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## The Effect of a Fish Oil and/or Probiotic Intervention from Early Pregnancy Onwards on Colostrum Immune Mediators: A Randomized, Placebo-Controlled, Double-Blinded Clinical Trial in Overweight/Obese Mothers

Jenni Soukka,\* Lauri Polari, Marko Kalliomäki, Lotta Saros, Teemu D. Laajala, Tero Vahlberg, Diana M. Toivola, and Kirsi Laitinen

Scope: Modifying the composition of colostrum by external factors may provide opportunities to improve the infant's health. Here, we evaluated how fish oil and/or probiotics supplementation modify concentrations of colostrum immune mediators and their associations with perinatal clinical factors on mothers with overweight/obesity.

Methods and results: Pregnant women were randomized in a double-blind manner into four intervention groups, and the supplements were consumed daily from early pregnancy onwards. Colostrum samples were collected from 187 mothers, and 16 immune mediators were measured using bead-based immunoassays.

Interventions modified colostrum composition; the fish oil+probiotics group had higher concentrations of IL-12p70 than probiotics+placebo and higher FMS-like tyrosine kinase 3 ligand (FLT-3L) than fish oil+placebo and probiotics+placebo (one-way analysis of variance, post-hoc Tukey's test). Although the fish oil+probiotics group had higher levels of IFN $\alpha$ 2 compared to the fish oil+placebo group, these differences were not statistically significant after correction for multiple testing. Multivariate linear model revealed significant associations between several immune mediators and the perinatal use of medication.

Conclusion: Fish oil/probiotics intervention exerted a minor effect on concentrations of colostrum immune mediators. However, medication during the perinatal period modulated the immune mediators. These changes in colostrum's composition may contribute to immune system development in the infant.

# **1. Introduction**

Breast milk contains multiple nutritional and non-nutritional factors including immunoglobulins, cytokines, and chemokines that are important for the development and maturation of an infant's immune system.<sup>[1,2]</sup> The composition of breast milk differs according to the stage of lactation, for example, colostrum (0-7 days' postpartum) is richer in immunoglobulins and immune derived cells compared to mature milk (from 14 days' postpartum). In addition, there is a range of mother and infant related factors that influence the composition of breast milk.[3] For example, it has been demonstrated previously that maternal diet composition is associated with composition of breast milk, the strongest evidence being for the association of fish consumption with higher docosahexaenoic acid concentration in breast milk.<sup>[4]</sup> The composition of breast milk can be modified by interventions focused on altering the maternal diet, as indicated by one study in which dietary counseling and provision of rapeseed oil-based food products was associated with higher total n-3 fatty acids in breast

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#### DOI: 10.1002/mnfr.202200446

L. Polari, D. M. Toivola Department of Biosciences Cell Biology Faculty of Science and Engineering Åbo Akademi University Turku FI-20520, Finland L. Polari, D. M. Toivola InFLAMES Research Flagship Center Turku FI-20520, Finland M. Kalliomäki Department of Pediatrics University of Turku Turku FI-20521, Finland milk.<sup>[5]</sup> However, there are still only few randomized controlled trials investigating if breast milk immunomodulatory properties could be modified by diet interventions.

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To date, some investigators have suggested that overweight mothers could secrete breast milk with altered types and concentrations of immune mediators in comparison to the milk secreted by normal weight mothers,<sup>[6,7]</sup> although this speculation has not been confirmed in all studies.<sup>[8]</sup> Indeed, obesity has been linked to a systematic low-grade inflammation<sup>[9]</sup> and prepregnancy obesity is a risk factor for complications and adverse outcomes during the pregnancy.<sup>[10]</sup> Further knowledge on the extent to which maternal adiposity, i.e., maternal weight status and body fat proportion, influence breast milk composition may bring further insight into how breast milk could be modified by dietary compounds with known immunomodulatory properties.

Fish oil includes long-chain n-3 polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Thus far, previous studies have reported an association between dietary LC-PUFA and reduced inflammation<sup>[11]</sup> and insulin resistance.<sup>[12]</sup> In addition, n-3 LC-PUFA supplementation has been associated with lower levels of inflammatory markers in placenta and maternal adipose tissue in overweight/obese pregnant women.<sup>[13]</sup> It has also been indicated that probiotics could have an anti-inflammatory effect<sup>[14,15]</sup> in non-pregnant individuals, but this effect is still being debated.<sup>[16]</sup> As part of our larger on-going trial, it has been demonstrated that particularly the combined use of probiotics and fish oil (including n-3 LC-PUFA) modified the blood serum lipids.<sup>[17]</sup> However, our previous report did not demonstrate an association between the combined use of probiotics and fish oil in incidence of gestational diabetes mellitus (GDM) and glucose metabolism,<sup>[18]</sup> nor biochemical marker of inflammation in mother's blood serum using high-sensitivity C-reactive protein (hsCRP) as a marker in the mothers.<sup>[19]</sup>

