Food Chemistry 356 (2021) 129628

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Human milk metabolome is associated with symptoms of maternal psychological distress and milk cortisol

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ARTICLE INFO

Keywords:
^{1}H NMR
Cortisol
Human milk
Lactation
Metabolomics
Stress

ABSTRACT

The composition of human milk is subject to considerable variation, but the effects of maternal stress are largely unknown. We studied differences in human milk metabolome between Finnish mothers ($n = 120$, secretors) with symptoms of prenatal symptoms of psychological distress and milk cortisol concentrations. Human milk samples acquired at 2.5 months postpartum were analyzed using targeted ^{1}H NMR metabolomics. Self-reported scores for depression (EPDS), overall anxiety (SCL-90), and pregnancy-related anxiety (PRAQ) were used to evaluate psychological distress. Prenatal psychological distress was positively associated with concentrations of short-chain fatty acids, caprate, and hypoxanthine ($q < 0.0012$). Milk cortisol was positively associated with lactate concentration ($q < 0.05$). Changes in the human milk metabolome were shown to be associated with maternal psychological distress and concentration of milk cortisol in a dissimilarly, suggesting alterations in bacterial and energy metabolism of the mother, respectively.

1. Introduction

Human milk is a dynamic secretion providing nutrients and other bioactive metabolites for the infant during the first months of postnatal life (Ballard & Morrow, 2013; Smilowitz et al., 2013). The human milk metabolome is influenced by many factors including maternal genotype and ethnicity, gestational and lactational age, circadian rhythm, and maternal body mass index (BMI) as well as maternal lifestyle factors including diet (Ballard & Morrow, 2013; Gay et al., 2018; Sundekilde et al., 2016). Early evidence suggested that human milk would not be merely passive reflection of maternal concentrations of bioactive substances and nutrients, but an actively and timely regulated secretion programming immuno- and cognitive development of infants (Di Benedetto, Bottanelli, Cattaneo, Maria Pariante, & Borsini, 2019). Human milk composition might, therefore, have a significant impact on infant development and future health (Di Benedetto et al., 2019). Indeed, human milk composition is associated with infant growth (Lagström et al., 2020) and risk for non-communicable disease such as obesity

Abbreviations: CV, coefficient of variation; DSS-d_6, 3-(trimethylsilyl)-1-propanesulfonic acid-d_6; EPDS, Edinburgh Postnatal Depression Scale; FDR, false discovery rate; FL, fucosyllactose; HC, high milk cortisol; HMO, human milk oligosaccharide; HPA, hypothalamus-pituitaryadrenal; LC/LD, low milk cortisol low distress; LDFT, lactodifucotetraose; LNFP, lacto-*N*-fucopentaose; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetraose; MCFA, medium-chain fatty acid; NMR, nuclear magnetic resonance; NOESY, Nuclear Overhauser effect spectroscopy; PCA, principal components analysis; PEMT, phosphatidylethanolamine *N*-methyl transferase; PRAQ, Pregnancy Anxieties Questionnaire; SCFA, short-chain fatty acid; SCL-90, Symptom Checklist-90 anxiety subscale; SL, sialyllactose; SPS, sum of prenatal (symptom) scores; TCA cycle, tricarboxylic acid cycle.

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https://doi.org/10.1016/j.foodchem.2021.129628
Received 25 November 2020; Received in revised form 5 March 2021; Accepted 13 March 2021
Available online 20 March 2021

(Verduci et al., 2014) as well as cognitive (Helland, Smith, Saarem, Saugstad, & Drevon, 2003), and socioemotional development (Nolvi et al., 2018).

Perinatal psychopathologies, which are present in over 25% of mothers, may affect milk lipids, hormones and immune components (such as secretory immunoglobulin A, sIgA), and have the potential to impact neurodevelopment and health of infants even beyond infancy (Di Benedetto et al., 2019; Moirasgenti, Doulougeri, Panagopoulou, & Theodoritis, 2019). The hypothalamic–pituitary–adrenal (HPA) axis is the major neuroendocrine system mediating the physiological effects of stress and the measures of HPA axis functioning, including cortisol, are often used to measure stress. Dysregulation of HPA axis is often associated with psychopathology and it has been suggested that alterations in HPA axis functioning could mediate some of the effects of early life stress on the offspring health and development (Cottrell & Seckl, 2009). Milk cortisol concentration is correlated with maternal plasma cortisol (Patacchioli et al., 1992) and the transmission of glucocorticoids through milk may serve as a signaling mechanism though which mothers prepare their infants to the postnatal environment, as the glucocorticoids can influence behavior and modify brain development of the offsprings (Hahn-Holbrook, Le, Chung, Davis, & Glynn, 2016; Hinde et al., 2015; Hollanders, Heijboer, van der Voorn, Rotteveel, & Finken, 2017). The concentration of milk cortisol is associated with gestational weeks at birth, infant weight gain, and temperament (Hahn-Holbrook et al., 2016; Nolvi et al., 2017). Cortisol measurements (e.g. hair cortisol) show associations with some, but not all types of maternal psychological distress (Aparicio et al., 2020; Mustonen et al., 2019; Nolvi et al., 2017).

The current study is part of the FinnBrain Birth Cohort Study examining the effects of early life stress on child brain development and health (Karlsson et al., 2017). Here, the correlation between different measures of maternal stress (psychological and hormonal) and the human milk metabolome was studied for the first time. The aim was to whether perinatal symptoms of depression and anxiety are reflected in the human milk metabolome (the low-molecular weight metabolites) and how. In order to increase the understanding of the biological significance of milk cortisol, the association between milk cortisol and human milk metabolome was also investigated.

2. Subjects and methods

2.1. FinnBrain birth Cohort study

The study group consisted of 429 Finnish mothers who breastfed their infants, recruited from a larger FinnBrain Birth Cohort Study (www.finnbrain.fi) described by Karlsson et al. (2017). The FinnBrain Cohort Study is an ongoing transgenerational prospective observational study conducted at the University of Turku, Finland. The participants for FinnBrain pregnancy cohort were recruited from South-Western Finland, between 12/2011 and 04/2015 (n = 3,808). Self-report questionnaires were collected online or by mail three times during the pregnancy, as well as at 3 months postpartum. The FinnBrain questionnaire data was linked with data from the Finnish Medical Birth Register (FMBR; maternal pre-pregnancy BMI, duration of gestation, mode of delivery, infant sex, birth weight and length, use of anesthesia, induction, parity), maintained by the Finnish National Institute for Health and Welfare (THL; www.thl.fi), and from the register from Statistics Finland (education). Breast milk sample donors were recruited by emails and phone calls.

