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Heat treatment of bovine colostrum: I. Effects on bacterial and somatic cell counts, immunoglobulin, insulin, and IGF-I concentrations, as well as the colostrum proteome

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ABSTRACT

The objective of this study was to investigate the effects of heat treatment on colostral low-abundant proteins, IgG and IgA, insulin, and insulin-like growth factor I (IGF-I), as well as bacteria and somatic cells. First-milking colostrum samples >8 L and Brix % > 22.0 were harvested from 11 Holstein cows on a commercial dairy in New York State and split into 2 aliquots using single-use colostrum bags. One aliquot of each pair was cooled on ice immediately after harvest (raw, R; n = 11), and the other was heat-treated for 60 min at 60°C (heat, H; n = 11). All samples were analyzed for IgG and IgA via radial immunodiffusion assay and insulin and IGF-I concentrations by radioimmunoassay. Total bacterial counts and somatic cell counts (SCC) were determined using standard plate culture techniques and flow cytometry, respectively. Samples from a subset of 5 pairs (n = 10) were further analyzed by nano liquid chromatography-tandem mass spectroscopy, after ultracentrifugation at 100,000 × *g* for 60 min at 4°C to enrich the low-abundant protein whey fraction. Data were analyzed using either paired *t*-test or Wilcoxon signed-rank test or using an online software package to analyze proteomics data. Outcomes of proteomics analysis were fold change ≥1.5 between pairs, and paired *t*-tests with false discovery rate–adjusted *P*-value < 0.05. The median reduction of IgA concentrations was 8.5% (range: 0–38.0%) due to heat treatment, whereas IgG concentrations did not change due to treatment. Insulin concentrations decreased by a median of 22% (7–45%), and IGF-I decreased by 10% (0–18%) in H samples. Heat treatment was associated with a median reduction of SCC of 36% (0–90%) in paired samples, as well as a median reduction in total bacterial count of 93% (45–100%) in H versus R samples. Proteomics analysis identified a total of 328 unique proteins that were present in all 10 samples. Nine of the 25 proteins that decreased by at least 1.5-fold in H compared with R were identified as complement proteins. We conclude that heat treatment of colostrum is associated with a reduction in the concentration of bacterial counts and SCC, IgA, insulin, and IGF-I. In addition, proteomics analysis of colostral whey identified several complement components and other proteins that decreased in abundance due to heat treatment. Although IgG concentrations were unaffected and a reduction in bacterial counts was achieved, the change in several immunologically active proteins and growth factors may have biologically important effects on the developing immune system of the neonate fed heat-treated colostrum.

Key words: colostrum, heat treatment, proteome, immunology

INTRODUCTION

Colostrum contains a particularly high concentration of proteins, consisting predominantly of Ig and casein (McGrath et al., 2016), and has long been recognized as the critical source of Ig to achieve adequate transfer of passive immunity in the agammaglobulinemic bovine neonate (McGuire et al., 1976). It also has a high nutrient value and delivers vitamins, minerals, and growth factors to the newborn calf (Quigley and Drewry, 1998; Blum and Hammon, 2000; Hammon et al., 2013). Due to the importance of transfer of passive immunity, quality of bovine colostrum is traditionally judged by direct measurement of Ig (Gelsinger et al., 2015) or by indirect estimation of Ig concentration—for example, with the use of a refractometer (Bielmann et al., 2010; Quigley et al., 2013). Although the importance of Ig in colostrum is clearly documented, colostrum is also rich in low-abundant proteins such as growth factors, cytokines, hormones, or enzymes, as recent proteomic

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analysis has shown (Nissen et al., 2012). Insulin and IGF are present in amounts substantially higher than those circulating in the blood of the dam (Malven et al., 1987). Although the possibility and the degree of systemic uptake by the neonate from colostrum is still questioned, they are likely to play biological roles in the gut: for example, by affecting gut maturation (Roffler et al., 2003) and enhancing glucose absorption capacity (Steinhoff-Wagner et al., 2011; Hammon et al., 2013).

With advancements in proteomics and liquid chromatography mass spectrometry technologies, we are now beginning to explore the global colostrum proteome in depth (Nissen et al., 2012; Nissen et al., 2017) and interpret the possible biological roles of newly identified and previously understudied components. The same is true for lipid fractions (Contarini et al., 2014) and complex sugars (Fong et al., 2011). In addition, recent advances in describing cell-free nucleic acids have shown that colostrum is also rich in microRNA (Sun et al., 2013; Ylioja et al., 2019; Van Hese et al., 2020). For many of these newly discovered colostrum components, short-term and long-term biological functions in raw colostrum have yet to be determined, as discovery of colostrum components is currently advancing.

Heat treatment of colostrum before feeding is a management approach on dairy operations to control bacterial contamination and the potential transmission of infectious agents such as *Mycoplasma bovis*, *Mycobacterium avium* ssp. *paratuberculosis*, and *Salmonella* spp. from the dam to the newborn calf via milk (Godden et al., 2006). Using a lower-temperature (60°C), long-term (60 min) heating process is a well-documented approach to maintain acceptable fluidity characteristics and successfully reduce or eliminate bacteria (Rebelein, 2010; Godden et al., 2019). Heat treatment with this approach has been shown to preserve the important IgG fraction (McMartin et al., 2006), although this may depend on initial IgG concentration, with a decrease observed particularly in samples with high initial concentration (Donahue et al., 2012). To the best of our knowledge, the effect of heat treatment on other Ig, such as IgA, has not been investigated to the same degree.

Recent work has shown that heat treatment can alter carbohydrate fractions (Fischer et al., 2018), but the effect of heat treatment on the proteome of bovine colostrum has only been described in a single pooled sample to date (Tacoma et al., 2017). Holder pasteurization (heat treatment at 63°C for 30 min) has been shown to reduce insulin and IGF-I concentrations in human colostrum (Goelz et al., 2009; Ley et al., 2011), but similar effects on bovine colostrum at 60°C for 60 min have yet to be determined.

To address current gaps in knowledge, the primary objective of this study was to investigate the effect of heat treatment on colostral low-abundant proteins. In addition, we extended the investigation of heat treatment on Ig to IgA because this Ig type, with importance in establishing mucosal immunity, is known to be more heat-labile than IgG (Mainer et al., 1997), as well as to insulin and IGF-I concentrations, representatives of well-described colostral growth factors affected by heat treatment in human colostrum. Last, given that the purpose of colostrum heat treatment is reduction of viable bacteria, we investigated the effect of heat treatment on colostral bacterial contaminants in the samples used in this study. We hypothesized that heat treatment for 60 min at 60°C would affect the abundance of all investigated components, and that the investigation of heat treatment effects on the colostrum proteome would allow us to formulate novel hypotheses regarding its possible biological effect.

MATERIALS AND METHODS

Animals

All animal procedures were reviewed and approved by the Cornell University Institutional Animal Care and Use Committee (protocol no. 2018-0021; Ithaca, NY). The study was performed between July and August 2018 on a commercial dairy farm in New York State, with written consent from the owner. Holstein cows were housed indoors year-round in freestalls 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, that were clinically healthy immediately postpartum, was eligible for enrollment. Exclusion criteria included colostrum harvested from cows having a dry period shorter than 28 d, those showing clinical signs of hypocalcemia, or those that had experienced dystocia.

Colostrum Harvest and Treatments

Calves were removed from dams within 10 min of birth and not allowed to suckle. Colostrum was harvested in a 4-stall herringbone parlor (DeLaval International AB, Tumba, Sweden) according to farm protocol. Colostrum of individual cows was harvested 3 times per day into sanitized buckets (allowing a maximum of 8 h from parturition to first milking) and gently mixed with a whisk before taking an aliquot with a disposable plastic pipet to test Brix percentage 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. Only the first milking of colostrum from each animal was used in this study. Colostrum (n = 11) was whisked carefully again to mix thoroughly, avoiding foam production, and then filled into 2 separate 4-L disposable bags (Perfect Udder, Dairy Tech Inc., Windsor, CO) with the help of a filler (Dairy Tech Inc.), which was washed and sanitized thoroughly between individual colostrum samples. Air was removed from the top of the bag, and the bag sealed.