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To our knowledge, no studies have systematically explored the combined effect of probiotics and n-3 LC-PUFA supplements on immune mediators in breast milk. It has previously been observed that both probiotics<sup>[20,21]</sup> and n-3 LC-PUFAs,<sup>[22]</sup> when they have been administered separately, would be able to modify the concentrations of immune mediators in breast milk. We aimed to investigate their combined effect on breast milk immune mediators. Our working hypothesis was that the combined use of probiotics and n-3 LC-PUFA supplements could modify inflammatory responses through different mechanisms in early breast milk. For example, it has been shown previously that maternal obesity is linked with a reduced gestational down-regulation of C-C motif chemokine 2 (CCL2) in the circulation.<sup>[23]</sup> CCL2 has also been associated with increased hepatic steatosis and elevated insulin resistance.<sup>[24]</sup> This could lead to a shift in the immune mediator milieu in colostrum towards an anti-inflammatory profile. Our aim was also to identify the potential effect of differ-

Our aim was also to identify the potential effect of different maternal and birth characteristics on immune mediators in colostrum. For example, the relationship between body fat mass and the types of immune mediators present in breast milk has not been addressed previously. The BMI is known to be associated with an increase in the release of immune mediators derived from adipose tissue into the circulation.<sup>[25]</sup> We speculated that this phenomenon could also be reflected in breast milk. In order to include the BMI aspect into our study, we applied an accurate measure of adiposity i.e. body fat mass.

In this study, we aimed to address 1) the potential efficacy of fish oil and/or probiotics in modifying immune mediator levels in the first milk, i.e., colostrum and 2) the association of maternal and birth characteristics with the composition of immune mediators in colostrum.

#### 2. Experimental Section

#### 2.1. Study Population and Design

The colostrum samples were collected for analysis of immune mediators from mothers participating in a mother-infant dietary single-center intervention trial (clinicaltrials.gov, NCT01922791) being executed by Turku University Hospital and University of Turku. The study design has previously been described in detail.<sup>[18]</sup> Briefly, the participants were recruited in maternal welfare clinics between October 2013 and July 2017. The women were randomized in a double-blind manner to four parallel intervention groups: fish oil+placebo, probiotics+placebo, fish oil+probiotics, and placebo+placebo during early pregnancy. Women were allocated into the groups according to the mother's parity and previous gestational diabetes mellitus with a stratified randomization being made with random blocks of four (Table 1). The randomization was conducted by an external statistician. The study staff and participants were blinded to the intervention. The study involved two visits to the study clinic during pregnancy. The colostrum samples (n = 187) were collected in the maternity hospital and frozen at -70 °C prior to the analysis of the immune mediators (Figure 1). The baseline information on all study participants is provided in the Supplement 5, Supporting Information. The graphical abstract summarizing this study was created with BioRender.com.

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 Table 1. Clinical characteristics of all overweight/obese mothers and according to their allocation to the intervention groups.