2.2. Ethics

The whole study protocol was approved by the Ethics Committee of the Hospital District of Southwest Finland. All participants gave written informed consent for their participation, data and sample use, and the study protocols were approved by The Joint Ethics Committee of University of Turku and Turku University Hospital (Breastfeeding study: 17.2.2013 §88 ETMK 121/1801/2013). Birth register data from the National Institute for Health and Welfare (THL) were used in this study.

2.3. Psychological distress

The symptoms of psychological distress were self-reported by the mothers at gestational weeks (gw) 14, 24, and 34, and at 3 months postpartum. The 10-item Edinburgh Postnatal Depression Scale (EPDS) (Cox, Holden, & Sagovsky, 1987), the Symptom Checklist-90 anxiety subscale (SCL-90) (Derogatis et al., 1973), and the 10-item Pregnancy Anxieties Questionnaire - Revised2 (PRAQ-R2) (Huizink et al., 2016) were used to assess symptoms of depression, overall anxiety, and pregnancy-related anxiety, respectively.

2.4. Milk cortisol

Milk cortisol was used here as a non-invasive marker of expressed glucocortinoid levels, as it has been shown to significantly correlate with plasma cortisol (Patacchioli et al., 1992). The hypothalamus–pituitaryadrenal (HPA) axis responsible for secreting cortisol has a distinctive diurnal rhythm, with a high cortisol peak in the morning that linearly decreases towards the night (Pundir et al., 2017). To avoid unwanted variation, breast milk was expressed in the presence of a study nurse at a controlled sample collection time. Milk samples were obtained as previously described (Nolvi et al., 2017) at 2.5 months postpartum, and stored at −70 °C until analysis. Milk cortisol assays were carried out at the Finnish Institute of Occupational Health using a validated luminescence immunoassay method (Nolvi et al., 2017).

2.5. Human milk samples

Out of 429 mother–infant dyads, a subset of samples for ^{1}H NMR analysis was drawn as follows: 1) 50 samples with the highest concentrations of milk cortisol; 2) 50 samples associated with the highest psychological distress (as per sum of prenatal symptom scores, SPS); and 3) 50 samples associated with both low concentration of milk cortisol and low psychological distress ("control") (Fig. 1). The EPDS and SCL-90 scores from gw 14, 24, and 34 were attributed to the sum of prenatal symptom scores. PRAQ questionnaire was included in the study protocol later during the sample collection leading to large number of missing values (58%) at gw 14 (Table 1), hence the PRAQ data was not included in the sum variable.

To increase statistical power, the samples were re-grouped based on the highest and lowest tertiles for milk cortisol and SPS to yield the following groups: 1) "high milk cortisol" (HC, n = 50), where milk cortisol ≥ 10.08 nmol/L and SPS = mean 20.6 ± SD 13.8; 2) "high distress" (HD, n = 53), where milk cortisol < 10.08 nmol/L and SPS = 51.8 ± 15.6; and 3) "low milk cortisol / low distress" (LC/LD, n = 40), where milk cortisol ≤ 7.69 nmol/L and SPS = 13.0 ± 8.3. Five out of the 150 samples were excluded for not fitting the in the three groups mentioned above (Fig. 1). One sample was excluded due to low volume and one due to contamination. One sample was excluded due to highly elevated concentrations of ketone bodies, resulting in 142 samples included in the ^{1}H NMR analyses.

2.6. ^{1}H NMR analysis

Milk samples were prepared for analysis according to the method described by Smilowitz et al. (Smilowitz et al., 2013). An aliquot of 180 μL, containing 10% of Chenomx internal standard solution with 3-(trimethylsilyl)-1-propanesulfonic acid-d_6 sodium salt (DSS-d_6) and 0.03% sodium azide (NaN_3) in D_2O, was placed in a 3-mm NMR tube. ^{1}H NMR spectra were acquired on a Bruker Avance 600 MHz NMR spectrometer equipped with a nitrogen-cooled PRODIGY TCI-cryoprobe and a SampleJet autosampler using a NOESY-presaturation pulse sequence