Bags of raw colostrum (treatment **R**) were immediately placed on ice for 30 min. Ice-cold raw colostrum was then stored in a refrigerator at 4°C for up to 24 h. The paired aliquot of each colostrum batch was heat-treated (treatment **H**) immediately after filling into bags as described above. The commercial pasteurizer (Dairy Tech Inc.) was run on the "Colostrum Normal" profile, with heat treatment at approximately 60°C and cool-down to approximately 43°C. Actual water temperature of the pasteurizer was monitored with a Bluetooth Thermo Recorder (TR42, T&D Corporation, Nagano, Japan) immersed in the water. Maximum temperature reached was 59.8°C for the 60-min pasteurization step, and the water temperature remained stable between 59.5 and 59.8°C until cool-down, which lasted approximately 25 min. When the water bath reached the programmed cool-down temperature of 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.

Colostrum Samples

Colostrum samples were taken for analysis from the H and R bags immediately before feeding but before re-warming of colostrum. Colostrum in bags was mixed thoroughly before aliquots were taken and either refrigerated for immediate analysis of SCC and bacterial counts, or snap-frozen in liquid nitrogen and stored frozen until further analysis. Frozen samples were stored short-term at −20°C (<24 h) until they were moved on dry ice to −80°C for long-term storage.

Milk Quality Analysis

Refrigerated colostrum samples were submitted within 24 h to the Quality Milk Production Services laboratory (Animal Health Diagnostic Center, Cornell University, Ithaca, NY) for quantification of bacteria by plate counts. In brief, the colostrum samples were mixed and diluted 10-fold with PBS. Fifty microliters of the dilutions were inoculated onto trypticase soy agar with 5% sheep blood and 0.1% esculin, modified Edwards medium, and MacConkey agar. MacConkey plates were incubated at 37°C for 24 h, and lactose fermenter colonies were counted. At 48 h of incubation Edwards medium plates were inspected, and *Streptococcus* and *Streptococcus*-like colonies were counted. At 48 h, trypticase soy agar plates were read to quantify staphylooccci, *Prototheca*, gram-positive bacilli, and fungi. Another aliquot was referred to DairyOne (Ithaca, NY) for analysis of SCC via flow cytometry (Fossomatic, Foss Analytics, Hillerød, Denmark).

IgA and IgG

Concentrations of colostral IgG and IgA were determined by radial immunodiffusion (**RID**) assay, according to the manufacturer's instructions, using commercially available kits (Triple J Farms, Bellingham, WA). All samples were run using IgG or IgA RID assays and standards from the same lot number. Briefly, colostrum samples were thawed at room temperature for 30 min and vortexed vigorously. To ensure that colostrum IgA concentration fell within the range of the standards provided, 1 mL of whole colostrum was diluted 4-fold with sterile 0.9% saline that was preheated to 37°C. Samples for IgG were diluted further to an 8-fold dilution by adding 1 mL of the above dilution to 1 mL of 0.9% saline preheated to 37°C. For both IgG and IgA RID assay, 5 μL of sample was pipetted into duplicate wells. Assay plates were incubated at room temperature for 24 h to allow samples to reach equilibrium in the agarose gel. Plates were then stored at 4°C until precipitate ring diameters were measured using an LED 10× Scale Loupe (TekcoPlus, Kowloon, Hong Kong) and MagniPros LED magnifying glass (South El Monte, CA). Mean Ig concentration and standard deviation (SD) were calculated from duplicates to determine coefficient of variation (CV). Samples with CV > 10% were re-assayed. Raw and heat-treated samples from the same batch of colostrum were analyzed on the same RID assay plate.

Ultracentrifugation

To increase the detectability of low-abundant proteins in colostrum, a high-speed centrifugation step was performed after samples were thawed, to produce casein-depleted colostral whey according to procedures described for bovine colostrum by Nissen et al. (2012). Complete protease and phosphatase inhibitor (Halt, Thermo-Fisher Scientific, Waltham, MA) was added to the sample at a final concentration of 1× immediately upon thawing. Samples were then vortexed and diluted 1:4 in sterile-filtered PBS and vortexed again. Five milliliters of diluted sample were filled to the top

of thin-wall polypropylene ultracentrifuge tubes (Beckman Coulter, Brea, CA), and spun at 100,000 × *g* for 60 min at 4°C in a swinging-bucket rotor (SW 55 Ti, Beckman Coulter). The fat layer was punctured, and the clear supernatant was transferred to a glass tube, mixed thoroughly, and then aliquoted and either submitted immediately for proteomics analysis or stored at −80°C for other analyses.

Immunoreactive Insulin and IGF-I Concentration

The concentrations of immunoreactive IGF-I and insulin were measured using RIA techniques. Colostral IGF-I concentration was determined in ultracentrifuged whey samples at the Endocrinology Laboratory of the Animal Health Diagnostic Center (Cornell University, Ithaca, NY) using an IGFBP-blocked human RIA technique (IGF-I RIA, Mediagnost, Reutlingen, Germany) suitable for heterologous use in bovine samples. To fall within the range of standards, samples were diluted to a final 200-fold dilution with assay buffer.

Colostral insulin concentration was determined in whey samples in the same laboratory using a rat RIA (RI-13K, Millipore Sigma, Burlington, MA) in samples with a final 40-fold dilution in assay buffer as previously described (Mann et al., 2016b).

Proteomics Analysis

Ultracentrifuged, defatted colostral whey of 5 randomly selected paired samples (10 samples total: H, n = 5; R, n = 5) was submitted immediately after centrifugation for proteomics analysis to the Cornell University Proteomics and Metabolomics Facility (Ithaca, NY). Concentration of protein in each sample was determined by running precast 10% Bis-Tris gels (NOVEX, Invitrogen, Carlsbad, CA) in electrophoresis with subsequent colloidal Coomassie staining. Gels were imaged on a Typhoon 9400 scanner followed by ImageQuant TL 8.1 (GE Healthcare, Chicago, IL). Quantification was based on an *Escherichia coli* lysate standard curve with a series of known amounts of *E. coli* lysates and loaded on the same gel (2.5, 5, 10, or 15 μg/lane; Figure 1), and the mean ± SD protein concentration of samples was 6.61 ± 0.68 μg/μl. Samples were subjected to tandem mass tag (**TMT**) 10-plex (TMT Isobaric Mass Label Reagent Set, Thermo-Fisher Scientific) as previously described for colostrum (Tacoma et al., 2017), with modifications (Yang et al., 2011, 2018; Zhang et al., 2019). In brief, a total of 50 μg of protein from each sample in PBS pH 7.4, 7 *M* urea, 2 *M* thiourea, 0.1% SDS, and 0.1 *M* tetraethylammonium

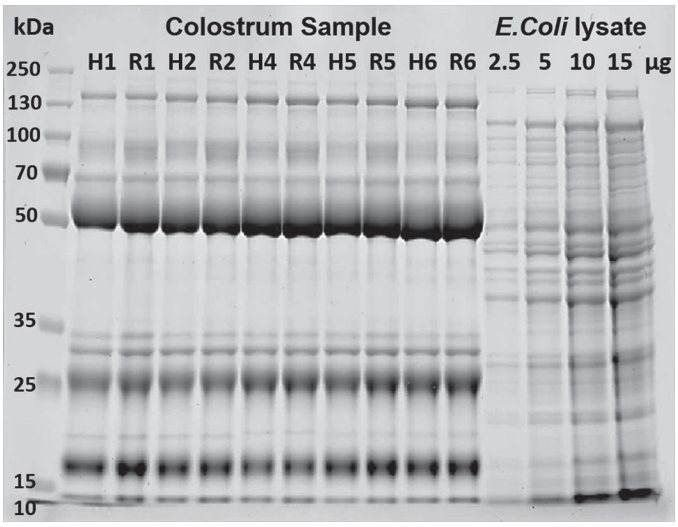


Figure 1. SDS-PAGE gel profiling of the 10 ultracentrifuged colostrum whey samples. Molecular mass ladder on the left; *Escherichia coli* lysate used for quantification on the right. Loading volume per lane = 1.5 μL. H = heat treated; R = raw. Stained with colloidal Coomassie blue. *E. coli* lysate standard curve $R^2 = 0.9981$.

bromide (TEAB) at pH 8.5 was reduced with 10 m*M* Tris (2-carboxyethyl) phosphine for 1 h at 34°C, alkylated with 18 m*M* iodoacetamide for 45 min at room temperature in the dark, and then quenched with a final concentration of 23 m*M* 1.4-dithiothreitol. Samples were diluted with 50 m*M* TEAB, pH 8.5, to a final concentration of 1 *M* urea. Each sample was digested with 5 μg of trypsin for 18 h (overnight) at 37°C, 1:10 wt/wt. Final sample volume was 63 μL.