Characteristics	п	All	Fish oil+	Probiotics +	Fish oil +	Placebo +	p value
			placebo	placebo	probiotics	placebo	
Age	45/45/44/53	$30.7 \pm 4.6$	30.8 ± 5.2	$30.4 \pm 4.3$	30.7 ± 5.3	31.0 ± 3.8	0.914 <sup>a)</sup>
Education (university or college)	or college) 45/44/44/52		31 (68.9)	26 (59.1)	27 (61.4)	32 (61.5)	0.790 <sup>b)</sup>
Prepregnancy BMI [kg m <sup>-2</sup> ]	nancy BMI [kg m <sup>-2</sup> ] 45/45/44/53		29.8 ± 4.1	29.2 ± 3.7	$28.8 \pm 3.6$	$28.9 \pm 3.3$	0.546 <sup>a)</sup>
Overweight		124 (66.3)	27 60)	29 (64.4)	30 (68.2)	38 (71.7)	0.643 <sup>b)</sup>
Obese		63 (33.7)	18 (40)	16 (35.6)	14 (31.8)	15 (28.3)	
Fat mass, late pregnancy [kg]	45/45/43/52	$37.0\pm0.6$	38.5 ± 1.4	36.7 ± 1.4	35.5 ± 1.3	37.4 ± 1.1	0.419 <sup>a)</sup>
Primipara	45/45/44/53	95 (50.8)	23 (51.1)	24 (53.3)	22 (50)	26 (49.1)	0.979 <sup>b)</sup>
Smoked before pregnancy	45/44/44/52	32 (17.3)	9 (20.0)	9 (20.5)	3 (6.8)	11 (21.2)	0.216 <sup>b)</sup>
Smoked during pregnancy	45/44/44/52	6 (3.2)	1 (2.2)	1 (2.3)	2 (4.6)	2 (3.9)	0.584 <sup>b)</sup>
Allergy, asthma, atopy	45/45/44/53	49 (26.2)	14 (31.1)	10 (22.2)	12 (27.3)	13 (24.5)	0.793 <sup>b)</sup>
Family history of diabetes	45/44/44/51	34 (18.5)	12 (26.7)	6 (13.6)	6 (13.6)	10 (19.6)	0.337 <sup>b)</sup>
Gestational diabetes mellitus	44/45/42/52	48 (35.1)	11 (25)	14 (31)	10 (23.8)	13 (25)	0.860 <sup>b)</sup>
Pregnancy weeks at delivery	45/45/44/53	$39.8\pm0.1$	$39.9\pm0.2$	39.8 ± 0.2	$39.8\pm0.2$	$39.7\pm0.2$	0.946 <sup>a)</sup>
Mode of delivery	45/45/44/53						
Vaginal		161 (86)	40 (89)	37 (82)	37 (84)	47 (89)	0.729 <sup>b)</sup>
Cesarean		26 (14)	5 (11)	8 (18)	7 (16)	6 (11)	
Child's sex: girl	45/45/44/53	90 (48)	22 (48.9)	21 (46.7)	20 (45.5)	27 (50.9)	0.952 <sup>b)</sup>
Premature (<37 GW)	45/45/44/53	8 (4.3)	1 (2.2)	2 (4.4)	2 (4.6)	3 (5.7)	0.868 <sup>b)</sup>
Postdate (>42 GW)	45/45/44/53	4 (2.1)	0 (0)	2 (4.4)	1 (2.3)	1 (1.9)	0.543 <sup>b)</sup>
Birth weight [g]	45/45/44/53	$3650\pm500$	$3610\pm400$	$3610\pm560$	$3660\pm560$	$3720 \pm 480$	0.707 <sup>a)</sup>
Baby admitted to neonatal intensive care unit	45/45/44/53	18 (9.6)	4 (8.9)	4 (8.9)	4 (9.1)	6 (11.3)	0.971 <sup>b)</sup>
Sampling days after birth	45/45/44/53	$3.3 \pm 1.3$	3.4 ± 1.5	$3.4 \pm 1.5$	$3.0 \pm 1.0$	$3.3 \pm 1.2$	0.350 <sup>a)</sup>
Storage time [days]	45/45/44/53	0.9 ± 1.2	0.9 ± 1.2	$0.8 \pm 0.9$	0.9 ± 1.4	0.9 ± 1.3	0.947 <sup>a</sup>
Antibiotics, labor	45/45/44/53	50 (27)	13 (29)	13 (29)	12 (27)	12 (23)	0.880 <sup>b)</sup>
Pain relief, labor	45/44/44/53	171 (92)	40 (89)	42 (95)	40 (91)	49 (92)	0.710 <sup>b)</sup>
Opioids, labor	45/45/44/53	22 (12)	7 (16)	7 (16)	4 (9.1)	4 (7.5)	0.483 <sup>b)</sup>
Epidural, labor	45/44/44/53	118 (63)	30 (67)	30 (68)	27 (61)	31 (58)	0.736 <sup>b)</sup>
Antibiotics, puerperium	45/45/44/53	10 (5.3)	2 (4.4)	4 (9.1)	1 (2.3)	3 (5.7)	0.568 <sup>b)</sup>
Pain relief, puerperium	45/45/44/53	169 (90)	42 (93)	40 (89)	38 (86)	49 (93)	0.649 <sup>b)</sup>
Opioids, puerperium	45/44/44/53	19 (10.2)	3 (6.7)	7 (15.6)	5 (11.4)	4 (7.5)	0.475 <sup>b)</sup>
Ibuprofen, puerperium	45/45/44/53	157 (84)	40 (89)	39 (87)	33 (75)	45 (85)	0.294 <sup>b)</sup>
Good compliance with the consumption of capsules	45/45/44/53	170 (91)	42 (93)	41 (91)	39 (89)	48 (91)	0.895 <sup>b)</sup>
Index of dietary quality, early pregnancy	45/44/44/53	$9.7\pm2.0$	9.3 ± 2.1	9.7 ± 2.1	9.5 ± 2.1	10.0 ± 1.7	0.334 <sup>a)</sup>
Index of dietary quality, late pregnancy	45/45/43/53	9.8 ± 2.0	$9.7\pm2.0$	9.8 ± 2.2	9.6 ± 2.1	10.1 ± 1.8	0.713 <sup>a)</sup>
Dietary pattern, early pregnancy	44/44/44/52						
"Healthy diet"		105 (57)	25 (57)	22 (50)	24 (54.5)	34 (65)	0.479 <sup>b)</sup>
"Unhealthy diet"		79 (43)	19 (43)	22 (50)	20 (45.5)	18 (35)	
Dietary pattern, late pregnancy	44/45/43/52						
"Heathy diet"		100 (54)	25 (57)	22 (49)	23 (53)	30 (58)	0.826 <sup>b)</sup>
"Unhealthy diet"		84 (46)	19 (43)	23 (51)	20 (47)	22 (42)	

