefore, it was expected that numerous compounds could not be annotated, or could only be identified with a formula or weight.

B

	Columns	Compounds detected (CV < 25%)	Annotated metabolites	Identified formula	Detected weight
Colostrum	RPLC	458	328	15	-
Serum	RPLC	1481	659	822	-
	HILIC	1879	780	920	179

Figure 1. Workflow of untargeted metabolomics data analysis of colostrum samples and serum samples of calves (A), and global output of detected compounds, annotated metabolites, identified formula and weight from RPLC and HILIC methods (B).

Effect of Heat Treatment on Metabolome Profiles in Colostrum

We observed a trend of separation in the metabolome profiles between raw and heated colostrum by PCA (Figure 2-A) and by sPLS-DA (Figure 2-B). Three metabolites, 8-hydroxy-deoxyguanosine, leucine-leucine, and phosphocreatine, were found to have a relatively high variable importance in projection (VIP) score in the sPLS-DA (Figure 1-C), as well as a *P*-value < 0.10 (FDR adjusted) in the t-test (Figure 2-D). Both 8-hydroxy-deoxyguanosine and leucine-leucine had a higher concentration in the heated colostrum, compared with the raw colostrum, while phosphocreatine had a higher concentration in the raw colostrum, compared with the heated colostrum. The most likely explanation for the altered metabolite concentrations is that the direct or indirect degradation of heat-labile molecules due to the heat treatment. A shown in our previous study, heat treatment resulted in the reduction of total bacterial count (Mann et al.,

2020a), which could affect the concentration of colostrum metabolites due to the different metabolic activity of an altered colostral microbial community. Further, heat treatment could also affect the structure of high-molecular-weight metabolites, for example, it was suggested that the heat-induced cleaveage of colostrum oligosaccharides from colostral lipids or proteins could increase the concentration of free oligosaccharides in the colostrum (Fischer et al., 2018). There is a limited number of studies that can provide plausible explanation for the concentration change of these 3 metabolites in particular, however, it could be expected that even more metabolites, rather than only 3, could be altered by heat treatment in a study with a larger sample size.