The TMT 10-plex labels (0.4 mg of dried powder) were reconstituted with 33 μL of anhydrous acetonitrile (**ACN**) before labeling and added to each of the tryptic-digested samples (in 100 μL of 0.1 *M* TEAB, pH 8.5), incubated for 1 h at room temperature, and then the 10 digested samples were pooled together per set. The pooled peptides of each set were then evaporated to 150 μL and subjected to cleanup by solid-phase extraction on MCX cartridges (Waters Corp., Milford, MA) before first-dimensional liquid chromatography (**LC**) fractionation via high-pH reverse-phase chromatography.

High-pH reverse-phase fractionation was carried out using a Dionex UltiMate 3000 HPLC system with the built-in micro fraction collection option in its autosampler and UV detection (Thermo-Fisher Scientific) as reported previously (Yang et al., 2011). Specifically, the TMT 10-plex tagged tryptic peptides were reconstituted in buffer A (20 m*M* ammonium formate, pH 9.5 in water) and loaded onto an XTerra MS C18 column (3.5 μm, 2.1 × 150 mm; Waters Corp.) with 20 m*M*

ammonium formate (NH_4FA), pH 9.5 as buffer A, and 80% ACN/20% 20 m*M* NH_4FA as buffer B. The LC was performed using a gradient from 10 to 45% of buffer B in 30 min at a flow rate of 200 μL/min. Forty-eight fractions were collected at 1-min intervals and pooled into a total of 8 fractions based on the UV absorbance at 214 nm and with multiple fraction concatenation strategy (Yang et al., 2011). Each of the 8 fractions was dried and reconstituted in 150 μL of 2% ACN/0.5% formic acid for nano liquid chromatography-tandem mass spectroscopy (**LC-MS/MS**) analysis.

Nano-Scale Reverse-Phase Chromatography and Tandem MS (Nano LC-MS/MS). The nano LC-MS/MS analysis was carried out using an Orbitrap Fusion (Thermo-Fisher Scientific) mass spectrometer equipped with a nanospray Flex Ion Source, using high-energy collision dissociation, similar to previous reports (Yang et al., 2011, 2018; Zhang et al., 2019) and coupled with the UltiMate3000 RSLCnano (Dionex, Sunnyvale, CA). Each reconstituted fraction (6 μL) was injected onto a PepMap C-18 RP nano trap column (3 μm, 20 mm, Dionex) at 15 μL/min flow rate for on-line desalting, and separated on a PepMap C-18 RP nano column (2 μm, 75 μm × 25 cm). The labeled peptides were eluted in a 120-min gradient of 4% to 35% ACN in 0.1% formic acid at 300 nL/min, followed by an 8-min ramping to 95% ACN-0.1% FA and a 9-min hold at 95% ACN-0.1% FA. The column was re-equilibrated with 2% ACN-0.1% FA for 25 min before the next run. The Orbitrap Fusion was operated in positive ion mode with nano spray voltage set at 2.1 kV and source temperature at 275°C. For global proteomics fractions, the instrument was operated in data-dependent acquisition mode using FT mass analyzer for 1 survey MS scan for selecting precursor ions, followed by 3-s "Top Speed" data-dependent high-energy collision dissociation-MS/MS scans for precursor peptides with 2 to 8 charged ions with normalized collision energy of 40%. All data were acquired under Xcalibur 3.0 operation software and Orbitrap Fusion Tune 3.0 (Thermo-Fisher Scientific).

Data Processing and Protein Identification. All MS and MS/MS raw spectra from the TMT10-plex were processed and searched using Sequest HT software within the Proteome Discoverer 2.2 (PD2.2, Thermo-Fisher Scientific). The *Bos taurus* NCBI 2019 database, containing 41,064 sequence entries, was used for database searches. The search parameters included 2 mis-cleavages for full trypsin with fixed carbamidomethyl modification of cysteine, fixed 10-plex TMT modifications on lysine and N-terminal amines and variable modifications of methionine oxidation, deamidation on asparagine or glutamine residues, and protein N-terminal acetylation. Identified peptides were filtered for maximum 1% false discovery rate (**FDR**) using the Percolator algorithm in PD 2.2, along with additional peptide confidence set to high and peptide mass accuracy ≤ 5 ppm. The TMT10-plex quantification method within Proteome Discoverer 2.2 software was used to calculate the reporter ratios with mass tolerance ±10 ppm applying the isotopic correction factors. Only peptide spectra containing all reporter ions were designated as "quantifiable spectra" and used for peptide/protein quantitation, and only proteins with 2 or more peptides were considered for further analysis. A protein ratio was expressed as a median value of the ratios for all quantifiable spectra of the unique peptides pertaining to that protein. A precursor co-isolation filter of 50% was also applied for minimizing ratio compression caused by co-isolation of precursor ions.

Statistical Analysis

Sample size determination was based on the number of calves needed to enroll to achieve the objectives of the companion paper (Mann et al., 2020). Experimental outcomes included differences between groups in colostral IgG, IgA, IGF-I, and insulin concentrations, correlations between Ig and Brix, as well as differences in the colostral whey proteome.

Treatment differences in concentrations between all 11 pairs of colostrum samples were analyzed using paired *t*-tests or Wilcoxon signed-rank test in the statistical software JMP (v. 11.0, SAS Institute, Cary, NC). The relationship between Brix measurements and IgG as well as IgA concentrations in raw colostrum were analyzed using Spearman (Brix × IgG) or Pearson (Brix × IgA, IgG × IgA) correlations.

Proteomics analysis was carried out in a subset of 5 pairs of colostrum samples. A protein's abundance had to be recorded in all 10 samples to be considered for statistical analysis. Fold changes and FDR-adjusted *t*-tests for protein ratios were analyzed using MetaboAnalyst v. 4.0 software (Chong et al., 2018). Data were uploaded as paired data, no filtering was applied, data were log-transformed, and Pareto scaling was applied to achieve normalization. Box plots and kernel density plots were visually assessed for normality after transformation and scaling. Settings for analysis were as follows. Paired fold change analysis: at least 1.5-fold change in at least 4 out of 5 pairs; paired *t*-tests: FDR-adjusted *P*-value ≤ 0.01; paired volcano plot: at least 1.5-fold change in at least 4 out of 5 pairs, plus FDR-adjusted *P*-value ≤ 0.05. For the paired *t*-test analysis, average fold change

across all pairs was added. Principal component analysis was performed and 3-dimensional plots generated, and all figures were generated in MetaboAnalyst v. 4.0.

RESULTS

Parity of the enrolled cows ranged from 1 to 4 with a median of 3; 2, 2, 4, and 3 cows were in first, second, third, and fourth parity, respectively. The median Brix percentage from 11 batches of colostrum was 27.0 (range: 23.0–31.5).