The results are presented as mean  $\pm$  SD or *n* (%). <sup>a)</sup> One-way ANOVA; <sup>b)</sup>  $\chi^2$ -test.

The Ethics Committee of the Hospital District of Southwest Finland (115/180/2012) has approved the study protocol and the study met the guidelines of the Declaration of Helsinki 2013. All participants provided written informed consent. The inclusion criteria were overweight (BMI  $\geq$ 25–30 kg m<sup>-2</sup>) or obesity (BMI > 30 kg m<sup>-2</sup>), early pregnancy (<18 gestational weeks), and the absence of chronic diseases. The exclusion criteria were diabetes before pregnancy, multifetal pregnancy, chronic diseases



Figure 1. Flow chart of the enrollment, allocation, and inclusion of samples for colostrum analyses.

affecting metabolic and gastrointestinal health, refusal to stop the intake of other probiotic or fish oil supplements, diagnosis or history of coagulopathy, and anticoagulant medication. Further, for the analyses of the colostrum samples, a delay of freezing as an exclusion criterion was applied based on the stability test (Supplement 1, Supporting Information). Based on this stability test and previous studies of cytokine thermal stability during storage,<sup>[26]</sup> the study decided that the inclusion criterion was fridge storage time of 0–5 days (one sample that was stored for 8 days was included in the study). The stability test was performed for IL-6, IL-8, CCL2, and TNF $\alpha$ . Samples were stored in the fridge (at +4 °C) for a median of 0 days.

#### 2.2. Dosage of Intervention Supplements

Consumption of the intervention supplements started in early pregnancy (baseline, 13.9 SD  $\pm$  2.1 gestational weeks) and continued until 6 months after delivery. The fish oil capsules (Croda Europe Ltd., Leek, UK) contained a total of 2.4 g of n-3 LC-PUFA, of which 1.9 g (79%) was DHA (22:6 n-3), 0.22 g (9.4%) EPA, and the rest consisted of other n-3 fatty acids. The probiotic capsules

contained Lacticaseibacillus rhamnosus HN001 (ATCC SD5675; DuPont, Niebüll, Germany) and Bifidobacterium animalis ssp. lactis 420 (DSM 22089; DuPont), each 10E10 colony-forming units. Both placebo capsules for fish oil and probiotics were identical to the intervention capsules. The probiotic placebo capsule contained microcrystalline cellulose and the placebo for fish oil capsule contained medium-chain fatty acids (capric acid C8 54.6% and caprylic acid C10 40.3%). The women were instructed to consume two fish oil capsules and one probiotic capsule every day and not to consume any other fish oil or probiotic supplements during the study. The compliance with consumption was reported good and it has been previously described in detail.<sup>[17]</sup> The compliance was evaluated two times: by phone call at 28 gestational weeks and by interview at the second study visit. A good compliance being defined as taking study capsules  $\geq 5$  days/week reported at both time points.

