Original Research

Composition and Variation of Macronutrients, Immune Proteins, and Human Milk Oligosaccharides in Human Milk From Nonprofit and Commercial Milk Banks

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Abstract

Background: When human milk is unavailable, banked milk is recommended for feeding premature infants. Milk banks use processes to eliminate pathogens; however, variability among methods exists.

Research aim: The aim of this study was to compare the macronutrient (protein, carbohydrate, fat, energy), immune-protective protein, and human milk oligosaccharide (HMO) content of human milk from three independent milk banks that use pasteurization (Holder vs. vat techniques) or retort sterilization.

Methods: Randomly acquired human milk samples from three different milk banks ($n = 3$ from each bank) were analyzed for macronutrient concentrations using a Fourier transform mid-infrared spectroscopy human milk analyzer. The concentrations of IgA, IgM, IgG, lactoferrin, lysozyme, α-lactalbumin, α antitrypsin, casein, and HMO were analyzed by mass spectrometry.

Results: The concentrations of protein and fat were significantly ($p < .05$) less in the retort sterilized compared with the Holder and vat pasteurized samples, respectively. The concentrations of all immune-modulating proteins were significantly ($p < .05$) less in the retort sterilized samples compared with vat and/or Holder pasteurized samples. The total HMO concentration and HMOs containing fucose, sialic acid, and nonfucosylated neutral sugars were significantly ($p < .05$) less in retort sterilized compared with Holder pasteurized samples.

Conclusion: Random milk samples that had undergone retort sterilization had significantly less immune-protective proteins and total and specific HMOs compared with samples that had undergone Holder and vat pasteurization. These data suggest that further analysis of the effect of retort sterilization on human milk components is needed prior to widespread adoption of this process.

Keywords

breastfeeding, human milk, Human Milk Banking Association of North America, infant development, late preterm infant, milk banking

Introduction

The benefits to providing mother's own human milk to premature infants are vast and well documented in the literature and include improved neurodevelopmental outcomes (Koo, Tank, Martin, & Shi, 2014); decreased risk of late-onset sepsis (Patel et al., 2013), necrotizing enterocolitis (Meinzen-Derr et al., 2009), and retinopathy of prematurity (Manzoni et al., 2013); and improved feeding tolerance resulting in decreased costs (Ahrabi & Schanler, 2013; Assad, Elliott, & Abraham, 2016). Many of these benefits are attributed to the nonnutritive, bioactive components in human milk such as IgA, lactoferrin, lysozyme, α-lactalbumin, and lipoprotein lipase, as well as human milk oligosaccharides (HMOs; Ballard & Morrow, 2013). The effects of these bioactive components emphasize that human milk provides more than just nutrition; it provides protection. When mother's own milk is unavailable, banked human milk is the next best option for feeding premature infants and is recommended by the American Academy of Pediatrics (Johnston & Landers, 2012).

To ensure quality control and safety, nonprofit donor milk banks adhere to the guidelines of the Human Milk Banking Association of North America (HMBANA, 2015). Briefly, these guidelines include screening mothers for health, no drug or tobacco use, negative tests for bloodborne pathogens (HIV, HTLV, hepatitis B or C, and syphilis), no regular use of

specific medications or herbal supplements, and pasteurizing milk to ensure the elimination of pathogens and postpasteurizing culturing for bacterial growth (HMBANA, 2015). Commercial (for-profit) milk banks adhere to the HMBANA guidelines as above but also extend their testing to bacterial content (before heat treatment), recreational drugs, prescription drugs, milk adulteration, and additional pathogen testing (varies among commercial milk banks) (https://www.prolacta.com/Data/Sites/14/media/PDF/mcc-140044-prolacta-company-profile.pdf, http://www.medolac.com/safety–quality.html). In addition, one bank routinely analyzes milk for final nutrition content after processing, and if the caloric content is below 20 calories/ounce, additional human milk components are added to achieve 20 calories/ounce (Prolacta Bioscience Inc., Monrovia, CA, personal communication, 27 August, 2015). Another difference between nonprofit and for-profit commercial milk banks is how they compensate mothers for their milk. Human Milk Banking Association of North America nonprofit donor milk banks do not financially compensate donors; however, for-profit commercial milk banks are known to pay women for their human milk.