SCC and Bacterial Culture

Somatic cell count and colostrum culture results for bacterial counts of the 22 samples are presented in Figure 2. Cultures for *Mycoplasma* spp., *Streptococcus agalactiae*, *Staphylococcus aureus,* gram-positive *Bacillus* spp., *Corynebacterium* spp., *Trueperella pyogenes*, and other organisms (*Prototheca* spp., yeast, mold, fungi) were negative for all samples. *Pseudomonas* spp. were detected in 2 samples of raw colostrum but not in the corresponding heat-treated samples. The median SCC in 11 raw colostrum samples was 470,000 (range: 300,000–1,300,000). Heat treatment was associated with a decline ($P = 0.01$) in SCC of 207,000 ± 68,000 cells (mean ± SE), corresponding to a median reduction of 36% (0–90%) in paired samples. Heat treatment was associated with a decline ($P = 0.001$) in total bacterial count of 13,162 ± 3,472 cfu/mL, corresponding to a median total bacterial count reduction of 93% (45–100%) in H versus R samples. When considering individual bacterial groups, heat treatment completely eliminated coliforms and other bacteria (mainly gram-negative *Bacillus* spp.) in the 9 and 10 out of 11 samples, respectively, in which they were present. *Streptococcus* spp. were eliminated in 6 of 11 samples and reduced by a median of 97% (0–99%) in the remaining 5 samples. The reduction of bacterial culture of *Staphylococcus* spp. by heat treatment was lowest among all measured types of bacteria with a median reduction of 60% (0–84%) compared with R of the 7 samples that contained *Staphylococcus* spp.

IgG and IgA Concentrations

Median concentration of IgG in the 22 samples was 97.2 (range: 69.7–189.0) mg/mL with a mean (±SE) difference of −1.7 ± 4.5 mg/mL in H versus R paired samples ($P = 0.36$), corresponding to a median reduction of 6.6% (0–16.0%) between pairs. Median concentration of IgA was 5.3 (3.3–9.4) mg/mL, and heat treatment led to a decline of IgA in paired samples of 0.72 ± 0.27 mg/mL ($P = 0.02$), corresponding to a median reduction of 8.5% (0–38%) between paired samples. Correlations were moderate between Brix measurements and Ig concentrations in R colostrum samples (Figure 3). Average sample CV for colostrum IgG and IgA were 2.64% (SD: 1.8) and 3.36% (2.5), respectively.

Immunoreactive Insulin and IGF-I

Median concentrations of insulin in colostral whey of all 22 samples was 855 (366–1,847) μIU/mL. Heat treatment decreased (mean ± SE) insulin concentration in paired samples by 227 ± 40 μIU/mL ($P < 0.001$), corresponding to a median decrease of 22.2% (7.0–44.9%) in H compared with R.

Median concentration of IGF-I in colostral whey was 751 (340–1,478) ng/mL. The concentration of IGF-I in paired samples decreased ($P = 0.005$) due to heat treatment by 80 ± 25 ng/mL, corresponding to a median decline of 10.2% (0–18%) in H versus R.

Results of Proteomics Analysis

Identified Proteins. A total of 328 proteins were identified after quality control steps in all 10 colostrum samples submitted for proteomics analysis. The median number of distinct peptide sequences in the protein group was 5 (2–130). The median amino acid length of the identified proteins was 92 (414–4,649). To annotate identified proteins in all tables, the protein name was entered into the UniProt search function (www.uniprot.org), and biological function was extracted for each protein. Proteins were subsequently grouped according to overlapping functions.

Principal Component Analysis. The exploration of principal component (**PC**) scores showed a clear separation of paired samples according to treatments along PC2 (Figure 4). Furthermore, samples within a group showed clear differences with separation along PC1 and PC3, representing the variability between individual animals. The top 30 features that differed in abundance between the 2 treatments are depicted as a heatmap (Figure 5). A heatmap of all identified features in all samples can be found in Supplemental Figure S1 (https://doi.org/10.3168/jds.2020-18618).

Paired t-Test. A total of 75 proteins differed in the FDR-adjusted paired *t*-test at a *P*-value of ≤ 0.01. Of these proteins, 45 decreased in H compared with R (Table 1), of which 11 were complement proteins and 6 were classified as innate immune response or coagulation proteins. Table 2 shows the 30 proteins that increased in H compared with R, out of which 6 were protease inhibitors, 6 were whey proteins including casein, and 3 were acute-phase proteins.

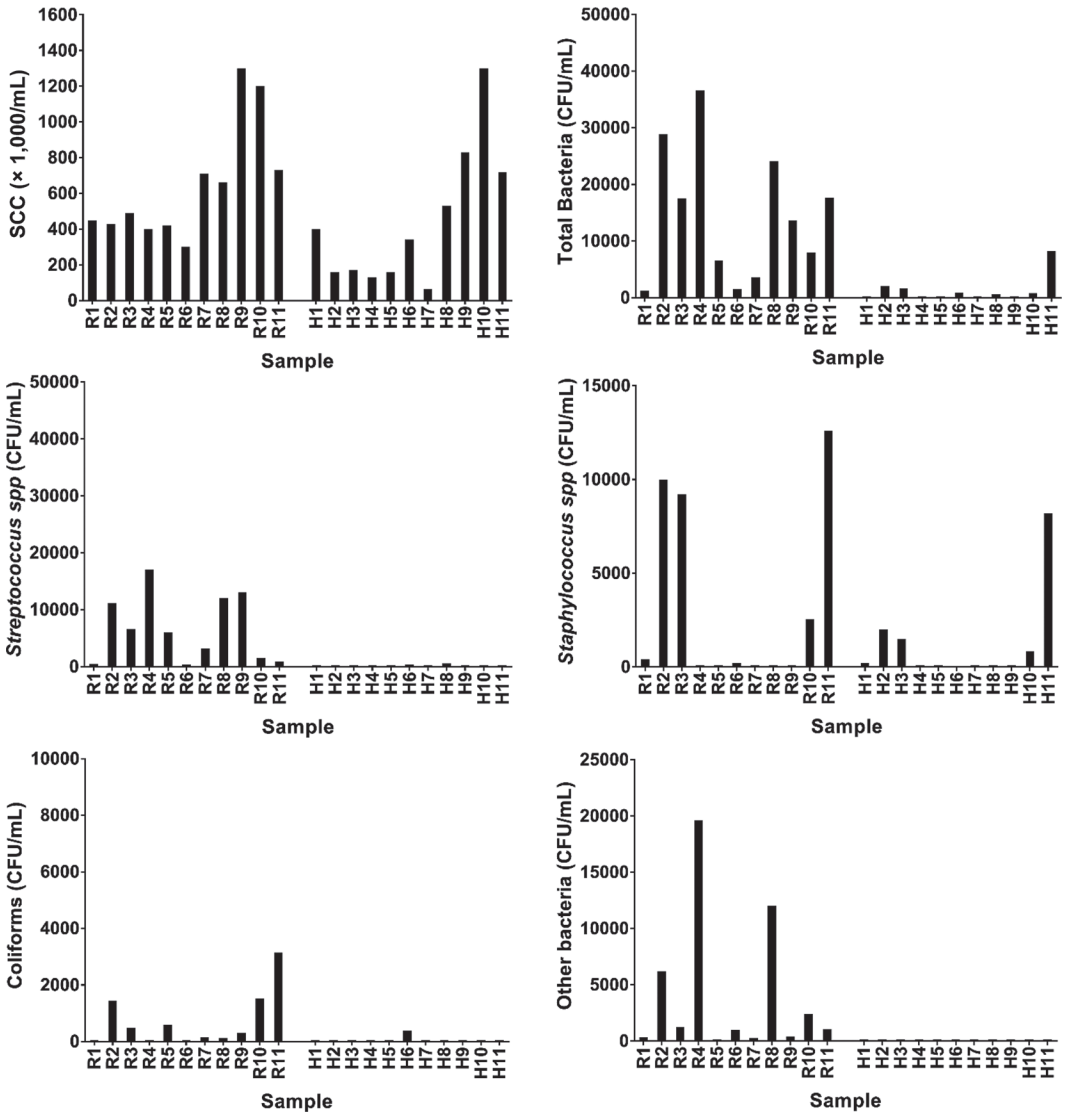


Figure 2. SCC (× 1,000/mL), total bacterial count (cfu/mL), *Streptococcus* spp. (cfu/mL), *Staphylococcus* spp. (cfu/mL), coliform count (cfu/mL), and other bacteria (cfu/mL) for 11 paired colostrum samples that were either heat treated for 60 min at 60°C (H; n = 11) or raw, refrigerated immediately after harvest (R; n = 11).