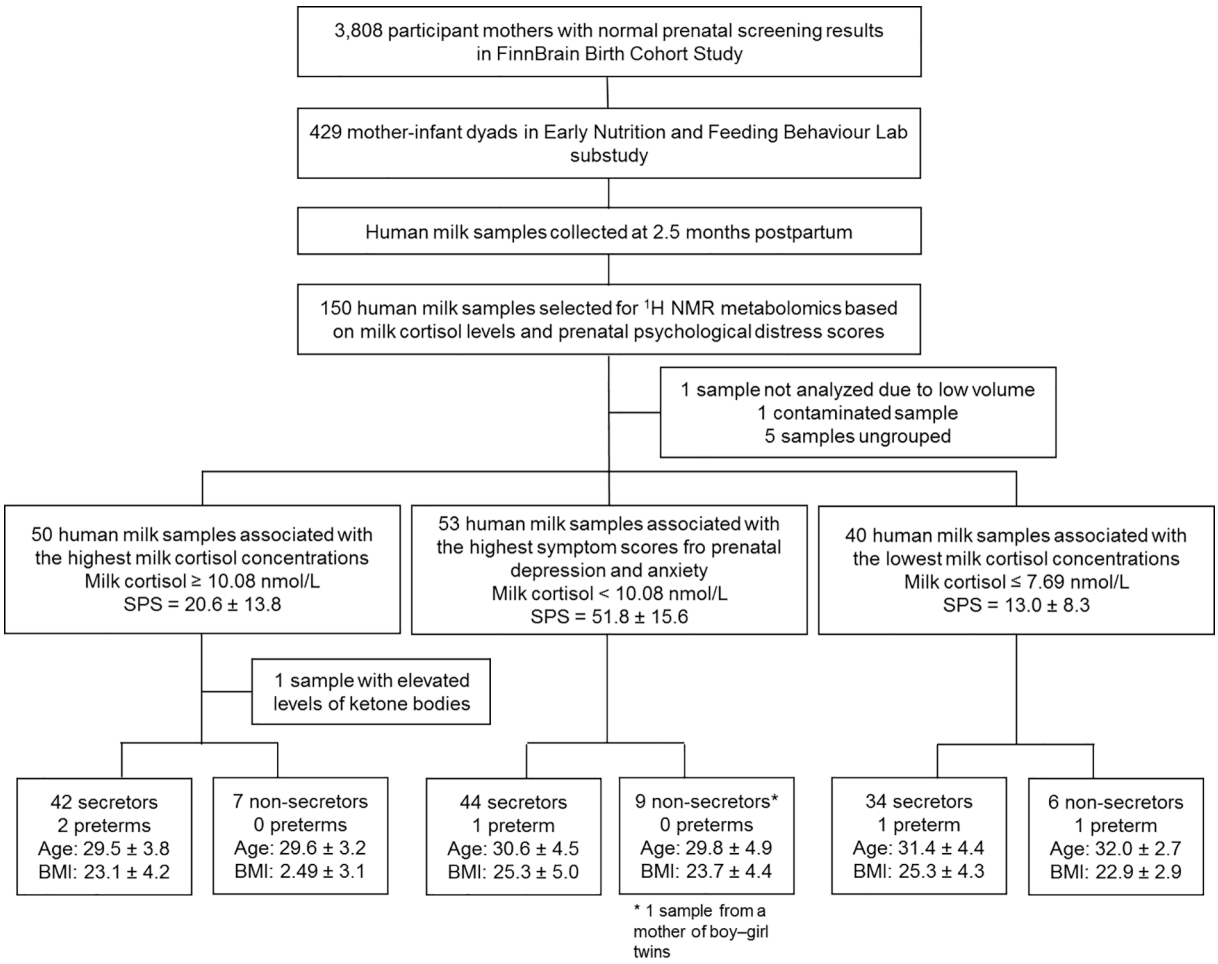

Fig. 1. Study flowchart. Secretor status was determined based on the ^{1}H NMR profile (presence of 2′FL). SPS, sum of prenatal (symptom) scores (for EPDS and SCL-90).

(*noesygppr1d*) with the following parameters: 512 transients, 4 dummy scans, spectral width 14 ppm, acquisition time 3.9 s, relaxation delay 5.0 s, and mixing time 100 ms. The Fourier-transformed spectra were phase-, baseline-, and shim-corrected (to 0.9 Hz) in Chenomx Processor (Chenomx NMR Suite 8.3, Chenomx Inc., Edmonton, AB, Canada). The NMR signals were assigned based on an in-house standard compound library (Chenomx Profiler) and literature. The metabolites were quantified in reference to the internal standard (DSS-d_6) in Chenomx Profiler. Maternal α-1,2-fucosyltransferase 2 (FUT2) secretor status affecting the human milk oligosaccharide (HMO) composition was assessed based on the presence/absence of 2′-fucosyllactose (2′FL) resonances in the spectra (secretor/non-secretor phenotype, respectively) (Sundekilde et al., 2016).

2.7. Statistics

Non-parametric missing value imputation by random forest method was primarily applied on missing data using the 'missForest' package in R (version 3.5.0), suitable for mixed-type data (Stekhoven & Buhlmann, 2012). Single missing values were replaced with the mean of the non-missing observations for respective variables. Assessment of normality was performed with IBM SPSS Statistics v25 (IBM Corp., Armonk, NY) using the Shapiro–Wilk test. The non-parametric Mann–Whitney *U* test was used to test the statistical differences between the three groups using GraphPad Prism v7.04 (GraphPad Software, Inc., San Diego, CA). Benjamini–Hochberg false discovery rate (FDR < 5%) adjustments (adjusted to the total number of metabolites observed) were also performed on the two-tailed *p*-values with GraphPad Prism. Non-parametric Cliff's Delta effect sizes were calculated in R using the 'effsize' package.

Principal component analysis (PCA) performed with MetaboAnalyst 4.0 (Chong et al., 2018) on log-transformed and Pareto-scaled data. Spearman's rank correlation analysis was performed with MetaboAnalyst on selected metabolites (2-aminobutyrate, 2-oxoglutarate, acetate, caprate, caprylate, formate, hypoxanthine, lactate, lactose, methanol, propionate, pyruvate) and background data (maternal age, BMI, parity, duration of exclusive breastfeeding, duration of non-exclusive breastfeeding, C-section y/n, anesthesia y/n, induction y/n, infant's weight and height at birth, milk cortisol, prenatal EPDS and SCL-90, PRAQ, postnatal EPDS and SCL-90) after log-transformation and auto-scaling. Linear regression analyses were performed in SPSS using log-transformed variables to test the association between lactate and milk cortisol. Maternal age, BMI, and gestational age were selected as covariates based on previous literature (Smilowitz et al., 2013; Spevacek et al., 2015).

3. Results and discussion

3.1. Participant characteristics

The characteristics of the mother–infant dyads ($n = 142$) included in this study are listed in Table 1. The genotype (secretor versus non-secretor) of each subject was estimated by assessing the presence or absence of the human milk oligosaccharide (HMO) 2′FL. Mothers

Table 1
Characteristics of the mother–infant dyads.