Nonprofit donor milk banks use the Holder pasteurization method (Updegrove, 2013), which entails heating human milk to 62.5ºC for 30 min, followed by rapid cooling. Although this is the current standard pasteurization process for donor milk banks in the United States, it is known that the macronutrient content as well as the activity and content of major bioactive components (immunoglobulins, lactoferrin, lysozyme, α-lactalbumin, and enzymes) are significantly reduced, whereas HMOs (Underwood & Scoble, 2015) and lactose are not affected (Vieira, Soares, Pimenta, Abranches, & Moreira, 2011). Alternate pasteurization methods have been proposed to destroy pathogens while also maintaining the structure and function of bioactive molecules in milk (Underwood & Scoble, 2015). Recently, a new commercially sterile banked human milk has become available with a shelf life of 3 years without refrigeration. This pooled banked human milk is sterilized using retort processing that is commonly used in the canning industry and uses high heat and high pressure and thus a much shorter treatment time. The pooled banked human milk is heated to 121ºC for 5 min, at 15 pounds per square inch above atmospheric pressure (*Co-op Donor Milk*, 2014). Compared with pasteurization methods that destroy heat-labile microorganisms, commercial sterilization decreases all microorganisms (including spores)

Key Messages

- Time, pressure, and temperature used during pasteurization or sterilization can influence human milk quality.
- Random milk samples that had undergone retort sterilization had significantly less total and specific human milk oligosaccharides and protective proteins compared with samples that had undergone Holder and vat pasteurization.
- Our findings are likely related to processing and reflect the individual variation of their donors.
- These data suggest the need for further evaluation of processing methods to ensure the highest nutritional and protective quality of banked human milk.

to undetectable levels. Currently, published data on the effects of retort sterilization on human milk bioactive proteins or HMOs are not available.

The objective of this study was to compare the macronutrient (protein, carbohydrate, fat, calculated energy), bioactive human milk protein (IgA, IgM, IgG, lactoferrin, lysozyme, α-lactalbumin, α antitrypsin, osteopontin, casein), and HMO content of pooled banked human milk from one donor milk bank that uses the Holder pasteurization method and two commercial milk banks—one that uses vat pasteurization (minimum of 63°C for not less than 30 min; details are proprietary) and one that uses retort sterilization. The secondary objective was to explore the intrasample variation for each milk component within each milk bank. We started with the null hypothesis that human milk processed by retort sterilization, Holder pasteurization, and vat pasteurization did not differ in content of nutrients and bioactive molecules.

Methods

Design

Three random samples from three different lots were acquired from one donor milk bank that uses the Holder pasteurization method and two commercial milk banks—one that uses vat pasteurization and one that uses retort sterilization for the comparison of the macronutrient (protein, carbohydrate, fat,

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Table 1. Description of Banked Human Milk Samples Used in This Study.

	Holder (*n* = 3)	Vat (*n* = 3)	Retort (*n* = 3)
Heat-treatment details	62.5°C × 30 min	63°C × ≥ 30 min	121°C × 5 min with 15 PSI
Milk bank	San Jose Mother's Milk Bank	Prolacta Milk Bank	Medolac Milk Bank
Volume/no. mothers per lot	6.3 L/2	1,200 L/250	3,785 L/200
Specified mother[a]	Yes	No	No
Homogenized	No	No	Yes
Unopened shelf-life	6 months, −20°C	2 years, −20°C	3 years, 23°C
Opened shelf-life	24 hours, 1°C-4°C	48 hours, 2°C-8°C	7 days, 2°C-8°C

Note. PSI = pounds per square inch.
[a]Yes = 11% milk from mothers delivering ≤ 36 weeks obtained at < 1 month of lactation mixed with 89% milk from mothers delivering at ≥ 37 weeks obtained in the first 2 months of lactation. No = pooled milk from a variety of donors (term and preterm, early and mature).

calculated energy), bioactive human milk protein (IgA, IgM, IgG, lactoferrin, lysozyme, α-lactalbumin, α antitrypsin, osteopontin, casein), and HMO content of pooled banked human milk. The intralot variation for each of the above molecules was also determined for each milk bank.

Setting

Three human milk samples from different lots were acquired between December 2014 and April 2015 from three milk banks that use different heat treatment methods: Holder pasteurized "hospital-grade" donor pooled milk (lots SC150217E, SC1502241, and SC1502244; Mother's Milk Bank, San Jose, CA), commercially available vat pasteurized pooled milk (lots CHM1410A, CHM1409A, and CHM1402B; Prolacta Bioscience Inc.), and commercially available sterilized pooled milk (lots 4059CA-1159-301, 4177CA-1421-305, and 4121CA-1439-305; Medolac Laboratories, Lake Oswego, OR).