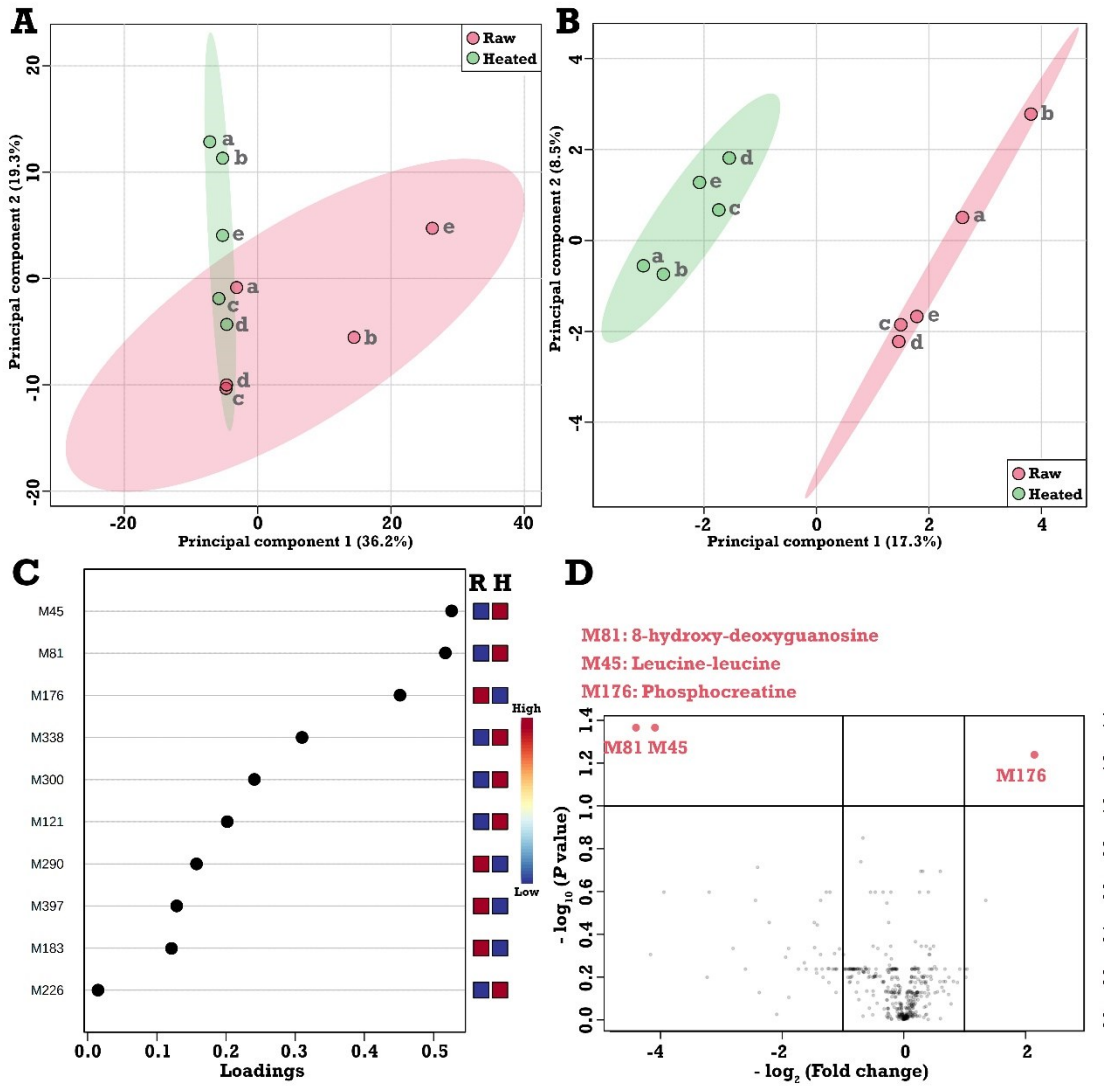

Figure 2. Effect of heat treatment on colostrum metabolome in pincinpal component

analysis (PCA) (A), and sparse partial least square discriminant analysis (sPLS-DA) (B). Top 10 metabolites with the highest variables in prejection (VIP) scores of sPLS-DA were listed (C), and the volcano plot shows the significantly different metabolites between treatments (D).

In (A) and (B), the same superscripts above score symbols (a-e) indicate colostrum pairs of different treatments (raw vs. heated).

Effect of Feeding Heat-Treated Colostrum on Serum Metabolome Profiles of Calves

To account for the time series data of the serum metabolome analysis, data of 0 h (before colostrum feeding) were used as a baseline, and the ratios of 8 h relative to 0 h were used to compare the time-course of metabolome changes. We first evaluated the serum metabolome data obtained by RPLC only, to be consistent with the colostrum data analysis approach. As shown in the Supplementary Figure 1, using the RPLC data only, no separation of the serum metabolome was observed in the PCA scores plot. Further, the PLS-DA model had a poor performance, reflected by its low accuracy (<0.5 with 1 to 5 components) and its negative Q2 revealed by crossed validation, which means that the model was not predictive or it was overfitted (Szymańska et al., 2012). To further explore any potential separtion of the serum metabolome between the two treatment groups, the additional metabolome data set obtained by HILIC were added to the analysis. Based on this combined data, we were still not able to observe a clear separation of serum metabolome between the calves fed raw colostrum vs. heat-treated colostrum using PCA (Figure 3-A) and PLS-DA (Figure 3-B). The poor performance of the PLS-DA model (Figure 3-C) suggested that the effect of feeding heated colostrum on the serum metabolome was non-significant or below the detectable limit with the current sample size (n=5).

