EFFECT OF PASTEURIZATION ON THE LIPID AND METABOLITE COMPOSITION OF DONOR HUMAN MILK

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INTRODUCTION

- ✓ Breastfeeding is the gold standard for infant nutrition.
- ✓ Preterm infants whose mothers are unable to provide sufficient own mother's milk, receive pasteurized donor human milk (DHM).
- ✓ We hypothesize that conventional pasteurization compromises DHM composition, and likewise its biological functionality.

OBJECTIVE

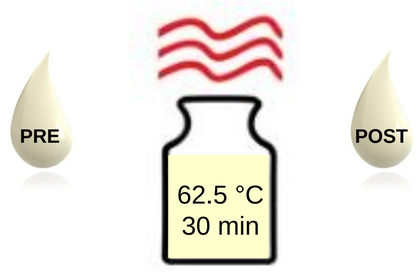
To assess the effect of conventional pasteurization on the composition and functionality of human milk (HM)

MATERIAL & METHODS

Donor Human Milk (DHM) samples

DHM samples provided by healthy volunteers admitted after the routine screening at the HM bank

Holder pasteurization



DHM aliquots (N=12) collected directly before and after Holder pasteurization

Sample preparation

1) Defrosting (33 °C, 10 min)

2) Single-phase extraction & dilution

+ 5 µL IS solution
+ 175 µL MeOH
+ 175 µL MTBE

45 µL HM/
H_2O (Blank extract)

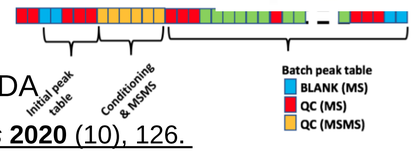
Untargeted analysis

UPLC-qTOFMS

- Agilent 1290 Infinity – iFunnel QTOF Agilent 6550 spectrometer
- Randomized sample order; QC samples analyzed every 6th sample and twice at beginning & end of batch; 3 blanks at the end of the sequence

MS² Data Dependent Acquisition (DDA)

- Untargeted DDA
- Untargeted iterated DDA
- Targeted dynamic iterated DDA



Ten-Doménech, I. *et al. Metabolites* **2020** (10), 126.

Data processing/analysis

MATLAB

Peak table extraction

xcms plus & CAMERA

Automated MS² peak annotation

hmdb (241952 MS²) · METLIN (25653 MS²) · LIPIDEX LipidBlast (*in-silico* ESI MS² spectral libraries of neutral and polar lipids)

Batch effect removal

QC-SVR (Quality Control-Support Vector Regression)

Kuligowski, J. *et al. Analyst* **2015** (140), 7810.

Filtering steps

i. Removal of features with RSD_{QC}% ≥ 20%
ii. Removal of features detected in blanks (i.e. features < 3x median area in blanks)

Network/pathway/enrichment analysis

MetDNA — Metabolite identification and Dysregulated Network Analysis

MetaboAnalyst 5.0

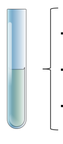
LION enrichment — Lipid Ontology

PIUMet — MIT BE BIOLOGICAL ENGINEERING

Reverse-phase (RP)	HILIC
ACQUITY BEH C18 column (2.1 mm × 50 mm, 1.7 µm)	SYNERGI-HYDRO C18 column (2.1 mm × 150 mm, 4 µm)
A: 5:1:4 IPA:MeOH:H_2O 5 mM NH_4Ac, 0.1% v/v HCOOH B: 99:1 IPA:H_2O 5 mM NH_4Ac, 0.1% v/v HCOOH	A: 99.8:0.2 H_2O:HCOOH B: ACN
ESI+ Raw data @ MendeleyData: DOI: 10.17632/fnzbxmkv83.1	ESI+ & ESI- Raw data: -

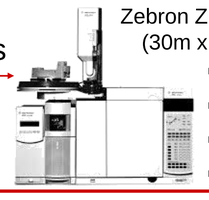
Fatty acid analysis (36 FAMEs by GC-MS)

Methanolic hydrochloric acid derivatization & GC-MS analysis



+ 250 µL HM
+ 300 µL IS in hexane
+ Derivatization reagents

FAMEs →



Zebron ZB-WAXPLUS column (30m x 0.25 mm, 0.25µm)

- **Injection volume:** 2 µL
- **Mode:** split
- **Gas flow:** Helium 1mL/min
- **SIM** ▪**EI:** -70eV

RESULTS & DISCUSSION

Data overview (untargeted analysis)

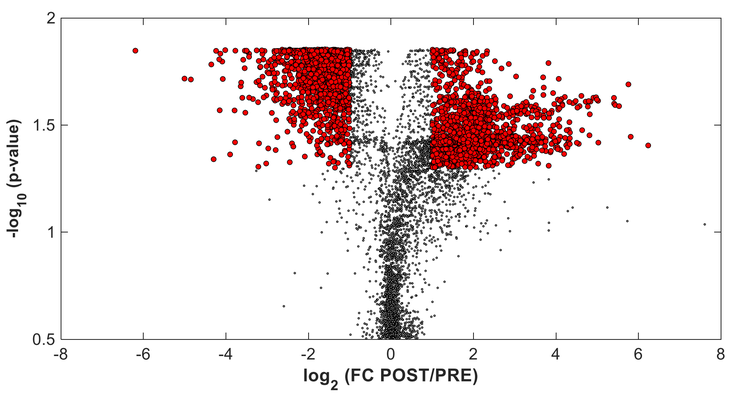
- ✓ Batch effect successfully corrected
- ✓ Tight clustering of QC samples/blanks
- ✓ Variance within HM samples > technical variance

*Shen X. *et al. Nature Communications*, **2019** (10), 1516.