Sample

General characteristics about the processing of milk by each milk bank are found in Table 1. The Holder pasteurized hospital-grade donor human milk was a mixture of 11% preterm milk (≤ 36 weeks at delivery + 1 month postnatal) and 89% term early mature milk (> 37 weeks, within 2 months postnatal) and included a small number of donors per lot, usually two (personal communication, 17 September 2015). The vat pasteurized and retort sterilized milk comprised a large volume of pooled milk from a large number of mothers, 200 to 250 per lot, without distinction of stage of lactation when milk was pumped (pooled volume of premature, early, and mature milk) (personal communication). One sample of unpasteurized human milk collected from a single donor, pumped at 6 months postnatal, was included as a reference.

Measurement

Macronutrient analysis by mid-infrared spectroscopy. The 10 human milk samples were thawed/warmed to 38°C using a water bath. Each sample was vortexed for 6 s and inverted 6 times to ensure uniform distribution. One-milliliter aliquots were removed for protein and HMO analyses. Total protein, carbohydrate, and fat were measured in triplicate for each sample using a Fourier transform mid-infrared spectroscopy human milk analyzer (Delta Instruments; Smilowitz, Gho, Mirmiran, German, & Underwood, 2014). The total energy was calculated from the measured carbohydrate, protein, and fat concentrations (9 kcal/g for fat and 4 kcal/g for protein and carbohydrate; Wojcik, Rechtman, Lee, Montoya, & Medo, 2009).

Protein analysis by mass spectrometry. Tryptic digestion and quantitation of milk proteins were performed as previously described (Wu, Ruhaak, & Lebrilla, 2016) with few modifications. For quantitation, lactoferrin, α-lactalbumin, IgA, IgG, IgM, α antitrypsin, and lysozyme standards were pooled together and used as a protein standard mixture. Briefly, milk proteins and the protein standard mixture were reduced with dithiothreitol, incubated for 50 min at 60°C in a water bath, and alkylated with iodoacetamide at room temperature in the dark prior to tryptic digestion. Digested proteins were purified with a C-18 96-well cartridge plate and peptides and glycopeptides were eluted with two volumes of 40% ACN in 0.1% TFA and one volume of 80% ACN in 0.1% TFA. The standard protein mixture was serially diluted in nano-pure water to obtain calibration curves for protein quantification. The tryptic digests were analyzed and quantified using an Agilent 1290 ultra-performance LC system coupled with Agilent 6490 triple quadrupole mass spectrometer. UPLC separation was done using an Agilent Eclipse plus C18 column. Milk protein abundances for lactoferrin, α-lactalbumin, IgA, IgG, IgM, α antitrypsin, and lysozyme are reported as concentrations (g/L) and osteopontin, β casein, κ casein, and α casein are reported as ion counts.

HMO analysis by mass spectrometry. The method used to measure HMOs by mass spectrometry is reported elsewhere (Xu et al., 2017). Briefly, the milk samples were defatted by centrifugation and proteins were precipitated out of solution. The supernatants containing mainly lactose and oligosaccharides

Table 2. Comparison of Mean and Relative Standard Deviations (%) of Macronutrient Composition of Human Milk Among the Different Banked Milk Groups.

	M (SD) [range]				Relative SD (%)		
	Retort	Vat	Holder	Human milk reference	Retort	Vat	Holder
	(n = 3)	(n = 3)	(n = 3)	(n = 1)	(n = 3)	(n = 3)	(n = 3)
Total protein (g/100 ml)	0.8 (0.04)ᵃ [0.74-0.82]	0.8 (0.01)ᵃ [0.76-0.77]	1.0 (0.1)ᵇ [0.9-1.11]	0.9	5.4	0.8	10.5
Fat (g/100 ml)	3.0 (0.6)ᵃ [2.3-3.3]	4.1 (0.2)ᵇ [3.9-4.6]	3.4 (0.9)ᵃᵇ [2.8-4.6]	4.3	19.2	4.8	26
Carbohydrate (g/100 ml)	7.0 (0.1) [7.0-7.2]	7.0 (0.1) [7.0-7.2]	7.2 (0.05) [7.2-7.3]	7.3	1.8	1.4	0.6
Calculated energy (kcal/ounce)	17.4 (1.7)ᵃ [15.5-18.6]	20.4 (0.4)ᵇ [20.0-20.8]	19.1 (2.5)ᵃᵇ [17.2-21.9]	21.5	9.6	2.0	12.8

Note. Different letter superscripts represent statistically significant differences among groups; $p \leq .05$.