As shown in our previous paper, the calves fed heated colostrum had increased plasma insulin concentrations and an altered serum protein and enzyme profile that could be associated with carbohydrate metabolism, with no differences in circulating glucose concentrations at the same time points (Mann et al., 2020b). However, the observed

alterations in the metabolome profiles between the raw and heated colostrum did not translate into any recognizable difference in the serum metabolome of calves. This was consistent with our previous proteomics findings in a sense that fewer serum proteins differed in abundance between the two groups of calves fed raw colostrum vs. heat-treated colostrum, compared with the number of colostrum proteins that differed in abundance between treatments (Mann et al., 2020b).

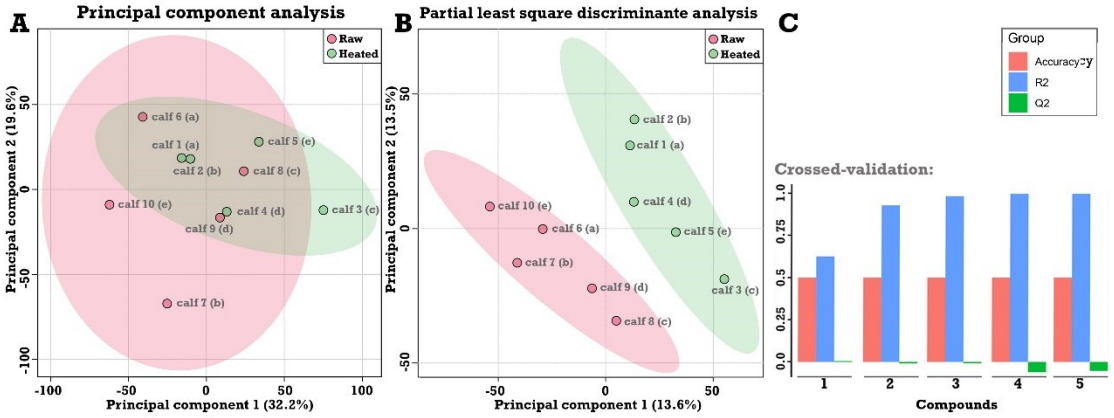

Figure 3. Based on the combined RPLC and HILIC data, possible differences of serum metabolome (ratio of 8 h to 0 h) of calves fed raw or heated colostrum were presented in pincinpal component analysis (PCA) (A), and partial least square discriminant analysis (PLS-DA) (B). Quality control of the PLS-DA model was tested by cross-validation with 5 components (C).

In (A) and (B), the same superscripts above score symbols (a-e) indicate colostrum pairs of different treatments (raw vs. heated).

Changes of Serum Metabolome Profiles from 0 h to 8 h Relative to Colostrum Feeding

We observed a clear time-course effect in the serum metabolome of calves, comparing the profiles of the samples taken just before (0 h) and 8 h after colostrum feeding, as shown in the heatmap (Supplementary Figure 2). In addition, the separation between 0 h and 8 h in the serum metabolome profiles was confirmed by PCA (Figure 4-A) and sPLS-DA (Figure 4-B). However, due to the limitation of the used untargeted metabolomics technology, a number of metabolites with high VIP scores in sPLS-DA

(Figure 4-C) could not be annotated by RPLC and HILIC. In the volcano plot, 10 features were found to be significantly different between 0 h and 8 h (Figure 4-D). Only two of the most significant metabolites were annotated, β-homovalin-β-homaalanine-β-homeleucine (M1 and M2423) and 1-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-1D-myo-inositol (M2725). The higher concentration of the tripeptide detected in the 8 h serum sample is most likely indicative of the increased absorption of oligopeptides in the small intestine in the early stage of neonatal live in calves (Gilbert et al., 2008). The significantly different serum metabolome profile between 0 h and 8 h is likely a combined effect of colostrum uptake and adaptation from intrauterine to postnatal life. The serum metabolome, considered to be a snapshot of the current metabolic activity, is likely affected by factors such as the timing of colostrum feeding (within an hour after birth in the current study), as well as colostrum quality, bacterial count, and sufficient quantity of colostrum fed to the calves (McGuirk and Collins, 2004). Further, the serum metabolome might change over time as a function of decreasing metabolic plasticity in the newborn (Bartol et al., 2013).