	All ($n = 142$)	Secretors ($n = 120$)[a]
Mothers		
Age, years	30.4 (20–44)[b]	30.5 (22–44); ($n = 114$)
Pre-pregnancy BMI, kg/m^2	24.4 (17.8–44.1); ($n = 141$)	24.4 (17.8–44.1); ($n = 119$)
Level of education[c]		
Low & mid, %	20 ($n = 135$)	23 ($n = 114$)
High school/ vocational, %	41 ($n = 135$)	45 ($n = 114$)
High, %	34 ($n = 135$)	32 ($n = 114$)
Parity	0.54 ($n = 141$)	0.51 ($n = 119$)
Primiparae, %	58 ($n = 141$)	60 ($n = 119$)
PRAQ score		
Gestational week 14	23.4 ($n = 60$)	23.7 ($n = 50$)
Gestational week 24	23.0 ($n = 134$)	23.2 ($n = 112$)
Gestational week 34	23.2 ($n = 135$)	23.3 ($n = 113$)
SCL-90 anxiety subscale scores		
Gestational week 14	4.0 ($n = 135$)	3.9 ($n = 114$)
Gestational week 24	5.0 ($n = 134$)	5.0 ($n = 112$)
Gestational week 34	3.8 ($n = 135$)	3.9 ($n = 113$)
3 months postpartum	3.7 ($n = 124$)	3.9 ($n = 104$)
EPDS scores		
Gestational week 14	5.8 ($n = 135$)	5.6 ($n = 114$)
Gestational week 24	5.8 ($n = 134$)	5.8 ($n = 112$)
Gestational week 34	5.6 ($n = 135$)	5.3 ($n = 113$)
3 months postpartum	5.4 ($n = 124$)	5.2 ($n = 104$)
Gestational age, weeks	40.0 (35.9–42.3); ($n = 141$)	40.0 (35.9–42.3); ($n = 119$)
Preterm (<37 gw), %	3.5 ($n = 141$)	3.4 ($n = 119$)
Infants		
Males, %	51 ($n = 143$)[d]	53
Weight at birth, kg	3.64 ($n = 142$)	3.66 ($n = 119$)
Height at birth, cm	50.8 ($n = 141$)	50.8 ($n = 118$)
Milk cortisol, nmol/L	7.78 (0.73–33.90); ($n = 141$)	7.69 (0.73–33.90); ($n = 119$)

[a] Secretor status based on the presence of 2'FL in the human milk sample as analyzed with NMR. $N = 120$ unless informed otherwise.
[b] Data given as mean (range) or as %.
[c] Education level (grouped as "Low and Mid", levels 1–5 in the Finnish education system: secondary school / vocational education or lower; "High school/ vocational", level 6: polytechnic education; "High", levels 7–9: university/ graduate school).
[d] The sample set includes a set of twins.

providing samples where 2′FL was below the detection limit of the NMR (~1 μM), were designated as non-secretors and mothers providing samples where 2′FL was above the detection limit were designated as secretors. In our sample set, 22 mothers (~16%) were classified as non-secretors, with low 2′FL, lacto-*N*-fucopentaose I (LNFP I), lactodifucotetraose (LDFT), lacto-*N*-neotetraose (LNnT), and fucose (Supplementary Fig. S1). This is a lower prevalence than in other populations previously studied (Austin et al., 2016; Smilowitz et al., 2013; Sundekilde et al., 2016; Xu et al., 2017).

Sixty-two metabolites, including ten HMOs, were quantified from the human milk samples (Supplementary Table S1). Comparing the metabolomes of the secretors versus the non-secretors, in addition to the known differences in milk HMOs (Smilowitz et al., 2013), the concentrations of betaine, creatinine, and dimethyl sulfone were statistically different based on the Mann–Whitney *U* test ($p = 0.016$, $p = 0.009$ and $p = 0.034$, respectively) but not after FDR-adjustment for multiple comparison ($q > 0.05$). Unlike in the study by Smilowitz et al. (Smilowitz et al., 2013), statistically significant differences in the concentrations of 6′-sialyllactose (6′SL) between secretors and non-secretors was not observed here. The non-secretors were excluded from the subsequent data analysis to minimize the metabolic variation not related to the study question.

Among the secretors ($n = 120$), the prenatal EPDS score (mean of gw 14, 24 and 34) correlated positively with the respective SCL-90 (Spearman $\rho = 0.73$, $p = 0.0000$), PRAQ ($\rho = 0.44$, $p = 0.54 \times 10^{-6}$; PRAQ data as the mean of the gw 24 and 34), and with the postnatal EPDS ($\rho = 0.63$, $p = 0.84 \times 10^{-14}$) and SCL-90 ($\rho = 0.59$, $p = 0.99 \times 10^{-12}$) at 3 months postpartum (Supplementary Fig. S2, Supplementary Table S2). The measured cortisol concentrations in the human milk samples ranged from 0.73 to 33.90 nmol/L (Table 1). Milk cortisol may be associated with postnatal anxiety and stress related to daily hassles (Aparicio et al., 2020) but here, the concentration of milk cortisol did not correlate significantly with any of the pre- or postnatal symptoms ($\rho \leq |0.12|$, $p \geq 0.21$) (Supplementary Fig. S2, Supplementary Table S2).

3.2. Metabolic changes associated with maternal psychological distress

In the secretor sample, the mothers' prenatal psychological distress (symptoms of depression and anxiety) was positively associated with elevated concentrations of short- and medium-chain fatty acids with medium-to-large effect sizes compared to both the LC/LD and the HC group (Table 2). The largest effect sizes were calculated for formate and propionate. Based on the Mann–Whitney *U* test, 2-aminobutyrate, 2-oxoglutarate, 3-hydroxyisobutyrate, 3′-sialyllactose (3′SL), caprylate, creatine, lactose, methanol, succinate, tryptophan and tyrosine were higher in the HD group compared to LC/LD ($p < 0.05$). After FDR-adjustment, only the fatty acids acetate, caprate, formate, and propionate, as well as hypoxanthine exhibited statistical significance ($q < 0.0012$, large effect size). The concentration of hypoxanthine was higher in both the HD ($p < 0.0001$, $q < 0.0012$, Cliff's Delta = 0.52) and the HC ($p < 0.05$, $q =$, Cliff's Delta = 0.31) groups compared to the control. Lactose content was shown to be higher in the HD group in comparison to HC group ($p = 0.0067$, $q = 0.0692$, Cliff's Delta = –0.34) and the LC/LD ($p = 0.0201$). Lactose content is reportedly associated with ethnicity, gestational age, maternal BMI, and infant sex (Gay et al., 2018; Sundekilde et al., 2016). Based on the Mann–Whitney *U* test, no difference in milk lactose was seen between mothers of male and female infants ($p = 0.402$). Lactose was not associated with gestational age or pre-pregnancy BMI either (Supplementary Table S2). Methanol concentrations were lower in the distress group compared to both LC/LD and HC group with medium effect sizes (Cliff's Delta $\geq |0.39|$) (Table 2, Fig. 2). Milk methanol, which may also originate from microbial metabolism in addition to diet (aspartame, pectin, alcoholic beverages) or transformation of *S*-adenosyl methionine (SAM) in some metabolic processes (Dorokhov, Shindyapina, Sheshukova, & Komarova, 2015), correlated negatively with formate, which is expected as a result of the metabolic elimination of methanol.