were dried and reconstituted in water and directly injected for UHPLC/MRM-MS analysis. A human milk pool was prepared by mixing 20 unique term human milk samples from a previous study (Lewis et al., 2015; Smilowitz, O'Sullivan, et al., 2013; Smilowitz, Totten, et al., 2013). The pool was extracted in the same way as above and reconstituted to 2 ml. Half of the reconstituted pool sample was used to build calibration curves for HMO quantitation and the other half was cleaned up by solid phase extraction using porous graphitized carbon cartridges (PGC-SPE) to remove lactose and other impurities. Detection and quantitation of HMOs were performed using an Agilent 6490 triple quadrupole mass spectrometer equipped with an Agilent 1290 infinity LC system, and a Waters ACQUITY UPLC BEH Amide column (2.1 × 100 mm i.d., 1.7-μm particle size). Electrospray ionization was used as the ionization source and was operated in positive ion mode. The MS parameters optimized by Hong, Ruhaak, Totten, Smilowitz, German, and Lebrilla (2014) for oligosaccharide analysis were used in this study with some modifications. At least 31 compositions were monitored, representing more than 100 HMO structures based on our previously published libraries (Wu, Grimm, German, & Lebrilla, 2011; Wu, Tao, German, Grimm, & Lebrilla, 2010).

Data Analysis

Data are expressed as mean (standard deviation). The relative standard deviation ($SD / M \times 100$) was calculated for each sample set to demonstrate the intravariation among samples from the same source. Kruskal–Wallis one-way analysis of variance was used to determine significant differences of each milk component among the three milk bank groups. When Kruskal–Wallis identified that the distribution of any milk component was significantly different among the groups, the Mann–Whitney U test was performed to determine which groups were statistically different. Alpha was set at $< .05$ to define statistical significance.

Results

Macronutrient Concentrations

The total protein concentration measured as the total nitrogen concentration was significantly ($p < .05$) higher in the milk pasteurized by the Holder versus retort or vat methods. The fat concentration and calculated energy were significantly ($p < .05$) higher in the milk samples that were pasteurized by the vat when compared with the retort method but not different when compared with the Holder method. Pasteurization method did not influence the total carbohydrate concentration. The intrasample variation for protein (10.5%), fat (26%), and carbohydrate (0.6%) was the greatest for samples that were pasteurized by the Holder method, followed by retort (protein, 5%; fat, 19%; carbohydrate, 1.8%); they were the lowest for the vat method (protein, 0.8%; fat, 5%; carbohydrate, 1.4%) (see Table 2).

Bioactive and Nutritive Proteins

There were significant differences for the concentrations and abundances of bioactive proteins in milk acquired from the three milk banks. The concentrations of IgA, IgG, and IgM were the lowest in the milk samples sterilized by the retort method ($p < .05$). Concentrations and abundances of lactoferrin, α-lactalbumin, α-1-antitrypsin, and casein (α, β, and κ) were the lowest in the milk samples that were sterilized by the retort method and highest in the milk samples pasteurized by the Holder method ($p < .05$). Abundances of osteopontin were the lowest in the samples sterilized by the retort method compared with those pasteurized by the vat and Holder methods ($p < .05$). The lysozyme concentration was the highest in samples that were pasteurized by the vat method ($p < .05$). The intrasample variation for IgA, lysozyme, α1-antitrypsin, α-casein, and β-casein was the lowest (range = 3.4-6.7%) for the samples pasteurized by the vat method and the highest (range = 9.8-48.3%) for the samples pasteurized

Table 3. Comparison of Mean and Relative Standard Deviations (%) of Immune Protective Proteins of Human Milk Among the Different Banked Milk Groups.