#### 2.3. Sample Collection and Preparation

The foremilk colostrum samples were collected by manual expression after lactation had commenced into 15 mL light

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<b>Table 2.</b> Concentrations (pg mL <sup>-1</sup>	) of colostrum immune mediators a	according to the intervention groups.
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Immune mediator	Fish oil+ placebo $(n = 45)$	Probiotics+ placebo (n = 45)	Fish oil+ probiotics $(n = 44)$	Placebo+ placebo $(n = 53)$	Unadjusted <i>p</i> value	Adjusted* <i>p</i> value
IL-1β	9.20 (5.7–23)	10.4 (5.5–22)	18.1 (9.9–32)	11.2 (4.3–34)	0.724 <sup>a)</sup>	0.856
IL-1RA	34.6 (8.1–98)	29.0 (9.7–67)	59.0 (14–260)	26.8 (9.4–170)	0.416 <sup>a)</sup>	0.676
IL-3	0.2 (0.0-0.4)	0.0 (0.0-0.2)	0.2 (0.0-0.4)	0.1 (0.0-0.4)	0.057 <sup>a)</sup>	0.185
IL-6	16.5 (8.1–31)	17.2 (8.9–48)	15.0 (5.7–31)	14.9 (7.1–42)	0.566 <sup>a)</sup>	0.818
IL-8	1180 (600–2300)	1450 (540–3000)	1770 (590–3100)	1180 (480–3200)	0.663 <sup>a)</sup>	0.856
IL-10	12.0 (2.8–140)	11.0 (2.8–61)	33.9 (8.2–120)	30.5 (4.7–140)	0.362 <sup>a)</sup>	0.676
IL-12p70	0.0 (0.0-0.8)	0.0 (0.0-0.4)	0.6 (0.0–1.4) <sup>d)</sup>	0.0 (0.0-1.1)	<b>0.049</b> <sup>b)</sup>	0.185
CCL2	7450 (3100–11 000)	6570 (3000–13 000)	6360 (1600–13 000)	5410 (2400–12 000)	0.948 <sup>a)</sup>	0.948
TNFα	13.5 (8.1–21)	12.2 (8.2–20)	16.1 (10-21)	13.5 (8.9–23)	0.889 <sup>a)</sup>	0.948
FLT-3L	4.04 (2.8-5.6)	4.12 (2.9-6.1)	5.37 (3.8–9.5) <sup>e,f)</sup>	4.22 (3.3-5.4)	<b>0.022</b> <sup>a)</sup>	0.185
IFNα2	11.5 (0.0–22)	17.1 (2.1–24)	25.8 (4.7–40) <sup>g)</sup>	13.9 (0.0–30)	<b>0.042</b> <sup>a)</sup>	0.185
IFNγ	0.9 (0.0–1.6)	0.4 (0.2–1.2)	1.1 (0.5–1.9)	0.7 (0.2–1.7)	0.13 <sup>a)</sup>	0.338
TGF-β3	0.718 (0.4–1.4)	0.531 (0.2–1.1)	0.692 (0.3–1.1)	0.595 (0.1–1.5)	0.366 <sup>a) c)</sup>	0.676

The results are presented as median with interquartile range (lower quartile-upper quartile). There were values below lowest standard value and thus extrapolated (number of samples extrapolated): IL-1 $\beta$  (13), IL-1RA (11), IL-3 (112), IL-6 (1), IL-8 (9), IL-10 (36), IL-12p70 (61), CCL2 (13), TNF $\alpha$  (34), FLT-3L (2), IFN $\alpha$ 2 (36), and IFN $\gamma$  (102). Also, some samples were below the detection limit (number of samples): IL-1 $\beta$  (10), IL-3 (75), IL-8 (2), IL-10 (7), IL-12p70 (103), TNF $\alpha$  (2), IFN $\alpha$ 2 (49), IFN $\gamma$  (32), and TGF- $\beta$ 3 (6). TGF- $\beta$ 3 values were relativized to placebo+placebo group and has no unit pg mL<sup>-1</sup>. <sup>a)</sup> One-way ANOVA; <sup>b)</sup> Kruskall–Wallis; <sup>c)</sup> Values have been relativized to placebo+placebo group; p = 0.032; <sup>e)</sup> Different from probiotics+placebo group, p = 0.032; <sup>e)</sup> Different from probiotics+placebo group, p = 0.032; <sup>e)</sup> Different from fish oil+placebo group, p = 0.031. CCL2 = C-C motif chemokine 2, FLT-3L = FMS-like tyrosine kinase 3 ligand, TNF = tumor necrosis factor, TGF = transforming growth factor. \*Adjusted for multiple testing using Benjamini–Hochberg method.

protected polypropylene tubes in the maternity hospital. The samples were cooled down to +6 °C and transferred to the study clinic. There the samples were aliquoted and stored at -70 °C for further analysis. The duration between the collection and freezing at -70 °C was recorded for each sample. The samples were thawed, mixed, and centrifuged four times, once at 2000 × *g* for 20 min and three times at 10 000 × *g* for 10 min in order to remove fat. Between each centrifugation, the clear defatted supernatant was collected and moved to a new tube prior to further centrifugation. After the final centrifugation, the clear supernatant was aliquoted into two tubes for further cytokine concentration analysis and for transforming growth factor (TGF)  $\beta$  analysis, respectively.