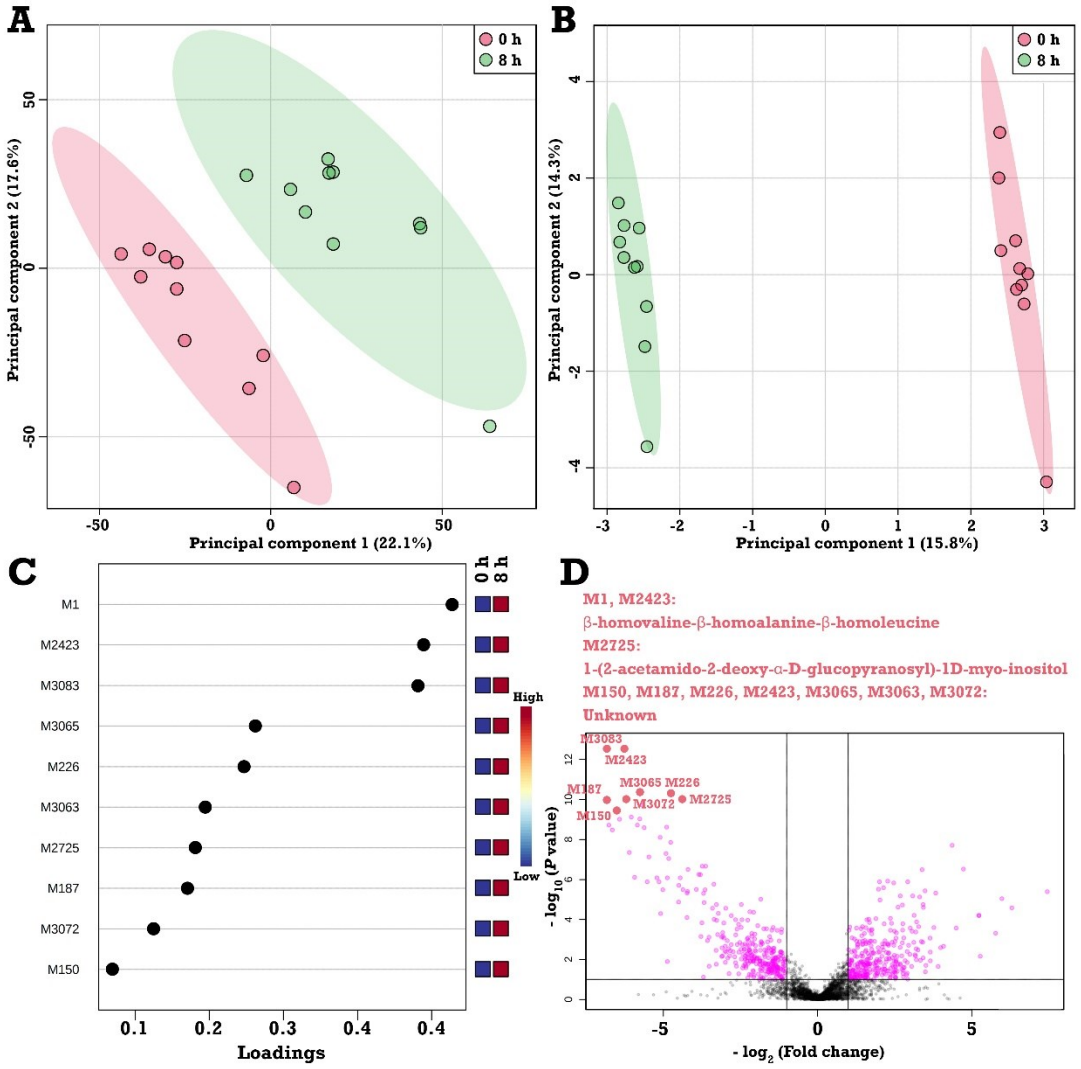

Figure 4. Different profiles of serum metabolome of calves between 0 h and 8 h relative to colostrum feeding in pincinpal component analysis (PCA) (A), sparse partial least square discriminant analysis (sPLS-DA) (B). Top 10 metabolites with the highest variables in prejection (VIP) scores of sPLS-DA were listed (C), and the volcano plot shows the significantly different metabolites between time points (D).