	M (SD) [range]				Relative SD (%)		
	Retort	Vat	Holder	Human milk reference	Retort	Vat	Holder
	(n = 3)	(n = 3)	(n = 3)	(n = 1)	(n = 3)	(n = 3)	(n = 3)
IgA (concentrations as g/L)	0.19 (0.03)[a] [0.16-0.22]	0.38 (0.02)[b] [0.36-0.39]	0.32 (0.15)[ab] [0.18-0.49]	0.32	15.1	4.1	47.6
IgM (concentrations as g/L)	0.01 (0.001)[a] [0.01-0.012]	0.02 (0.002)[b] [0.02-0.024]	0.03 (0.005)[c] [0.026-0.036]	0.02	9.1	10.5	17.0
IgG (concentrations as g/L)	0.05 (0.01)[a] [0.04-0.06]	0.08 (0.01)[b] [0.07-0.09]	0.08 (0.02)[b] [0.06-0.09]	0.13	20.9	12.3	21.1
Lysozyme (concentrations as g/L)	0.05 (0.01)[a] [0.04-0.06]	0.09 (0.01)[b] [0.08-0.09]	0.03 (0.01)[a] [0.02-0.04]	0.05	24.3	6.7	48.3
Lactoferrin (concentrations as g/L)	0.5 (0.10)[a] [0.39-0.59]	1.12 (0.14)[b] [1.00-1.28]	1.45 (0.12)[c] [1.35-1.58]	1.15	20.3	12.5	8.1
α-lactalbumin (concentrations as g/L)	0.95 (0.06)[a] [0.88-1.00]	3.70 (0.58)[b] [3.03-4.09]	4.44 (0.10)[c] [4.36-4.55]	3.90	6.8	15.6	2.2
α-antitrypsin (concentrations as g/L)	0.01 (0.001)[a] [0.014-0.016]	0.02 (0.001)[b] [0.021-0.023]	0.03 (0.003)[c] [0.027-0.033]	0.04	9.7	5.4	10.3
Osteopontin (abundances as 1×10^4)	0.85 (0.058)[a] [0.787-0.903]	1.24 (0.13)[b] [1.12-1.38]	1.70 (0.76)[b] [1.15-2.57]	1.9	6.8	10.6	44.7
α-casein (abundances as 1×10^4)	0.93 (0.22)[a] [0.71-1.15]	1.9 (0.14)[b] [1.71-1.98]	2.7 (0.85)[c] [2.06-3.67]	2.0	24.1	7.5	31.1
β-casein (abundances as 1×10^5)	2.7 (0.14)[a] [2.55-2.83]	3.4 (0.12)[b] [3.26-3.46]	3.8 (0.38)[c] [3.47-4.22]	3.3	5.2	3.4	9.8
κ-casein (abundances as 1×10^4)	3.8 (0.44)[a] [3.28-4.11]	6.7 (0.22)[b] [6.48-6.92]	10.0 (0.52)[c] [9.63-10.6]	4.8	11.7	3.3	5.2

Note. Different letter superscripts represent statistically significant differences among groups; $p \leq .05$.

by the Holder method with the retort in between (range = 5.2-24.3%). The intrasample variation for α-lactalbumin (2%) and lactoferrin (8%) was the lowest in samples pasteurized by the Holder method. The intrasample variation for osteopontin was the lowest in samples pasteurized by the retort method (see Table 3).

Human Milk Oligosaccharides

The concentration of total HMOs was about 2-fold higher and approximately 50% higher in samples pasteurized with Holder compared with retort and vat methods, respectively ($p < .05$). The total HMO concentration in the samples that underwent vat pasteurization was similar to the human milk reference. In a similar way, HMOs containing fucose, sialic acid, and nonfucosylated neutral (without fucose and sialic acid) sugars were the lowest in the milk samples that were sterilized by the retort method and highest in the milk samples pasteurized by the Holder method ($p < .05$). As a percentage of total HMOs, sialylated HMOs were the lowest in the milk samples that were sterilized by the retort method and highest in those pasteurized by the Holder method. Conversely, percentage fucosylated HMOs were the lowest in the milk samples that were pasteurized by the Holder

method and highest in the retort sterilized samples. The specific HMO compounds that are targets of fucosyltransferases coded for by the Lewis gene [FUT3, α(1-3/4) linkages], such as 3-fucosyllactose (3'FL), lacto-N-fucopentaose II, III, V (LNFP II, III, V), and fucosyltransferase 2 (FUT2), or the secretor gene [FUT2, α(1-2) linkages], such as 2-fucosyllactose (2'FL), lactodifucotetraose (LDFT), and lacto-N-fucopentaose I (LNFP I) (Smilowitz, O'Sullivan, et al., 2013; Thurl, Henker, Siegel, Tovar, & Sawatzki, 1997), were not significantly different among the three milk banks. One of the three samples of the Holder pasteurized donor milk did not contain any 2'FL, LDFT, and LNFP I and thus was classified as nonsecretor milk (Smilowitz, O'Sullivan, et al., 2013; Thurl et al., 1997). Thus, the intrasample variation for the secretor HMOs 2'FL, LDFT, and LNFP I was the highest for the samples that were pasteurized by the Holder method (range = 87-94%). The greatest intrasample variability for total HMOs and sialylated (as both abundance and composition) and nonfucosylated neutral HMOs was for samples that were sterilized by the retort method (9.5-21%) (see Table 4).

An unexpected finding was the color of the retort sterilized donor milk. The color of the milk was noted to be a distinct tan, quite different from the white or cream color of the other samples, as shown in Figure 1.