Volcano Plot (Combined Fold Change and Paired t-Test) and Fold Change. When we explored fold change between groups and differences in paired *t*-tests, H was associated with 16 proteins decreased by at least 1.5-fold in all paired samples and an additional 9 proteins decreased by at least 1.5-fold in 4 sample pairs (Figure 6). None of the identified proteins increased by at least 1.5-fold in H. Among the 25 total proteins decreased by at least 1.5 or higher fold change, 9 were identified as complement proteins.

When both analyses were combined in a Volcano plot, the same 25 proteins shown in Figure 6 remained in the analysis (Supplemental Figure S2, https://doi.org/10.3168/jds.2020-18618). These are shown with their respective FDR-adjusted *P*-value in Supplemental Table S1 (https://doi.org/10.3168/jds.2020-18618).

DISCUSSION

This study describes the effect of heat treatment according to current common practices on a commercial dairy farm using single-use colostrum bags and a commercial pasteurizer. The most recent comprehensive study in the United States showed that this practice has been adopted on 8.7% of 104 dairy operations in 13 states included in convenience sampling from 2014 to 2015 (Urie et al., 2018). Raw colostrum quality, as qualified by IgG concentrations (≥50 g/L) and bacte-

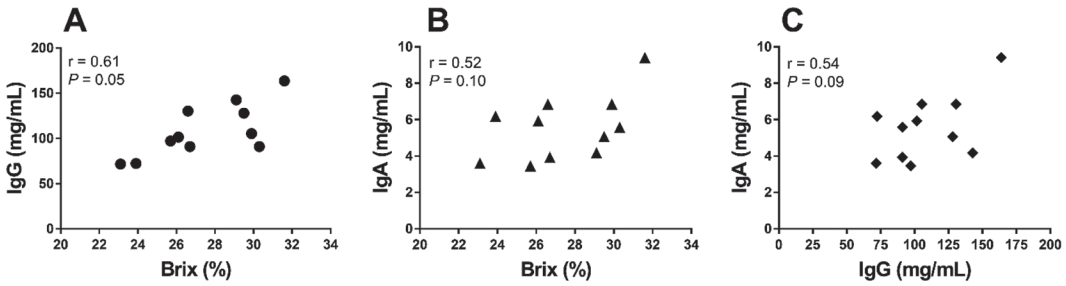


Figure 3. Relationship (correlation coefficient r; *P*-value) between Brix measurements (%), determined by digital refractometer, and IgG and IgA concentrations, determined by radial immunodiffusion, in raw bovine colostrum samples (n = 11).

rial counts, were considered adequate for all samples (Godden et al., 2019). The mean of approximately 93 g/L IgG for the colostrum sample were comparable to the average reported in a large Irish study (Conneely et al., 2013), but concentrations were higher on average than reported in a recent nationwide US survey (Morrill et al., 2012).

Bacterial Contamination

None of the colostrum samples exceeded industry goals for a maximum of 100,000 cfu/mL for total plate count, 10,000 cfu/mL for total coliform count, 50,000 cfu/mL for streptococci, and 50,000 cfu/mL for staphylococci (McGuirk and Collins, 2004) at time of feeding. Bacterial culture in this study targeted the most common contaminants of colostrum rather than specific pathogens. We confirmed that heat treatment resulted in reduction of total bacterial count and *Streptococcus* spp., coliform, and gram-negative *Bacillus* spp. counts, but was not as efficient in reducing *Staphylococcus* spp. to the same degree. This is consistent with findings by Rebelein (2010) in bovine colostrum heat-treated in a batch pasteurizer, and highlights that staphylococci in colostrum might be more heat-tolerant than other bacteria. Elizondo-Salazar et al. (2010) also found an incomplete reduction in staphylococci; however, at 60°C heating for 60 min, streptococci were even more resistant to killing.

SCC

Colostrum from uninfected glands was found to have a geometric mean SCC of 891,000/mL (Maunsell et al., 1998). The median SCC of our samples was well below this previously reported mean, with only 2 samples having higher SCC. Somatic cell counts, reflecting the maternal leukocyte populations and epithelial cells in colostrum, were reduced drastically due to heat treatment. In this study, we did not investigate the viability and composition of colostral leukocytes before and after heat treatment. Heat exposure may differentially affect individual immune cell types; however, Godden and colleagues (2019) reported that heat treatment killed most colostral leukocytes. Given recent data that suggests an important biological role for maternal leukocytes in the newborn calf (Donovan et al., 2007) and influence on lymphocyte development in the neonate (Reber et al., 2008), the effect of heat treatment on maternal leukocytes in colostrum should be further elucidated.

Immunoglobulins

Few studies are available describing colostrum IgA concentrations. Median colostral concentrations of IgG and IgA reported here were 30 and 20% greater, respectively, than those previously reported (Mach and Pahud, 1971). Apparent differences may reflect normal variability between herds or may be reconciled by differences in sample preparation. Where we analyzed whole colostrum, Mach and Pahud (1971) analyzed defatted and casein-depleted whey.

Conclusions of previous studies regarding loss of Ig in heat-treated colostrum are inconsistent. In one study, the effect of IgG loss when heating colostrum to 60°C depended on the quality of colostrum, with no decreases observed in moderate-quality colostrum and approximately 1.2% loss seen in high-quality colostrum, defined as ≥73 mg/mL IgG (McMartin et al., 2006). Donahue et al. (2012) confirmed that no loss was observed for moderate-quality colostrum (50 to 59.9 mg/mL), whereas 8.8% loss was observed at ≥70 mg/mL, which increased to 9.8% loss at ≥80 mg/mL. The 6.5% loss of IgG observed here is similar to findings by Donahue et al. (2012), higher than those reported by McMartin et al. (2006), but significantly lower than those reported by Elizondo-Salazar et al. (2010), who found over 30% reduction in total IgG at 60°C for 60 min. In the study by Elizondo-Salazar, however, 15-mL aliquots of colostrum samples were heated in a laboratory water bath, which likely differs from heat treatment of larger volumes in designated containers used on