#### 2.4. Immune Mediator Assays

The cytokine analyze was performed with multiplex panel kits (Human Cytokine/Chemokine Magnetic Bead Panel, 13multiplex; Merck Millipore, Saint-Quentin-en-Yvelines, France) for IL-8 (CXCL8), CCL2 (MCP-1), IL-1*β*, IL-1RA, IL-2, IL-3, IL-6, IL-10, IL-12p70, IFNα2, IFNγ, TNFα, and Flt-3L. Samples for this were not diluted. Standards (7) and quality controls were provided by the supplier. To measure TGF $\beta$ 1, 2, 3, the samples were first thawed and then diluted 1:30 according to the manufacturer's instructions. After the dilutions, the latent TGF $\beta$  was activated by acidic pre-treatment (1N HCl, 60 min) and then neutralized with assay buffer (Milliplex Assay Buffer, Merck Millipore, Saint-Quentin-en-Yvelines, France). The neutralization for this dilution was verified with pH meter (Fisherbrand pH-Fix 0-14, AO1789C). Multiplex panel kits (TGF $\beta$  1,2,3 Magnetic Bead Kit; Merck Millipore, Saint-Quentin-en-Yvelines, France) were used and the assays were performed similar to the cytokine panel, according to the manufacturer's instructions. Luminex 200 apparatus and Luminex xPONENT software (build 3.1.) (Luminex Corporation, Austin, TX, USA) were used for data acquisitions. The signals which were below obtained lowest standard, but above obtained with buffer, were extrapolated. These are shown in **Table 2**.

#### 2.5. Clinical Characteristics and Diet

At the first gestational visit (13.9 SD  $\pm$  2.1 gestational weeks), the woman's height and weight were measured, and her prepregnancy BMI was calculated using height and self-reported pre-pregnancy weight obtained from the records held in maternal welfare clinics. In addition, information on health, smoking habits and obstetric medical history was obtained for the participants. The second gestational visit was performed in late pregnancy (35.2 SD  $\pm$  0.9 gestational weeks). Body composition, including the body fat mass, was measured at both gestational visits with air displacement plethysmography.<sup>[18]</sup> The details of pregnancy, delivery, and medication during labor and puerperium were obtained from medical records.

Dietary patterns were derived from 3-day food diaries, which were collected at both gestational visits. The formation of dietary patterns and food diary collection have been previously described in detail.<sup>[27]</sup> In brief, the list of food consumption was collected, and each food was classified into individual food groups. The food grouping was based on the groups included in the Finnish food composition database (www.fineli.fi) provided by the Finnish Institute for Health and Welfare. Nutritionally similar groups were combined with each other. Principal component analysis (PCA) was used to reduce 22 food groups into a smaller number of components. Received scree plot and Eigenvalues

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Table 3. The results of multivariable linear model; only significant clinical characteristics are reported (effects of all clinical characteristics included in model are reported in supplement 3, Supporting Information).