Table 4. Comparison of Mean and Relative Standard Deviations (%) of Human Milk Oligosaccharides Among the Different Banked Milk Groups.

	M (SD) [range]				Relative SD (%)		
	Retort	Vat	Holder	Human milk reference	Retort	Vat	Holder
	(n = 3)	(n = 3)	(n = 3)	(n = 1)	(n = 3)	(n = 3)	(n = 3)
Total HMOs (concentrations as g/L)	6.64 (0.80)[a] [5.73-7.18]	8.24 (0.11)[b] [8.14-8.35]	12.58 (0.81)[c] [12.0-13.5]	8.05	12.0	1.3	6.5
Fucosylation (concentrations as g/L)	4.77 (0.44)[a] [4.27-5.09]	5.59 (0.14)[b] [5.46-5.74]	7.33 (0.79)[c] [6.50-8.07]	5.7	9.1	2.5	10.7
% Fucosylation	71.97 (2.33)[a] [70.4-74.6]	67.91 (1.22)[b] [66.5-68.7]	58.22 (4.26)[c] [53.4-61.5]	70.86	3.2	1.8	7.3
Sialylation (concentrations as g/L)	0.59 (0.12)[a] [0.45-0.67]	0.84 (0.03)[b] [0.82-0.88]	2.24 (0.28)[c] [2.00-2.55]	0.58	20.7	3.3	12.7
% Sialylation	8.87 (0.84)[a] [7.90-9.38]	10.25 (0.44)[b] [9.98-10.8]	17.78 (1.25)[c] [16.4-18.9]	7.15	9.5	4.3	7.0
NF-neutral (concentrations as g/L)	1.39 (0.27)[a] [1.07-1.54]	1.95 (0.11)[b] [1.84-2.07]	3.50 (0.60)[c] [2.95-4.15]	1.88	19.5	5.8	17.3
% NF-neutral	20.74 (1.73) [18.8-22.0]	23.68 (1.33) [22.6-25.2]	27.92 (5.35) [24.5-34.1]	23.67	8.3	5.6	19.2
2'FL (abundances as 1 × 10⁴)	6.21 (0.26) [5.93-6.42]	6.95 (0.90) [5.98-7.74]	5.44 (4.74) [0.0- 8.73]	5.36	4.1	12.9	87.2
LDFT (abundances as 1 × 10⁴)	1.13 (0.41) [1.08-1.16]	1.17 (0.24) [0.91-1.39]	1.26 (1.19) [0.01-2.36]	1.40	3.7	20.4	93.7
LNFP I (abundances as 1 × 10⁴)	2.08 (0.43) [1.63-2.48]	3.35 (0.38) [3.03-3.77]	2.26 (1.98) [0.0-3.68]	2.16	20.7	11.4	87.5
3'FL (abundances as 1 × 10⁴)	8.14 (0.76) [7.39-8.90]	8.93 (0.33) [8.67-9.30]	8.82 (1.46) [7.92-10.5]	9.01	9.2	3.6	16.5
LNFP II, III, V (abundances as 1 × 10⁴)	6.76 (0.79) [6.00-7.57]	7.17 (0.84) [6.63-8.13]	11.0 (1.43) [9.43-12.2]	8.83	11.7	11.6	13.0

Note. Different letter superscripts represent statistically significant differences among groups; $p \leq .05$. HMO = human milk oligosaccharide; 2'FL = 2-fucosyllactose; LDFT = lactodifucotetraose; LNFP = lacto-N-fucopentaose; 3'FL = 3-fucosyllactose; NF = non-fucosylated HMO.

Figure 1. Photograph of human milk samples provided by the milk banks used in this study. From left to right: Holder pasteurization, retort sterilization, and vat pasteurization.

Discussion

The macronutrient concentrations and calculated energy of banked human milk are dependent on many variables, such as the gestational age of the infant at birth (Gidrewicz & Fenton, 2014), lactation stage, and timing of milk collection, as well as maternal diet, nursing frequency, and the volume of milk produced (Nommsen, Lovelady, Heinig, Lönnerdal, & Dewey, 1991). Storage and processing also affect human milk macronutrient concentrations (Garcia-Lara et al., 2013). In the present study, we found significant differences for the macronutrient concentrations and calculated energy among the three milk banks. The protein concentration of the Holder pasteurized donor milk samples used in this study was similar to a previous analysis of donor milk macronutrient concentrations (Wojcik et al., 2009). Protein content was significantly greater in the Holder pasteurized donor milk samples, as this milk comprised both premature and early term milk in which protein content is generally higher (Gidrewicz & Fenton, 2014).