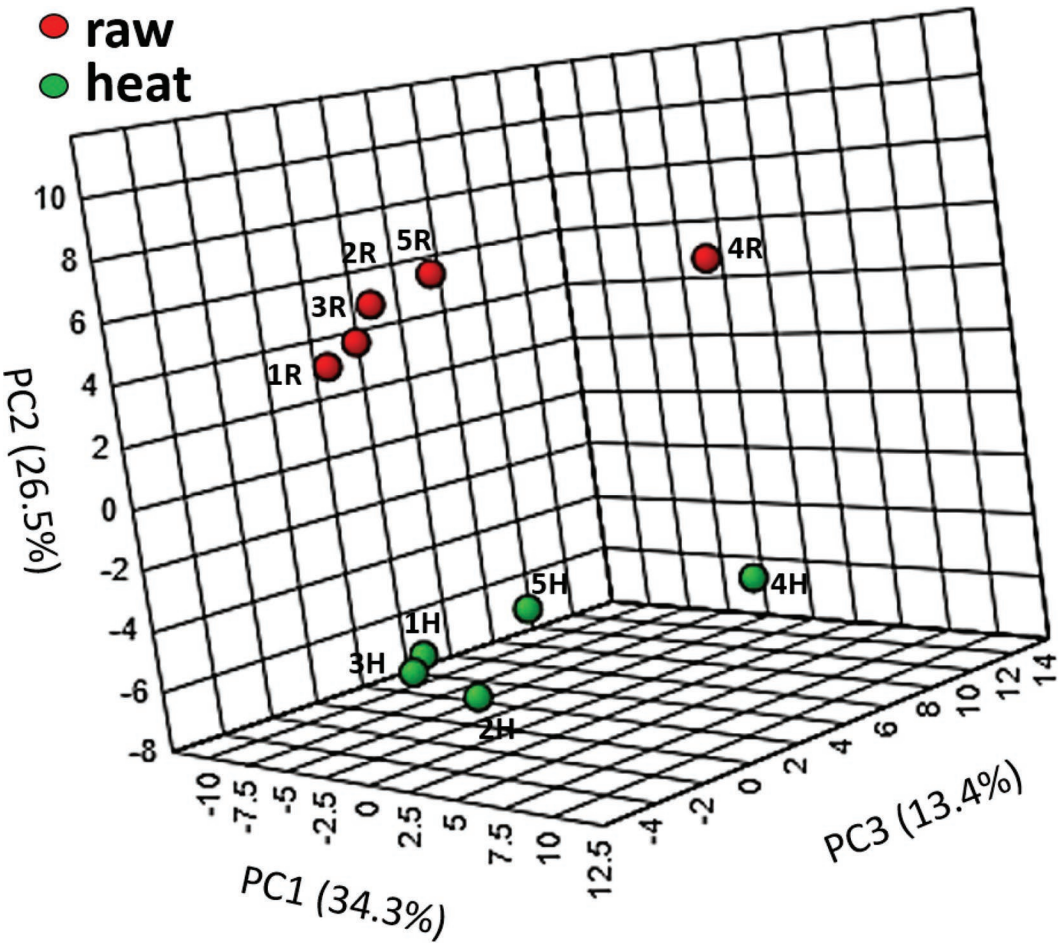


Figure 4. Principal component (PC) analysis showing the structure of the data that best explains the variance as a scatterplot of the eigenvectors and clear separation between pairs of colostrum samples (n = 5) according to treatments, as well as individual difference among individual samples. The first 3 PC explain 74.2% of the variability of the data. Heat-treated colostrum (H; n = 5) was heated at 60°C for 60 min; raw colostrum (R; n = 5) remained untreated before storage at −80°C.

farm. Our results confirm that IgA is less heat-tolerant than IgG (Mainer et al., 1997) in heat-treated bovine colostrum, albeit by a small increment that has yet to be determined as biologically significant. Although less is known about the role of colostral IgA in the bovine neonate compared with the role of IgG, colostral and milk IgA has an important role in intestinal health of other mammalian species, where IgA binds enteric pathogens, prevents bacterial colonization in the intestinal epithelium, and thereby avoids mucosal infections (Harris et al., 2006; Brandtzaeg, 2010; Hurley and Theil, 2011). Destruction of IgA by heat exposure may decrease the innate mucosal defense of the bovine neonate. Given the inconsistencies in results between the discussed studies, the effect of heat treatment on Ig concentrations in colostrum should to be revisited.

Correlations of Brix percentage and RID IgG measurements in raw colostrum were moderate (r = 0.61) and slightly lower than those reported by Elsohaby et al. (2018) or Quigley et al. (2013; r = 0.75). The correlation may have been underestimated in this study, considering the smaller sample set. Interestingly, colostral IgG and IgA correlated only moderately with each other. This may be due to IgA production stemming largely from plasma cells within the mammary tissue (Hurley and Theil, 2011), whereas IgG is mostly derived from serum via specific transport into colostrum (Larson et al., 1980).

Immunoreactive Insulin and IGF-I

Immunoreactive insulin and IGF-I were detected by RIA in colostral whey. We confirmed here that colostrum contains insulin at concentrations of approximately 1,000 IU/mL (Mann et al., 2016b), or 50 to 100 times higher than in circulating plasma of the dam

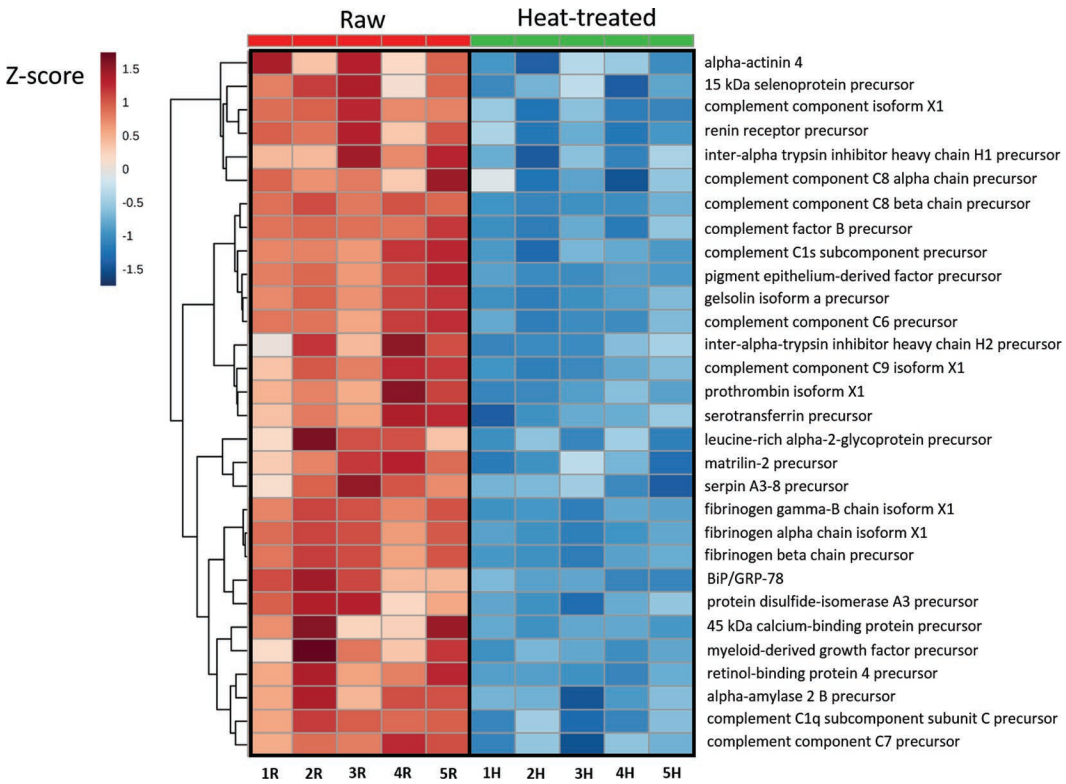


Figure 5. Heatmap of the top 30 proteins identified in liquid chromatography-tandem mass spectroscopy proteomics analysis, ranked by false discovery rate–adjusted *P*-values to differ in abundance between paired colostrum samples that were either heat treated (H; n = 5; 60 min at 60°C) or raw, refrigerated immediately after harvest (R; n = 5). Z-scores indicate values that range from low abundance (blue) to high abundance (red).

in the weeks preceding calving (Mann et al., 2016a). Concentrations of IGF-I are also higher in bovine colostrum, although concentrations reported here were approximately half of previous observations measured by RIA (Ronge and Blum, 1988; Hammon et al., 2013). This difference may have been caused by differences in analytical sensitivity, for example due to different antibodies used, or different degrees of success in blocking IGF-binding protein for adequate detection of IGF-I. Heat treatment at 63°C for 30 min has been shown to decrease both insulin and IGF-I concentrations in human colostrum by 46 and 39%, respectively (Goelz et al., 2009; Ley et al., 2011). To the best of our knowledge, the effect of common industry heat treatment practices for bovine colostrum on insulin and IGF-I concentrations has not been described in detail. Here, heating bovine colostrum at lower temperatures and for a longer time than that used for human colostrum was associated with a less-severe decline. Loss of measured concentrations was still obvious and of potential biological relevance at 22 and 10%, respectively, although the biological implications were not directly tested in this study. Given the role that these growth factors may play in gut maturation and nutrient absorptive function of calves (Ontsouka et al., 2016), the decline in the measured proteins may be of interest in future evaluations of successful colostrum programs.