One of the most significant metabolic changes associated with psychological distress was the increase of short-chain fatty acids (SCFAs) which are products of the anaerobic bacterial fermentation of unabsorbed or undigested carbohydrates (fiber) in the human intestine. SCFAs are important mediators of gut homeostasis and serve as signaling molecules of the host metabolism (Thorburn, Macia, & Mackay, 2014). They are absorbed from gut lumen by the colonocytes, transported to portal circulation, and to some degree, absorbed into systemic circulation (Dalile, Van Oudenhove, Vervliet, & Verbeke, 2019). As SCFAs can also be delivered via breast milk, they may have a role in the development of the infant's immune system (Thorburn et al., 2014).

Acetate and propionate, both elevated in the breast milk associated with high distress, can be produced from lactate e.g. by Actinobacteria (*Propionibacterium*) or Firmicutes (*Eubacterium*, *Veillonella*) (Jost, Lacroix, Braegger, & Chassard, 2015). A potential explanation for the observed correlation between prenatal distress and human milk SCFA concentrations could be alterations in maternal microbiota composition. Future studies should consider incorporating gut and breast milk microbiota samples as well as dietary assessment to elucidate potential mechanisms.

SCFAs are also known to improve intestinal barrier function, prevent translocation of bacteria and host-microbial interaction metabolites as well as directly affect immune cells, suggestive of their possible role as

Table 2
Human milk metabolites statistically different between sample groups (secretors; $p < 0.05$). Unadjusted p-values (Mann–Whitney U test), q-values (Benjamini–Hochberg FDR-adjusted p-values), and Cliff's Delta effect sizes are presented.

	HC ($n = 42$) vs. LC/LD ($n = 34$)			HD ($n = 44$) vs. LC/LD ($n = 34$)			HC ($n = 42$) vs. HD ($n = 44$)		
	p	q	Effect size	p	q	Effect size	p	q	Effect size
2′FL	0.1238	0.4174	−0.2073 (small)	0.5797	0.7813	0.0814 (negligible)	**0.0334***[a]	0.1883	−0.2691 (small)
2-Aminobutyrate	0.7652	0.8626	−0.0406 (negligible)	**0.0464***	0.1692	0.264 (small)	**0.0291***	0.1883	**−0.3858 (medium)**
2-Oxoglutarate	**0.0067****	0.098	**0.3606 (medium)**	**0.0255***	0.1624	0.2955 (small)	0.4873	0.6568	0.0877 (negligible)
3′SL	0.0623	0.2971	0.25 (small)	**0.0362***	0.1692	0.2774 (small)	0.9983	0.9983	0.0005 (negligible)
3-Hydroxyisobutyrate	0.9518	0.9835	−0.0084 (small)	**0.0441***	0.1692	0.2660 (small)	0.0568	0.2201	−0.2376 (small)
Acetate	0.1096	0.4001	0.215 (small)	**<0.0001****	**<0.0012****	**0.5749 (large)**	**0.0003*****	**0.0047****	**−0.4421 (medium)**
Butyrate	**0.0393***	0.2155	0.2759 (small)	**0.0395***	0.1692	0.2727 (small)	0.8315	0.8738	−0.027 (negligible)
Caprate	**0.0013****	**0.0403***	**0.4258 (medium)**	**<0.0001****	**<0.0012****	**0.51337 (large)**	0.4873	0.6568	−0.0877 (negligible)
Caprylate	**0.0079****	0.098	**0.3536 (medium)**	0.0362*	0.1692	0.2774 (small)	0.7423	0.8368	0.0417 (negligible)
Choline	**0.0286***	0.197	0.2927 (small)	0.2793	0.4948	0.1444 (negligible)	0.2601	0.4607	0.1418 (negligible)
Citrate	0.225	0.5167	−0.1639 (small)	0.6629	0.7861	0.0588 (negligible)	**0.0327***	0.1883	−0.2673 (small)
Creatine	0.9731	0.9891	0.0049 (negligible)	**0.0131***	0.116	0.3275 (small)	**0.0315***	0.1883	−0.2689 (small)
Formate	0.1434	0.4286	0.1968 (small)	**<0.0001****	**<0.0012****	**0.6083 (large)**	**<0.0001****	**<0.0021****	**−0.5162 (large)**
Fumarate	**0.0417***	0.2155	0.2724 (small)	0.562	0.7743	0.0775 (negligible)	0.0757	0.245	0.2224 (small)
Hypoxanthine	**0.0159***	0.1408	0.3113 (small)	**<0.0001****	**<0.0012****	**0.5194 (large)**	0.0789	0.245	−0.2273 (small)
Lactate	**0.0002*****	**0.0124***	**0.4811 (large)**	0.497	0.7184	0.0909 (negligible)	**<0.0001****	**<0.0021****	**0.48052 (large)**
Lactose	0.8233	0.8955	0.0308 (negligible)	**0.0201***	0.1558	0.3075 (small)	**0.0067****	0.0692	**−0.3377 (medium)**
Leucine	0.4976	0.8017	−0.0917 (negligible)	0.0691	0.2142	0.2413 (small)	**0.0375***	0.1938	−0.2603 (small)
Methanol	0.4813	0.8017	0.0952 (negligible)	**0.0057****	0.0589	**−0.3899 (medium)**	**0.0011****	**0.0136***	**0.4214 (medium)**
Niacinamide	**0.0099****	0.1023	0.3158 (small)	0.0858	0.2418	0.2052 (small)	0.2494	0.4607	0.1374 (negligible)
Pantothenate	0.722	0.8492	−0.0483 (negligible)	0.11	0.2965	0.2126 (small)	**0.0329***	0.1883	−0.2668 (small)
Propionate	0.455	0.8017	−0.0595 (negligible)	**<0.0001****	**<0.0012****	**0.5441 (large)**	**<0.0001****	**<0.0021****	**−0.6001 (large)**
Pyruvate	**0.0049****	0.098	**0.3739 (medium)**	0.4414	0.6657	0.1029 (negligible)	0.0502	0.2203	0.2451 (small)
Succinate	0.4494	0.8017	0.1022 (negligible)	**0.0262***	0.1624	0.2941 (small)	0.1683	0.3598	−0.1732 (small)
Tryptophan	0.1279	0.4174	0.2045 (small)	**0.0457***	0.1692	0.2647 (small)	0.8281	0.8724	−0.0276 (negligible)
Tyrosine	0.4069	0.8017	0.1120 (negligible)	**0.0442***	0.1692	0.2667 (small)	0.2091	0.4317	−0.158 (small)
Urea	**0.0374***	0.2155	−0.2787 (small)	0.6557	0.7861	−0.0601 (negligible)	0.0909	0.2424	−0.2121 (small)
Uridine	**0.0248***	0.1922	0.2997 (small)	0.7313	0.8097	0.0461 (negligible)	0.1325	0.3262	0.1889 (small)

[a] * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001. $p < 0.05$, $q < 0.05$ and medium-to-large effect sizes are marked in bold.