The fat concentration and calculated energy were significantly highest in the vat pasteurized milk samples and lowest in the retort sterilized milk samples. This is likely due to the former's manufacturing quality control for testing milk macronutrient concentration and adjusting the fat content to ensure that each lot delivers 20 calories/ounce. This is supported by the very low intrasample variation observed among the vat pasteurized milk samples. The lower concentrations of energy and fat in the retort sterilized samples were reported by Ding, Asula, and Tan (2015), in which mean energy and fat concentrations were 17.4 kcal/ounce and 3.0 g/dl, respectively, pre- and postprocessing. This could have clinical implications, given the challenges of achieving optimal growth in premature infants. Growth and tolerance were reported recently with the use of retort sterilized banked milk for premature infants with an average gestational age of 31 weeks (Serke & Greenwell, 2016). This abstract reported a mean weight gain of 16.3 g/kg/day when 27 infants were fed 71% of their feedings as sterilized donor human milk. This weight gain velocity meets the minimum goal for intrauterine growth (15 g/kg/day; Ehrenkranz et al., 1999); however, it does not meet the optimal weight gain goal of ≥ 18 g/kg/day to support improved neurodevelopmental outcomes as reported by Ehrankranz et al. (2006).

The purpose of using donor milk for premature infants is to provide an alternate source of immunoprotection when the infant's own mother's milk is not available. Thus, the donor milk chosen should deliver high concentrations of immunoprotective proteins. This study demonstrated that the majority of immunoprotective proteins measured were significantly lower in the retort sterilized milk compared with the vat or Holder pasteurized donor milk. Premature infants are born with an immature acquired immunity and thus rely on maternal antibodies for defense against pathogens. Immunoglobulins are known to be heat sensitive, with IgM being the most affected by thermal treatment (Goelz et al., 2009). The immunoglobulin concentrations of the retort sterilized donor milk samples were significantly less, with IgM being most affected. It is possible that this is due to thermal degradation during the retort sterilization process. Hurley and Theil (2011) found significant reductions in bovine IgG when heated to 95°C for 15 s. Lactoferrin is one of the major whey proteins found in human milk and has important antimicrobial, antioxidant, and anti-inflammatory actions (Rollo, Radmacher, Turcu, Myers, & Adamkin, 2014). Our data showed about 2- to 3-fold less lactoferrin in retort sterilized milk compared with vat and Holder pasteurized milk. The low lactoferrin level in the retort sterilized donor milk group is likely due to the oxidation of lactoferrin caused by the high thermal treatment, as the denaturation temperature of lactoferrin is 67°C with a peak at 71.9°C (Mata, Sánchez, Headon, & Calvo, 1998). Mata and colleagues (1998) also showed that a 20-min heat treatment at 80°C reduces the lactoferrin content by up to 50%. The higher concentration of lactoferrin in the Holder pasteurized donor milk compared with vat pasteurized donor milk could likely be due to the high proportion of early term milk (Mehta & Petrova, 2011) used to make hospital-grade donor milk.

Alpha-lactalbumin is a major protein in human milk, making up approximately 25% to 35% of the total protein content (Lönnerdal & Lien, 2003). In addition to its important nutrition role, it also contributes to immune protection by binding and facilitating the uptake of antibiotics by gut bacteria and inhibiting bacterial reproduction (Marks, Clementi, & Hakansson, 2012). Our data show that α-lactalbumin in the retort sterilized donor milk was 74% to 78% less than vat and Holder pasteurized donor milk, respectively. Data from the dairy industry show that α-lactalbumin is very stable up to 80°C but is completely denatured with heat treatment of 96°C for 30 min (Larson & Rolleri, 1955). Based on data presented by Singh and Creamer (1991), holding human milk at a temperature of 121°C for 5 min would denature approximately 75% of the α-lactalbumin, which is consistent with our findings.

Caseins represent about half of the protein content in human milk and contribute to the antimicrobial activity of human milk, specifically κ-casein. We found significant lower abundances of β-casein and κ-casein in the retort sterilized versus vat pasteurized versus Holder pasteurized samples. Heat treatment of milk above 80°C can disrupt casein micelle structure through the interaction with denatured whey proteins. The lowest abundances observed in the retort sterilized samples are likely due to interactions between denatured whey proteins and caseins at the extreme temperature of 121°C.