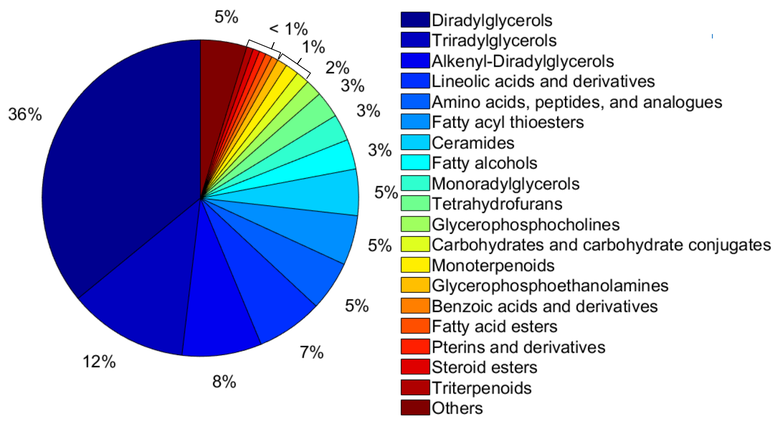
	RP	HILIC ESI+	HILIC ESI-
Total features	18401	1826	893
Total features after batch effect removal and filtering steps	7109	466	379
Annotated features by HMDB, Metlin or LipidBlast	704	16	5
Annotated features by MetDNA*	204	52	41
Total annotated features/ (%)	786 (11%)	50 (11%)	25 (7%)

Lipidomic analysis

- ✓ 3259 features with Fold Change >2 or <0.5 (p_{FDR}<0.05)



- ✓ 298 out of 3259 (9%) features are annotated



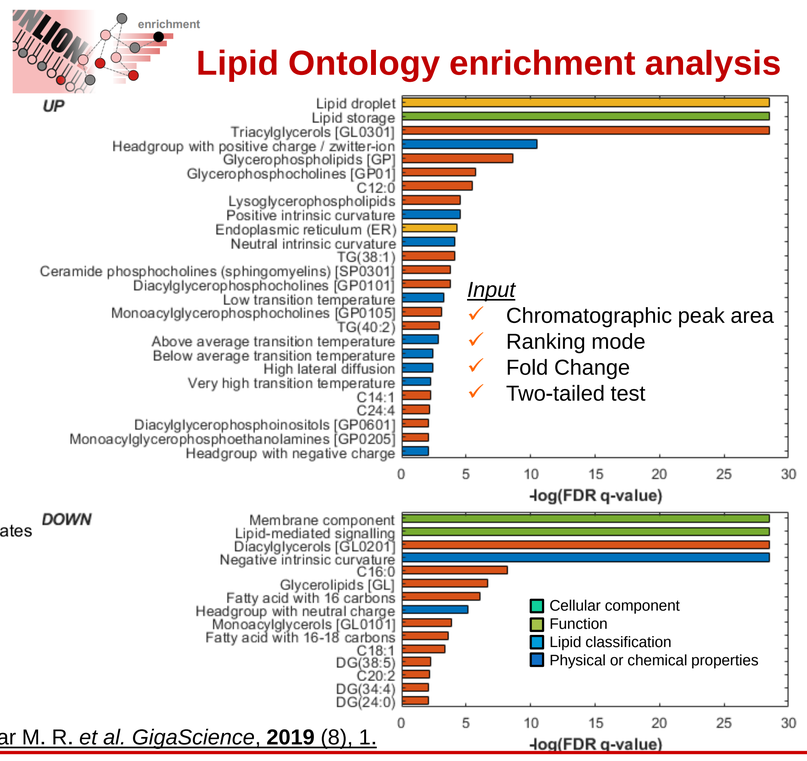
Molenaar M. R. *et al. GigaScience*, **2019** (8), 1.