Characteristics	Immune mediator	Estimated marginal mean	95% confidence interval		Group	95% confidence interval		p value
			Lower bound	Upper bound	effect	Lower bound	Upper bound	
Maternal allergy	IL-12p70							0.038
No (n = 138)								
Yes ( <i>n</i> = 49)								
Smoked during pregnancy	IL-1RA							0.013
No ( <i>n</i> = 179)		33	21	50				
Yes ( <i>n</i> = 6)		248	50	1200	0.88	0.18	1.6	
Dietary pattern, early pregnancy	CCL2							0.026
"Healthy diet" ( $n = 105$ )		7050	4500	10200				
"Unhealthy diet" ( $n = 75$ )		9360	6400	12900	13	1.6	24	
Fat mass, late pregnancy	IFNγ	Continuous variable			-0.014	-0.027	-0.001	0.036
Pain relief, labor	IL-8							0.039
No (n = 15)		689	386	1225				
Yes ( <i>n</i> = 171)		1300	1100	1500	0.28	0.015	0.54	
Opioids, labor	IL-10							0.017
No ( <i>n</i> = 164)		13.8	7.1	26.8	0.45	0.080	0.82	
Yes ( <i>n</i> = 22)		4.9	1.7	14.0				
Epidural, labor	IFNγ							0.004
No ( <i>n</i> = 68)		0.78	0.5	1.2	0.34	0.11	0.56	
Yes ( <i>n</i> = 116)		0.36	0.3	0.5				
Epidural, labor	IL-3							0.001
No ( <i>n</i> = 68)		0.16	0.11	0.23	0.17	0.07	0.28	
Yes ( <i>n</i> = 118)		0.10	0.07	0.15				
Antibiotics, labor	τΝFα							0.029
No ( <i>n</i> = 137)		14	12	17				
Yes ( <i>n</i> = 50)		20	15	24	0.68	0.070	1.3	
Pain relief, puerperium	IL-3							0.005
No (n = 18)		0.10	0.06	0.15				
Yes ( <i>n</i> = 168)		0.17	0.12	0.24	0.24	0.24	0.24	
Antibiotics, puerperium	IL-6							0.028
No ( <i>n</i> = 176)		11.7	8	16				
Yes ( <i>n</i> = 10)		28.6	13	65	0.39	0.042	0.74	
Child's birth weight over 4 kg	CCL2							0.033
No ( <i>n</i> = 135)		9460	6600	12 800	14	1.1	27	
Yes (n = 45)		6960	4200	10 400				

The models included intervention group and other variables significantly associated (p < 0.05) with immune markers in the univariable analyses. Results are reported as back-transformed estimated marginal means. Group effects are shown as log-transformed (IL-1RA, IL-3, IL-6, IL-8, IL-10, FLT3L, IFN $\gamma$ ) or square root-transformed mean differences (CCL2, TNF $\alpha$ , IFN $\alpha$ 2).

(>1.5) both from principal component analysis, were used in the selection of the components. The first two components were selected and rotated with Varimax rotation. Based on the loadings of different food group variables, these two components were translated into dietary patterns. The dietary patterns were named "Healthy diet" and "Unhealthy diet."

The quality of diet was evaluated by validated index of diet quality questionnaire<sup>[28]</sup> depicting the dietary intake with reference to that recommended.<sup>[29]</sup> The score <10/15 represents a poor dietary quality and a score  $\geq$ 10/15 refers to a good dietary quality.<sup>[28]</sup>

#### 2.6. Statistical Analysis

The results of this study were predefined secondary outcomes of the main study (the primary outcome has been reported earlier<sup>[18]</sup>). All variables were reported as medians with interquartile ranges. Categorical variables were summarized with counts (*n*) and percentages. To make the result more readable, the backtransformed estimated marginal means were used in **Table 3**. Baseline characteristics between the intervention groups were compared using one-way analysis of variance (ANOVA) and  $\chi$ 2-test.



The distributions of immune mediators were positively skewed, and therefore the log transformed values were used for Flt-3L, IFN $\gamma$ , IL-1 $\beta$ , IL-1RA, IL-3, IL-6, IL-8, and IL-10 in the statistical analyses. To account for zero values, log transformations were performed by adding half of the lowest measured concentration to each respective immune marker value. Correspondingly, the square root transformed values were used for CCL2, TNF $\alpha$ , and IFN $\alpha$ 2. Since this study could not obtain normality for the values of cytokines IL-2 and IL-12p70 via transformations, they were analyzed with nonparametric methods. TGF $\beta$  values were relativized to placebo+placebo group due to interassay baseline variation.

Comparisons of immune mediators between intervention groups were performed using one-way analysis of variance (ANOVA), with further post-hoc comparisons conducted with Tukey's test. For IL-2 and IL-12p70, the comparisons were executed using Kruskal–Wallis test and Mann–Whitney *U* test with Bonferroni correction in pairwise comparisons. *P*-values for statistical significances of the multiple tests were corrected using Benjamini–Hochberg method.

The univariable analysis (*t*-tests) for independent samples was used to compare immune mediators and binomial factors (education, allergy, family history of diabetes mellitus, weight status, primiparous, gestational diabetes mellitus, smoking before and during pregnancy, dietary patterns, premature, postdate, mode of delivery, admittance to neonatal intensive care unit, child's gender, macrosomy, small for gestational age, medication during labor, and puerperium). In addition, multivariable linear models were devised. Linear models included intervention group and other variables significantly associated (p < 0.05) with immune markers in univariable analyses. As a result, adjustments were made for the intervention group and those variables which were statistically significant concerning the cytokine.