Human milk oligosaccharides, the third most abundant component in human milk (~10-20 g/L; Chaturvedi et al., 2001; Coppa et al., 1993), are a group of complex sugars that are nondigestible by the human infant and support the competitive growth of protective bifidobacterial strains within the intestine (Zivkovic, German, Lebrilla, & Mills, 2011). In addition, HMOs act as receptor analogs to inhibit the adhesion of pathogens to the infant's epithelial surface and interact directly with host immune cells (Boehm & Stahl, 2007). The total HMO concentration and composition of all the banked, pooled milk samples were comparable with the reported normal range of 7 to 12 g/dl (Boehm & Stahl, 2007). However, the Holder pasteurized donor milk group had statistically higher total HMO and fucosylated, sialylated, and nonfucosylated neutral compositions compared with retort sterilized and vat pasteurized samples. This finding may result from the inclusion of early term milk (Chaturvedi et al., 2001; Coppa et al., 1993; Coppa et al., 1999) in the Holder pasteurized samples. Furthermore, our data are supported by other findings whereby Holder pasteurization did not affect total HMO concentration (Bertino et al., 2008). Another possible explanation for the low concentration of HMOs in the retort sterilized samples may result from a Maillard reaction, as observed by the brownish color of the retort sterilized milk.

The Maillard reaction is a chemical reaction between amino acids and reducing sugars, specifically lactose and lysine, and occurs at temperatures ≥ 120ºC (Van Boekel, 1998). A possible occurrence of a Maillard reaction in the retort sterilized donor milk is supported by reduced lysine content (~20%), as reported by the manufacturer (http://www.medolac.com/uploads/8/8/1/4/8814177/sm-17_rev_01_01.28.2015_amino_acid_retention_a_key_indicator_of_protein_quality.pdf). Yet, we did not measure Amadori products of the Maillard reaction between lactose at the reducing end of HMOs and lysine and thus cannot confirm that a Maillard reaction occurred during retort sterilization.

The percentage fucosylated HMOs was the lowest in the milk samples that were pasteurized by the Holder method, which is explained by the presence of one sample containing nonsecretor milk that was pasteurized by the Holder method that did not contain any 2'FL, LDFT, and LNFP I (the HMOs that contain 2'-fucosylated sugars). These findings are noteworthy as fecal bifidobacteria, which prefer consumption of 2'-fucosylated HMOs, have been shown to be established earlier postnatally and in more infants breastfed by secretor mothers compared with nonsecretor mothers (Lewis et al., 2015). The presence of one nonsecretor milk sample explained the high intrasample variation for the secretor HMOs in the milk samples that were sterilized by the retort method and highest in the milk samples pasteurized by the Holder method. In addition, the intrasample variation for sialylated HMOs was the highest in the retort sterilized samples. These observations may have clinical relevance, given evidence that fucosylated HMOs and sialylated HMOs differ in the capacity to shape the infant intestinal microbiota (Boehm & Stahl, 2007; Lewis et al., 2015; Yu, Chen, & Newburg, 2013) and brain development (Bode, 2012) and even influence growth and lean mass (Alderete et al., 2015).

Limitations

One major limitation of this study is that we were not able to determine the effect of heat treatment alone on milk composition and variation without the influence of other confounding variables (e.g., gestational age, lactation stage, number of donors). To do so, we would need to apply the different heat treatments used by each milk bank to multiple identical samples that are derived from one original sample (not feasible given the proprietary nature of the vat process). Despite this limitation, we did demonstrate important information about three milk banks that vary in their use of processing methods and currently provide milk to premature infants across the United States. The second limitation of this study is the small number of samples included per milk bank. As this was a pilot study to determine if there were any differences among the three milk banks and the variation among three random samples from each bank for macronutrients and bioactive compounds, further confirmatory studies are indicated. In particular, the effect of the novel retort sterilization on the

bioactive proteins and immune properties of human milk merits further investigation.

Conclusion

This is the first study to evaluate the macronutrient, bioactive protein, and HMO content of milk from a nonprofit milk bank and two commercial milk banks that provide pasteurized/sterilized human milk to premature infants in neonatal intensive care units. The observed lower concentrations of several macronutrients, bioactive proteins, and HMOs (total and sialylated) suggest rejection of the null hypothesis and the need for further study of the effect of retort sterilization on the nutrient content of sterilized donor human milk and possible Maillard reaction compounds and how these influence clinical outcomes in premature infants. This study highlights the beneficial effect of including early stage lactation milk done by the nonprofit donor milk bank, as it provides increased amounts of bioactive proteins and HMOs, and the potential benefit of pooled milk from large numbers of women to overcome the deficiencies in HMOs in milk from mothers with mutations in the FUT2 gene.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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