CONCLUSIONS

Colostrum contains several hundreds of small molecules, as demonstrated by an LC-MS based quantification in this study. The concentration of the majority of these molecules seemed to be unaffected by a commonly used heat treatment procedure, however, we found 3 of the 458 colostrum metabolites to have an altered concentration

after heat treatment. Further studies are warranted to assess the repeatability and biological relevance of these concentration changes. Nevertheless, feeding the studied raw and heated colostrum batches to newborn calves did not trigger any detectable alterations in their serum metabolome profiles 8 hours after feeding. If any potential effects on the serum metabolome exist these were below the detection limit of the current study. Future research with an increased number of observations could help explore any minor effects, and studies with shorter or extended sampling intervals could help explore any shorter or longer term effects. The serum metabolome of calves was confirmed to undergo significant changes within the first 8 hours after the first feeding, which is likely a combined metabolic effect of colostrum uptake and adaptation to postnatal life.

ACKNOWLEDGEMENTS

We thank the farm and farm personnel who assisted with this project and allowed us to use the animals in this study. This work was supported by the Institute of Biotechnology Seed Grant Program of Cornell University (Ithaca, NY). We thank the Metabolomics Facility of Cornell University (Ithaca, NY).

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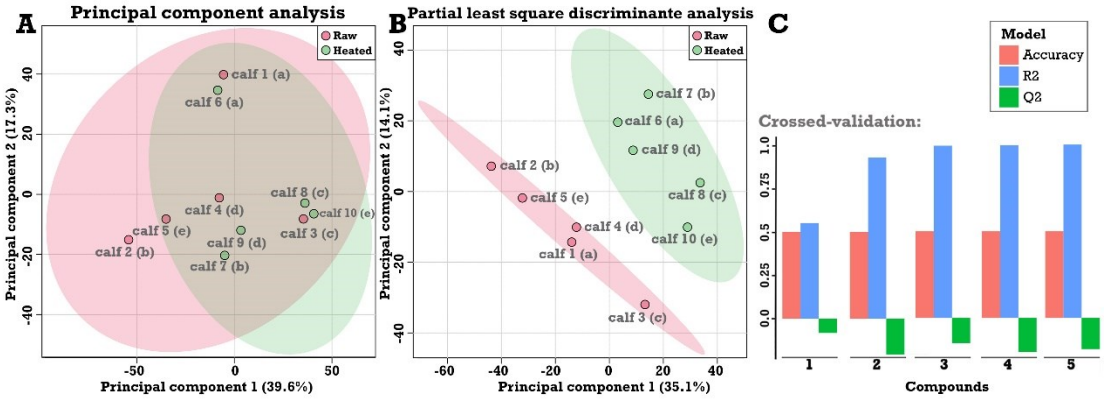
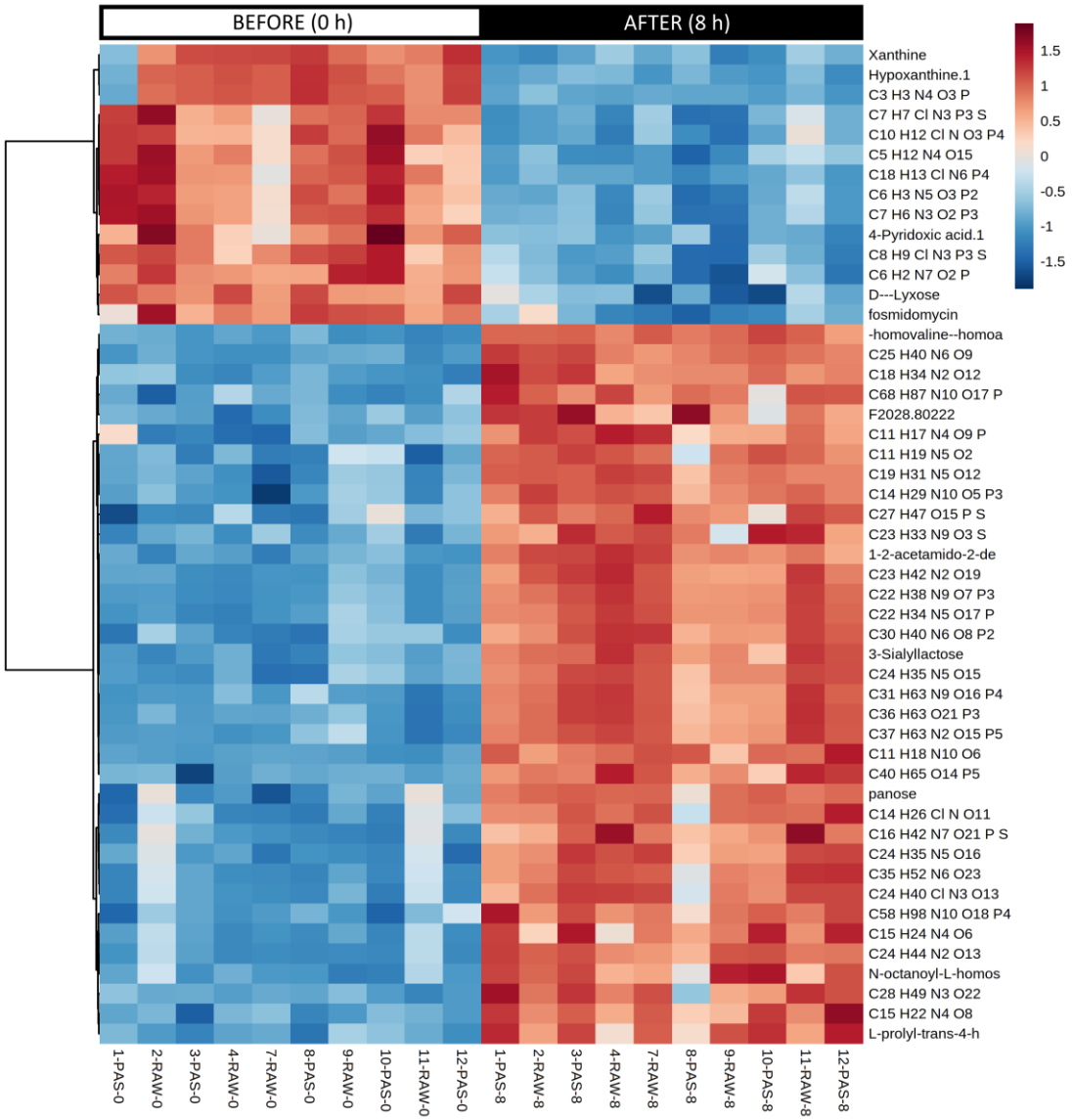
Supplementary Material



Supplementary Figure 1. Based on the RPLC data, possible differences of serum metabolome (ratio of 8 h to 0 h) of calves fed raw or heated colostrum were presented in pincinpal component analysis (PCA) (A), and partial least squares discriminant analysis (PLS-DA) (B). Quality control of the PLS-DA model was tested by cross-validation with 5 component (C).

In (A) and (B), the same superscripts above score symbols (a-e) indicate colostrum pairs of different treatments (raw vs. heated).



Supplementary Figure 2. Heatmap (relative concentrations) of serum metabolome of calves immediately before (0 h) and 8 h after colostrum feeding. Top 50 metabolites/features found to be different between 0 h and 8 h by t-test. Red indicates relatively higher concentrations, while blue indicates relatively lower concentrations.