MetaboAnalyst 5.0

Input
- ✓ t-test of 7109 features
- ✓ Accuracy 10 ppm
- ✓ Cut-off: p<0.005

Pathway name	Hits (total)	Hits (sig.)	p-value
Steroid hormone biosynthesis	6	4	0.017
Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	4	3	0.028
Linoleic acid metabolism	7	4	0.034
Biosynthesis of unsaturated fatty acids	17	7	0.036
Mucin type O-glycan biosynthesis	2	2	0.041

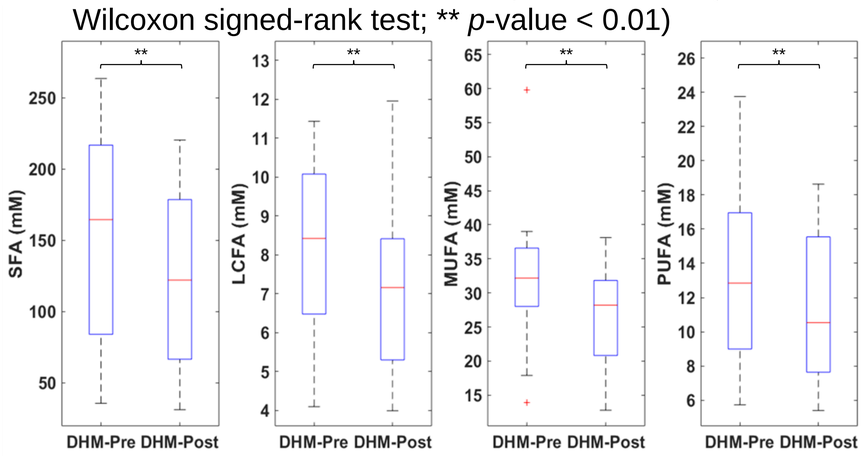
Lipid Ontology enrichment analysis



Input
- ✓ Chromatographic peak area
- ✓ Ranking mode
- ✓ Fold Change
- ✓ Two-tailed test

Fatty acid analysis

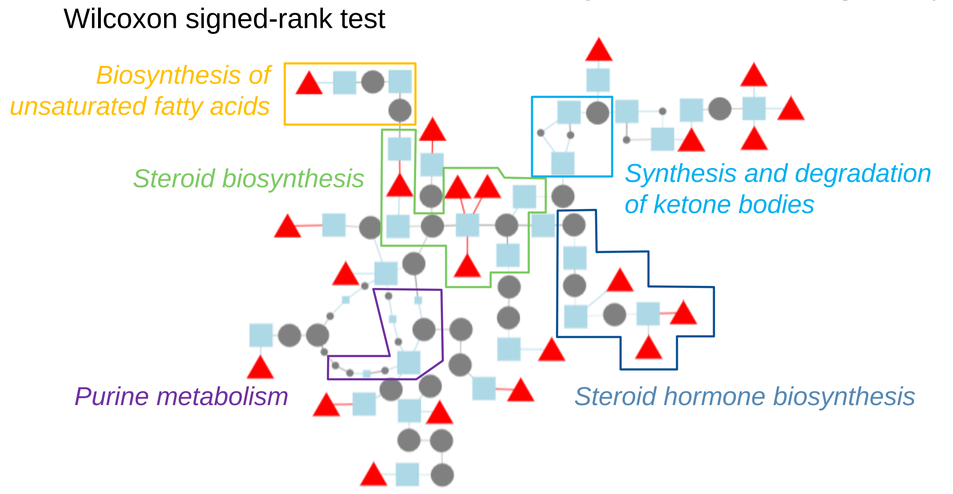
- ✓ 7 FAMEs < LOD
- ✓ Abundance of C6:0 > C10:0 > C8:0 > Oleic (C18:1n9c) > C12:0 > C16:0 > Linoleic (C18:2n6c) > others
- ✓ FAMEs' content decreased after pasteurization (One-tailed Wilcoxon signed-rank test; ** *p*-value < 0.01)



SFA (Saturated fatty acid)
LCFA (Long chain fatty acid)
MUFA (Monounsaturated fatty acid)
PUFA (Polyunsaturated fatty acid)

Integrative lipidomic and metabolomic analysis (PIUMet)

Input
- ✓ RP: 1033 features with significant difference (*p*<0.01) Wilcoxon signed-rank test
- ✓ HILIC ESI+ & ESI-: 35 features with significant difference (*p*<0.01) Wilcoxon signed-rank test



Pirhaji, L. *et al. Nature Methods*, **2016** (13), 770.

CONCLUSIONS & OUTLOOK

- ✓ More than 700 lipids and metabolites of different classes including triacylglycerols (TGs), diacylglycerols (DGs), phospholipids (PLs), sphingolipids, FAs, and carbohydrates, among others, have been annotated.
- ✓ Significant changes in HM composition before and after pasteurization, especially for DGs, TGs, alkenyl-DGs and lineolic acids and derivatives have been observed. FAs' content decreased after pasteurization.
- ✓ Due to pasteurization, several metabolic pathways seem to be altered (e. g. steroid hormone biosynthesis and the biosynthesis of unsaturated fatty acids).
- ✓ Lipid ontology enrichment analysis showed that the terms "lipid droplet" and "lipid storage", mainly associated with TGs, were enriched in pasteurized DHM, whereas the terms "membrane component" and "lipid-mediated signalling", associated with DGs, PLs, lyso-PLs and sphingomyelins, seemed to be more prominent in raw DHM.
- ✓ Future research aiming at a better conservation of DHM components and functionality is encouraged (e.g., High-Temperature Short-Time (HTST) pasteurization)

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