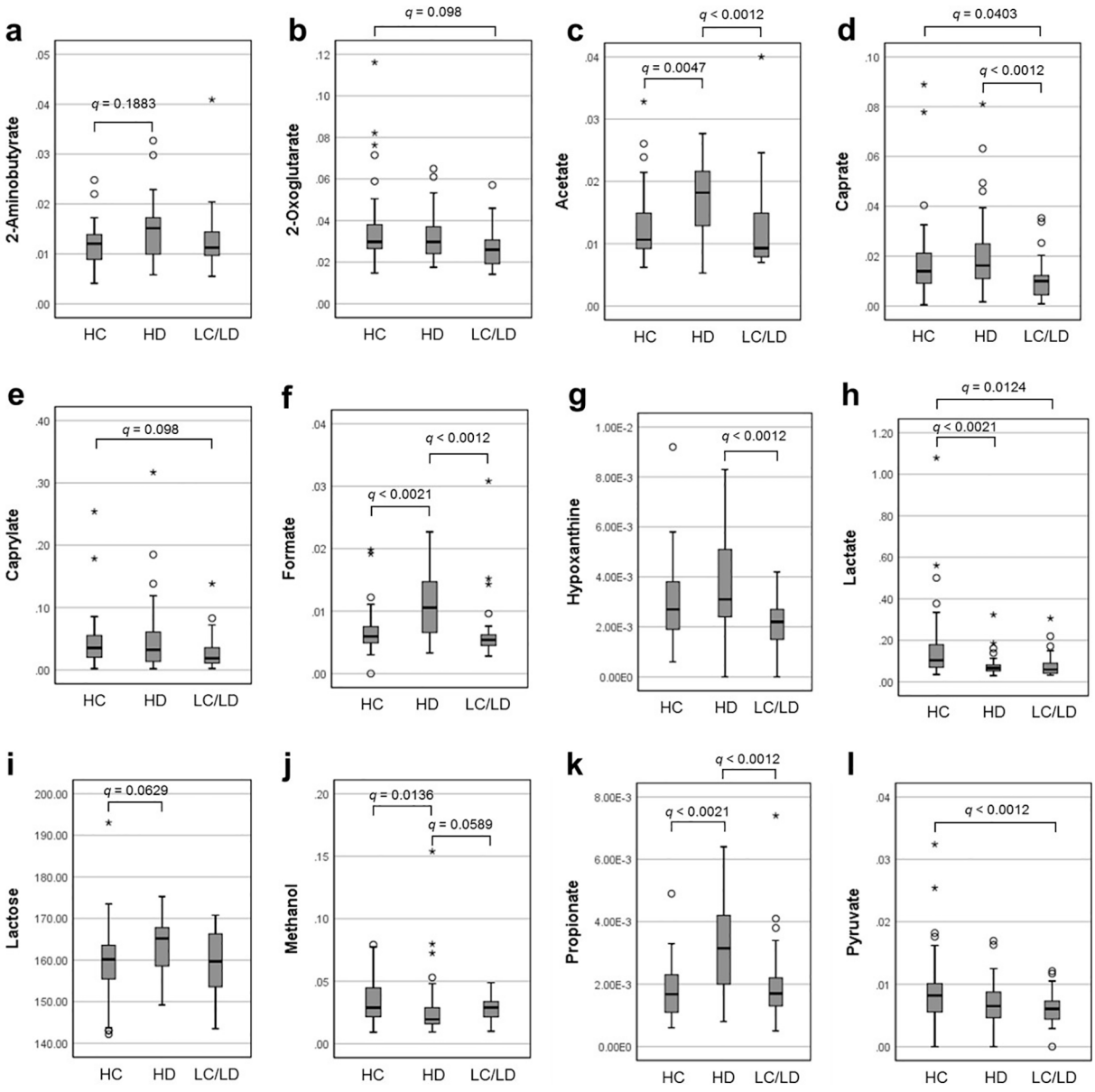

Fig. 2. Box-and-whisker plots of concentrations (μmol/L) of the metabolites in secretor milk exhibiting medium-to-large Cliff's Delta effect sizes: (**a**) 2-aminobutyrate, (**b**) 2-oxoglutarate, (**c**) acetate, (**d**) caprate, (**e**) caprylate, (**f**) formate, (**g**) hypoxanthine, (**h**) lactate, (**i**) lactose, (**j**) methanol, (**k**) propionate, and (**l**) pyruvate. Data are presented in the box plot as median (horizontal line) ± interquartile range (IQR). Whiskers extends to the 1.5 IQR values. The circles and asterisks represent outliers and extreme outliers, respectively. Benjamini–Hochberg FDR-adjusted *p*-values from Mann–Whitney *U* test (*q*) are used to mark significance between groups. HC, "high milk cortisol" group; HD, "high distress" group; LC/LD, "low milk cortisol / low distress" group.

anti-inflammatory agents (Thorburn et al., 2014). The role of inflammation and oxidative stress as underlying pathophysiological mechanisms related to distress is supported by the significant increase of hypoxanthine (a metabolic marker of oxidative stress associated with e.g. smoking and alcohol consumption) in the milk of the distressed mothers. Hypoxanthine can improve the anti-microbial properties of the milk against pathogens by increasing endogenous milk xanthine oxidase activity (Stevens et al., 2000).