Proteomics

This study represents one of the largest sample sets of the colostrum proteome with and without heat treatment to date, and allows us to explore the effects of heat treatment beyond the targeted analytes previously discussed. This novel data contributes to the advancement of our understanding of non-Ig low-abundant proteins in colostrum and the effect of a common management practice on their abundance. Initial PC analysis clearly showed clustering of samples according to treatment. Effects on decrease and increase of protein abundance due to heat treatment will be discussed separately later.

Naturally occurring proteins are often not thermally stable (Somero, 1995). It is therefore not surprising that a greater number of proteins decreased in abundance due to heat treatment compared with the number that increased. Components involved in local or systemic immune responses are of particular interest when considering possible biologic activity in the

bovine neonate. Heat treatment of human breast milk decreased cytokine and growth factor concentrations (Ewaschuk et al., 2011), and loss of immune factors through heat treatment is thought to have significant implications in human neonates. The most consistent and prominent effects of heat treatment in this study included decreases in complement components, fibrinogen, trypsin inhibitors, and several enzymes. Although they are considered low-abundant proteins, several of the proteins we identified that were reduced by heat treatment are found in higher concentrations in colostrum compared with milk (serotransferrin, complement components C9 and C3, factor B, and prothrombin; Zhang et al., 2015; Fahey et al., 2020). A previous study also found reduction of complement component C9 due to heat treatment (Tacoma et al., 2017). Complement components in particular have been described in several recent proteomic analyses of colostrum (Nissen et al., 2012; Nissen et al., 2017).

Complement components play a crucial role in the neonate's innate immune defense (Korhonen et al., 2000). In addition to microorganism killing and opsonization, complements also help to remove antibody-antigen immune complexes to tightly control immune activation

Table 1. Proteins (n = 45) identified in liquid chromatography-tandem mass spectroscopy proteomics with $P \leq 0.01$ of the false discovery rate (FDR)-adjusted paired *t*-test, that decreased in abundance in colostrum that was heat treated for 60 min at 60°C (n = 5), compared with paired samples left untreated and immediately refrigerated (n = 5)

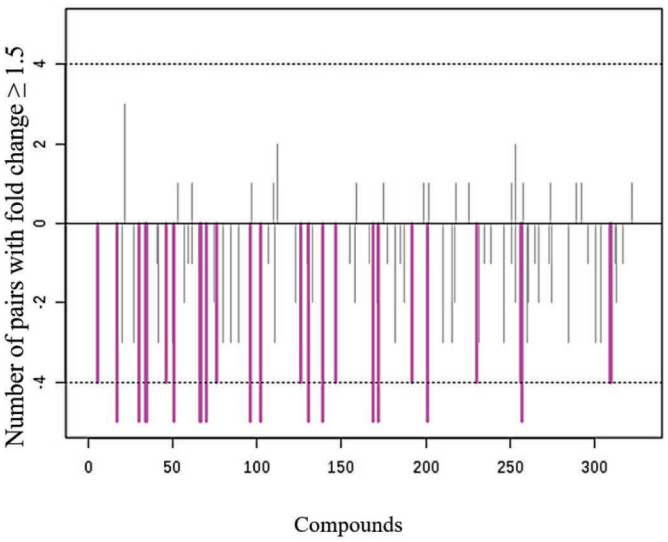
Biological function	Protein	FDR-adjusted *P*-value	Mean fold change all pairs
Complement (n = 11)	Complement C1q subcomponent subunit C precursor	0.0026	0.44
	Complement C1r subcomponent isoform X1 (predicted)	0.0026	0.62
	Complement C1s subcomponent precursor	0.0028	0.61
	Complement C2 isoform X1 (predicted)	0.0047	0.78
	Complement C5a anaphylatoxin precursor	0.0047	0.67
	Complement component C6 precursor	0.0023	0.42
	Complement component C7 precursor	0.0044	0.65
	Complement component C8 α chain precursor	0.0026	0.55
	Complement component C8 β chain precursor	0.0013	0.37
	Complement component C9 isoform X1 (predicted)	0.0026	0.56
	Complement factor B precursor	0.0013	0.45
Immune response/coagulation (n = 6)	Monocyte differentiation antigen CD14 isoform X1	0.0042	0.66
	Fibrinogen α chain isoform X1 (predicted)	0.0023	0.38
	Fibrinogen β chain precursor	0.0026	0.39
	Fibrinogen γ-B chain isoform X1 (predicted)	0.0023	0.34
	Prothrombin isoform X1 (predicted)	0.0026	0.37
	Coagulation factor V precursor	0.0100	0.75
Trypsin inhibitor (n = 4)	Inter-α-trypsin inhibitor heavy chain H1 precursor	0.005	0.63
	Inter-α-trypsin inhibitor heavy chain H2 precursor	0.0073	0.70
	Inter-α-trypsin inhibitor heavy chain H4 isoform X1	0.0028	0.58
	Inter-α-trypsin inhibitor heavy chain H4 isoform X2	0.0028	0.58
Enzyme (n = 4)	α-Amylase 2B precursor	0.0026	0.63
	Neutral α-glucosidase AB	0.0085	0.83
	L-Lactate dehydrogenase B chain isoform LDHBx	0.0026	0.69
	Fructose-bisphosphate aldolase A isoform X1 (predicted)	0.0070	0.65
Transport			
Iron	Serotransferrin precursor	0.0026	0.63
Lipids	Apolipoprotein A-IV isoform X1 (predicted)	0.0028	0.57
Steroid hormone	Sex hormone-binding globulin isoform X1 (predicted)	0.0084	0.89
Vitamin A	Retinol-binding protein 4 precursor	0.0026	0.50
Matrix protein/cytoskeleton (n = 4)	Matrilin-2 precursor	0.0026	0.64
	Collagen α-1XII chain isoform X1 (predicted)	0.0052	0.59
	Gelsolin isoform a precursor	0.0013	0.41
	Dystroglycan isoform X1 (predicted)	0.0078	0.86
Cell motility/adhesion (n = 2)	Leucine-rich α-2-glycoprotein precursor	0.0055	0.74
	Thrombospondin-1 precursor	0.0013	0.76
Protease inhibitor (n = 2)	Plasma serine protease inhibitor precursor	0.0044	0.71
	Serpin A3–8 precursor (serin protease inhibitor)	0.0084	0.80
Protein folding/ER stress (n = 2)	15 kDa selenoprotein precursor	0.0013	0.66
	Binding immunoglobulin protein (BiP) (GRP-78)	0.0026	0.62
Cellular receptor	Renin receptor precursor	0.0026	0.59
Membrane protein/folding	Neuropilin-2 isoform X1 (predicted)	0.0045	0.76
Redox signaling	Thioredoxin domain-containing protein 5 precursor	0.0028	0.75
Acetylgalactosamine transfer	β-1,4-N-acetylgalactosaminyltransferase 3	0.0026	0.72
Adipokine	Pigment epithelium-derived factor precursor	0.0017	0.46
Growth factor	Myeloid-derived growth factor precursor	0.0079	0.52

(Ricklin et al., 2010). Complement components are degraded by trypsin, but this can be prevented by trypsin inhibitors, which are naturally abundant in colostrum (Brock et al., 1975). The confirmation of abundance of different trypsin inhibitors in bovine colostrum in this study and others (Piñeiro et al., 1975; Zhang et al., 2015), as well as our finding of reduced abundance of trypsin inhibitors due to heat treatment is therefore of interest. Trypsin inhibitors reduce proteolysis and are thought to protect colostrum components from proteolytic cleavage, to preserve their activity and absorption through the neonatal gut (Hernández-Castellano et al., 2014). Notably, exogenous trypsin decreases the natural bactericidal activity of bovine colostrum (Brock et al., 1975). The bactericidal capacity of heat-treated human colostrum is lower than that of untreated colostrum (Wesolowska et al., 2019), but the specific role of complement components has not been investigated to our knowledge. Fibrinogen is also present in colostrum at concentrations higher than those of mature milk (Yamada et al., 2002), and may have a beneficial role in enhancing the neonate's immune system efficiency (Hernández-Castellano et al., 2014). The importance of our findings warrants further investigation regarding the biological significance of reduced abundance of complement and other immunologically active components for the neonatal calf.

Although none of the detected proteins showed at least a 1.5-fold increase between pairs, several proteins were identified in paired *t*-test as elevated in heat-treated colostrum. Although it is biologically implausible to increase protein due to heat in an environment that does not allow for evaporation, the effect may have been caused by a relative increase in heat-stable proteins due to the decrease of other (heat-labile) proteins under heat treatment, keeping the total amount of analyzed protein the same (Tacoma et al., 2017). Another plausible mechanism is that protein might be spared from degradation due to loss of substrates or inhibitors that would normally lead to a natural decline (e.g., increase in protease inhibitors due to heat inactivation of proteases). Alternatively, release of protein from a bound form to one that may have facilitated detection, such as from the milk fat fraction, from an intracellular location, or released from lipoproteins, could be a possible explanation (Tacoma et al., 2017). This could explain the relative increase in serum amyloid-3, an acute-phase protein expressed in mammary cells (McDonald et al.,

Table 2. Proteins (n = 30) identified in liquid chromatography-tandem mass spectroscopy proteomics with $P \leq 0.01$ of the false discovery rate (FDR)-adjusted paired *t*-test, that increased in abundance in colostrum that was heat treated for 60 min at 60°C (n = 5), compared with paired samples left untreated and immediately refrigerated (n = 5)

Biological function	Protein	FDR-adjusted *P*-value	Mean fold change all pairs
Protease inhibitor (n = 6)	α-2-Macroglobulin precursor	0.0028	1.08
	Antithrombin-III precursor (serpin C1)	0.0041	1.12
	Serpin A3–1	0.0079	1.36
	Serpin A3–2 precursor	0.0028	1.24
	Serpin A3–3 precursor	0.0074	1.25
	Serpin A3–5 (predicted)	0.0039	1.23
Milk protein (n = 6)	β-Casein isoform X1 (predicted)	0.003	1.21
	β-Lactoglobulin precursor	0.0078	1.27
	Serum albumin precursor	0.0024	1.18
	Glycosylation-dependent cell adhesion molecule 1 precursor	0.0100	1.23
	α-S1-casein isoform X13 (predicted)	0.0044	1.15
	α-S1-casein isoform X7 (predicted)	0.0100	1.13
Acute-phase protein (n = 3)	α-1-acid glycoprotein precursor	0.0028	1.26
	Haptoglobin precursor	0.0075	1.24
	Serum amyloid A3 precursor	0.0052	1.40
Complement	Complement C3 isoform X1 (predicted)	0.0033	1.11
Enzyme: amine oxidation	Primary amine oxidase, liver isozyme precursor	0.0026	1.15
Enzyme: antimicrobial activity	Lactoperoxidase isoform X1 (predicted)	0.0049	1.17
Enzyme: peptide cleavage	Putative N-acetylated-α-linked acidic dipeptidase	0.0026	1.14
Ca homeostasis	Calumenin isoform X1 (predicted)	0.0074	1.21
Cell receptor	Immunoglobulin λ-like polypeptide 1 isoform X1	0.0074	1.14
Coagulation	Vitamin K-dependent protein S precursor	0.0013	1.25
Immune response	IgM precursor	0.0047	1.16
Protein transport	Golgi membrane protein 1 isoform X1 (predicted)	0.0072	1.26
Unknown/unspecific function (n = 6)	HHIP-like protein 2 (predicted)	0.0042	1.18
	Neutrophil gelatinase-associated lipocalin (predicted)	0.0026	1.24
	Osteopontin isoform X1 (predicted)	0.0044	1.23
	Protein CutA isoform X1 (predicted)	0.0074	1.16
	Protein HP-25 homolog 1 precursor	0.0089	1.18
	Protein HP-25 homolog 2 precursor	0.0026	1.23



Biological function	Protein	Pairs with 1.5-fold decreased abundance in group H
Complement	Complement C1q subunit C precursor	5
	Complement component C6 precursor	5
	Complement component C8 α chain precursor	5
	Complement component C8 β chain precursor	5
	Complement component C9 isoform X1	5
	Complement factor B precursor	5
	Complement C1r isoform X1 (predicted)	4
	Complement C1s subcomponent precursor	4
	Complement component C7 precursor	4
Immune response/coagulation	Fibrinogen α chain isoform X1 (predicted)	5
	Fibrinogen β chain precursor	5
	Fibrinogen γ-B chain isoform X1 (predicted)	5
	Prothrombin isoform X1 (predicted)	5
Matrix protein/cytoskeleton	Alpha-actinin-4	4
	Collagen α-1XII chain isoform X1	4
	Gelsolin isoform a precursor	5
Transport: Vitamin A	Retinol-binding protein 4 precursor	5
Transport: Lipids	Apolipoprotein A-IV isoform X1 (predicted)	4
Transport: Iron	Serotransferrin precursor	4
Enzyme	L-lactate dehydrogenase A isoform X1 (predicted)	4
	Alpha-amylase 2B precursor	4
Growth factor	Myeloid-derived growth factor precursor	5
Adipokine	Pigment epithelium-derived factor precursor	5
Cellular receptor	Renin receptor precursor Bos taurus	5
Trypsin inhibitor	Trypsin inhibitor heavy chain H4 isoform X2	5

Figure 6. Fold change analysis of colostrum pairs analyzed by liquid chromatography-tandem mass spectroscopy proteomics analysis that were either heat treated for 60 min at 60°C and then refrigerated (H; n = 5) or raw, refrigerated immediately after collection (R; n = 5). Left-hand panel: The y-axis depicts the number of pairs with ≥1.5-fold change between pairs and the direction of fold change in H vs. R. (Negative values show pairs with a negative fold change for each protein; positive values show number of pairs with a positive fold change for each protein.) None of the listed proteins had a ≥1.5-fold increase in any pair. For a protein to be considered in this analysis, the minimum number of pairs with a fold change of ≥1.5 was set at 4. Right-hand panel: Table showing identified proteins in fold change analysis with numbers of pairs in each direction of change.

2001) and concentrated in bovine colostrum (Fahey et al., 2020).

A limitation of this study is that we focused on analysis of the defatted, ultracentrifuged whey proteome. According to Nissen et al. (2012), the sample preparation technique employed here would yield the highest number of unique proteins among all fluid fractions, and our study identified a total of 328 proteins in all 10 whey samples, which represents a large number compared with previous studies and increases the internal validity of our findings. This makes us confident in the chosen sample preparation and analysis. Nevertheless, we were unable to explore proteins associated with the lipid fraction of colostrum in the current study setup. Furthermore, it would be desirable to study the effects of heat treatment on a larger number of colostrum samples, including those of lower quality. Lastly, we focused on describing the abundance of a large number of colostral proteins following heat treatment, by direct measurement or proteomics approach, but we did not test whether the measured proteins retained their biological activity. It is therefore possible that biological function of the various molecules is impaired to a different extent by heat exposure than changes in abundance alone would indicate.

CONCLUSIONS

The findings discussed herein confirm that heat treatment of bovine colostrum succeeds in reducing viable bacteria but also decreases the abundance of certain immunological and growth factors, as well as the abundance of enzymes in the process. These include insulin, IGF-I, IgA, complement proteins, fibrinogen, and trypsin inhibitors. The immunological effects on the gastrointestinal and possibly systemic defense and growth-promoting mechanisms of newborn calves following heat treatment of colostrum in light of these

findings should extend beyond the consideration of Ig and transfer of passive immunity. However, given that the benefit of heat treatment lies in the control of bacterial contamination and specific infectious agents, the desire to control these challenges may be more critical for certain herds. Extending our knowledge, particularly of the role of complement proteins in the initial immune response of the newborn, is warranted in light of our findings, and this knowledge is of potential interest for other mammalian species as well.

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