Maternal prenatal distress was related to several metabolites with potential to affect neurodevelopment. The distress-related changes in our data showed an increase in important neurotransmitter precursors, albeit only at $p < 0.05$ level and with Cliff's Delta effect sizes of 0.26–0.29. Choline is an essential micronutrient for neurodevelopment and the precursor for acetylcholine, and can function as a methyl donor in cellular methylation reactions. Choline can epigenetically modify the HPA axis reactivity and promote the methylation of cortisol-regulating genes already *in utero* (Jiang et al., 2012). In human milk, choline is present mainly as *O*-phosphocholine and *sn*-glycero-3-phosphocholine, and to some extent, as free choline. The other neurotransmitter precursors in our data, tryptophan (precursor for serotonin) and tyrosine, were positively associated with distress, which is counter-intuitive as tryptophan concentrations in the body are usually decreased with depression, anxiety and sleep disorders. Host tryptophan metabolism can be influenced by the gut microbiota (O'Mahony, Clarke, Borre, Dinan, & Cryan, 2015), and dietary tryptophan can be catabolized by lactobacilli to indole-3-aldehyde, an aryl hydrocarbon receptor (AhR) agonist. AhR-dependent gene expression following agonist binding includes genes involved in the production of mediators important for gut homeostasis (Thorburn et al., 2014). Tryptophan affects the levels of 6-sulfatoxymelatonin in breastfed infants which can promote infant sleep (Cubero et al., 2005). Additionally, the SCFA propionate that was elevated in the high distress group, is a substrate for gluconeogenesis,

and it is also associated with the synthesis of the neurotransmitter serotonin (Dalile et al., 2019).

Prior studies report varying associations between perinatal psychopathologies and human milk composition (Di Benedetto et al., 2019; Moirasgenti et al., 2019; Aparicio et al., 2020). For example, prenatal depression has been linked to the decrease of long-chain polyunsaturated fatty acids in the milk (Di Benedetto et al., 2019) but the association between maternal stress and the immunological factors in human milk is not fully evident. Aparicio et al. (2020) did not find any consistent relationship between postnatal psychosocial distress (or milk cortisol) and the milk concentrations of innate or acquired immunity factors, chemokines, growth factors, and immunoglobulins. However, postpartum-specific stress (correlating positively with serum cortisol) could be associated with lower milk sIgA concentration, thus potentially reducing the immunological benefits of human milk (Moirasgenti et al., 2019) but other reports reviewed by Di Benedetto et al. (2019) indicate that the association between perinatal stress and milk sIgA is not unambiguous.

3.3. Metabolic changes associated with cortisol concentration in milk

The data suggests a negative association between milk cortisol and lactose content (Fig. 2). Based on the statistical comparison with the LC/LD group by the Mann–Whitney *U* test, the metabolites associated with high milk cortisol in secretors were 2-oxoglutarate, butyrate, caprate, caprylate, choline, fumarate, hypoxanthine, lactate, niacinamide, and pyruvate (Table 2). Higher concentrations of pyruvate ($p = 0.0049$, $q = 0.098$, Cliff's Delta = 0.37) and lactate ($p = 0.0002$, $q = 0.0124$, Cliff's Delta = 0.48) compared to the LC/LD group suggest that these metabolites may be produced glycolytically, and induced by cortisol (Table 3, Fig. 2). Lactate was the only metabolite associated with milk cortisol with a large effect size. Milk lactate may vary according to maternal age, BMI, and gestational age (Smilowitz et al., 2013; Spevacek et al., 2015), however, lactate was still associated with milk cortisol after adjusting for these potential confounders (Table 3).

The medium-chain fatty acids (MCFAs) caprylate (8:0) and caprate (10:0) were shown to positively correlate with both milk cortisol and distress. The concentration of 2-oxoglutarate were comparably higher in the HC group with medium effect sizes. A moderate negative correlation between 2-oxoglutarate and the duration of exclusive breastfeeding was observed ($\rho = -0.34$, $p = 1.26 \times 10^{-4}$; Supplementary Fig. S2 and Table S2). Other relationships ($\rho \geq |0.3|$) between the human milk metabolites and the background variables included in the correlation analysis were not seen (Supplementary Table S2).

The metabolic changes attributed to the mother's altered HPA axis activity as indicated by milk cortisol were clearly different from those associated with psychological distress. Cortisol generally takes part in mobilizing glucose, fatty acids, and amino acids from endogenous storage and delivers them to the circulation. Lactate, the only milk metabolite that was associated with milk cortisol with a large effect size, may be derived from pyruvate as a result of anaerobic glycolysis and be linked to stress hyperlactatemia (Garcia-Alvarez, Marik, & Bellomo, 2014). Lactate may serve as a substrate for gluconeogenesis and provide energy for tissues and organs (Garcia-Alvarez et al., 2014). In microbial metabolism, lactate is produced as an intermediate metabolite e.g. by *Bifidobacterium*, *Escherichia*, *Lactobacillus*, and *Staphylococcus* (Jost et al., 2015). However, pyruvate and 2-oxoglutarate, both associated with milk cortisol with a medium effect size, indicate that elevated maternal cortisol affects the tricarboxylic acid (TCA) cycle and energy metabolism (Fig. 3). Pyruvate and 2-oxoglutarate may provide protection against oxidative stress and exhibit neuroprotective effects on hippocampal neuronal cells *in vitro* (Sawa et al., 2017).

Urea is actively pumped into human milk and it serves a major source of readily available non-protein nitrogen, with increasing proportions with gestational and lactational age (Atkinson, Schnurr, Donovan, & Lönnerdal, 1989). Urea is also linked to the TCA cycle via the aspartate–argininosuccinate shunt. Cortisol has a significant negative relationship with maternal nitrogen balance (Motil et al., 1994), and accordingly our results showed that urea was negatively associated with milk cortisol. As proposed by Motil et al., cortisol can modulate nutrient

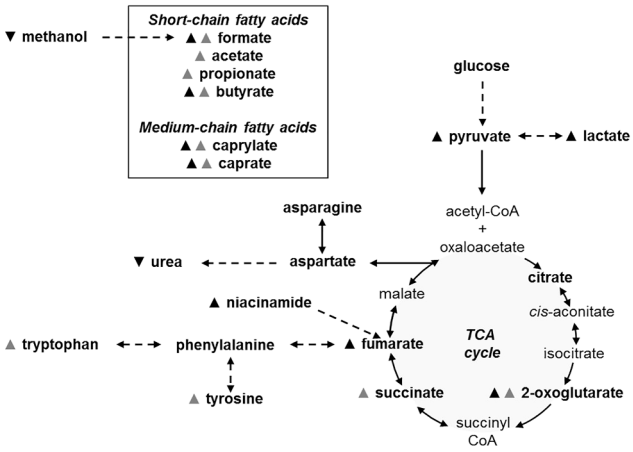

Fig. 3. Metabolic changes associated with milk cortisol (black triangle) and symptoms of psychological distress (gray triangle). Upward symbol (▲) indicates an increase and downward symbol (▼) a decrease compared to the "low milk cortisol / low distress" group ($p < 0.05$, Mann–Whitney *U*). Quantified metabolites are shown in bold.

Table 3
The association of lactate concentration and milk cortisol on the basis of (multiple) linear regression analyses. Milk cortisol is used as a continuous independent variable, and it is adjusted for maternal age, maternal pre-pregnancy BMI, and the duration of gestation in model II).

Dependent variable[a]	Model (R^2; adjusted R^2)	Constant / predictors	B (95% CI)[b]	SE B[c]	β[d]	*p*
Lactate	I (0.170; 0.163)	Constant	−1.305 (−1.405; −1.205)	0.050		**0.000**
		Milk cortisol	0.301 (0.180; 0.423)	0.061	0.412	**0.000**
	II-A (0.010; −0.015)	Constant	−0.644 (−9.760; 8.471)	4.602		0.889
		Maternal age	−0.474 (−1.348; 0.399)	0.441	−0.101	0.285
		Pre-pregnancy BMI	0.019 (−0.693; 0.730)	0.359	0.005	0.985
		Gestational age	0.095 (−3.579; 3.769)	1.855	0.005	0.959
	II-B (0.183; 0.155)	Constant	0.668 (−7.668; 9.004)	4.208		0.874
		Maternal age	−0.061 (−0.876; 0.753)	0.411	−0.013	0.882
		Pre-pregnancy BMI	0.416 (−0.253; 1.084)	0.338	0.109	0.221
		Gestational age	−1.009 (−4.392; 2.373)	1.707	−0.051	0.556
		Milk cortisol	0.319 (0.191; 0.448)	0.065	0.437	**0.000**

[a] Data represents the secretors ($n = 120$). All variables are log-transformed. $p < 0.05$ is marked in bold.
[b] Unstandardized coefficients (B) with their 95% confidence interval (CI) in parentheses.
[c] Standard error (SE) for unstandardized coefficient (B).
[d] Standardized coefficient beta (β).

partitioning toward milk production (Motil et al., 1994). Stress-induced changes in the endocrine system may therefore affect metabolism also at the mammary gland level. This is supported by the observed increase of MCFAs associated with stress conditions as they are exclusively synthesized *de novo* in the alveolar cells of the mammary gland from glucose or acetate (Neville & Picciano, 1997). Caprate especially exhibited a medium-to-large effect size in association to both high milk cortisol and distress. From the infant nutrition point-of-view, MCFAs are quickly oxidized into ketone bodies via acetyl-CoA, fueling the developing infant brain.

Our previous study showed that lauric (12:0) and myristic acid (14:0) in the triacylglycerol-rich fraction of the human milk lipids, and myristic and docosenoic acid (22:1$n-9$) in the phospholipid-rich fraction are positively associated with milk cortisol (Linderborg et al., 2020). The linkage between lipids and cortisol may be mediated via cytokines, but no clear relationship between immunological factors and milk cortisol has been reported (Di Benedetto et al., 2019; Aparicio et al., 2020).

4. Conclusion

We characterized significant metabolic changes associated with self-reported maternal psychological distress and milk cortisol concentrations. Maternal psychopathologies have previously been acknowledged to affect human milk composition and infant growth, health and brain development (Di Benedetto et al., 2019). Still, some of the basic metabolites in human milk have remained understudied in this respect until now. The metabolic changes associated with altered HPA activity as per increased milk cortisol concentrations, or with pre- and postnatal symptoms of depression and anxiety, can explain some of the high biological variations reported earlier for several human milk metabolites, including formate and acetate (Smilowitz et al., 2013). The metabolic changes associated with self-reported symptoms of depression and anxiety or milk cortisol were shown to be dissimilar, pointing toward stress-induced changes in microbiome–gut–brain axis and energy metabolism, respectively. It seems that many differences seen in the milk metabolome in this study are related to oxidative stress, and some observed changes in metabolite concentrations can be associated to better cognitive or other health outcomes of infants based on literature. Our observations warrant for further investigations of host–gut microbiota metabolic interactions in relation to pre- and postnatal psychological distress conditions as well as diet, and their longitudinal impact on infant metabolism, development and health, and also raise new questions on the effects of maternal stress on the milk microbiota.

CRediT authorship contribution statement

Maaria Kortesniemi: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization, Funding acquisition. **Carolyn M. Slupsky:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition. **Anna-Katariina Aatsinki:** Conceptualization, Methodology, Investigation, Writing - review & editing. **Jari Sinkkonen:** Methodology, Resources, Writing - review & editing. **Linnea Karlsson:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition. **Kaisa M. Linderborg:** Conceptualization, Writing - review & editing. **Baoru Yang:** Conceptualization, Resources, Writing - review & editing, Supervision, Funding acquisition. **Hasse Karlsson:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition. **Henna-Maria Kailanto:** Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Support for this research was provided by the Academy of Finland (134950 and 253270, HK; 308176, LK; Profi 4 funding 318894 supporting MK), the Jane and Aatos Erkko Foundation (HK, LK), the Signe and Ane Gyllenberg Foundation (HK, LK), the Finnish Cultural Foundation (HMK, MK), and the Finnish Food and Drink Industries' Federation (MK). During the study, MK received a personal grant from University of Turku for a research visit to the University of California - Davis. Funding for CS was through the USDA National Institute of Food and Agriculture Hatch Project 1021411. Ella Muhli and Laura Tuunanen are thanked for their technical contribution in NMR sample preparation, and Sirpa Hyttinen (National Institute for Health and Welfare) for her work on the milk cortisol assays. Juho Pelto, Teemu Kemppainen, and Yu Hasegawa are thanked for their assistance with the data handling. We want to thank all the participating families and the FinnBrain staff and assisting personnel.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.129628.

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