Correlations between continuous maternal and birth factors (mother's BMI before pregnancy, fat percentage and fat mass in early and late pregnancy, index of diet quality in early and late pregnancy, age, weight gain during intervention, duration of pregnancy, duration of labor, child's birth weight, and bleeding during labor) and colostrum cytokine concentrations were analyzed with Pearson correlation coefficient for parametric variables. For skewed variables, the Spearman correlation coefficient was used. Statistical computations were carried out using the IBM SPSS Statistics for Windows, version 27 (Chicago, IL, USA). A significance level of 0.05 was used in statistical analyses after correction for multiple testing. Further statistical analyses were conducted using the R Statistical Software (version 4.0.3).<sup>[30]</sup>

Correlations between the inflammation markers were calculated using Spearman rank-order correlation (**Figure 2**). *P*-values for statistical significances of these multiple tests of correlations were corrected using Benjamini–Hochberg. The R-package *corrplot*<sup>[31]</sup> was used for visualizing the lower diagonal of the variable correlation matrix. In addition, most variables and individuals were plotted as a heatmap (**Figure 3**) using the *Complex-Heatmap* R-package.<sup>[32]</sup> Binary indicators were used for 2-level (no/yes, boy/girl) type of variables, while square root transformation was first applied to the inflammation markers. After this, a row-wise *z*-score transformation was applied to scale the variables by subtracting the mean and then dividing by the stan-



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**Figure 2.** Spearman correlation matrix was examined between colostrum immune mediator concentrations. Statistically significant correlations (corrected using Benjamin–Hochberg method) are indicated in circles. The size and color grading are used to describe the strength of the relationship between variables. Statistically non-significant values are marked by a cross.

dard deviation. Hierarchical clustering was subsequently used for both the rows and columns, with Euclidean distance as the dissimilarity metric and complete linkage as the cluster aggregation strategy. A height cut strategy was then used for the hierarchical clustering to identify interesting subgroups of variables and individuals. Subgroups of interest in the height cut variables and individuals were then manually inspected.

#### 3. Results

#### 3.1. Study Population and Clinical Characteristics for Intervention

As shown in Table 1, the clinical characteristics of the women and the pregnancy outcome variables did not differ between the intervention groups. The majority of the participants were overweight, primipara, and well-educated. The mean age of the women was  $30.7 \pm 4.6$  years and BMI 29.2  $\pm 3.7$  and the mean weight of the infants was  $3650 \pm 500$  g. The overall compliance with consumption of supplements was good (91%). We compared the participants who provided a sample and those who did not provide a sample, these results are shown in Supplement 5 table, Supporting Information. Differences were observed in baseline characteristics in prepregnancy BMI, smoking before pregnancy, pregnancy weeks at delivery, birth weight, antibiotics during labor, and pain relief during puerperium between participants who provided a sample and those who did not have a sample available.





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**Figure 3.** Heatmap and hierarchical clustering of the study population, immune mediators, and clinical characteristics. Euclidean distance was used as the dissimilarity metric and complete linkage as the cluster aggregation strategy. A height cut strategy was used in the hierarchical clustering (e.g., subclusters 1–7 of individuals on the left-hand side). Sections (a–e) were then defined visually from the heatmap.

#### 3.2. Intervention Modulates the Colostrum Immune Mediator Concentrations

A proportion of samples did not show detectable levels for some of the analytes, with 40% of IL-3, 60% of IL-12p70, 74% of IL-2, 72% of TGF- $\beta$ 1, and 98% of TGF- $\beta$ 2 concentrations falling below the detection limits. The levels of immune mediators were found to correlate positively with each other (Figure 2). The strongest correlations were detected between the following pairs: FLT-3L and IFN $\alpha$ 2; IL-10 and IFN $\alpha$ 2; IL-6 and CCL2; FLT-3L and TNF $\alpha$ . No inverse correlations were observed between the delay of freezing and immune mediator concentrations (range R = -0.008-0.267, p > 0.05). However, a positive correlation with IL-10 was found (R = 0.168, p = 0.022) which could be due to an outlier.

The colostrum immune mediators of mothers are presented according to the intervention groups in Table 2. The concentrations of IL-12p70 (p = 0.049), FLT-3L (p = 0.022), and IFN $\alpha 2$  (p = 0.042) were statistically significantly different between the intervention groups (Supplement 2 figure, Supporting Information). These differences were attributable to the higher concentrations of IL-12p70 in the fish oil+probiotics group as compared to the probiotics+placebo group, FLT-3L in the fish oil+probiotics+placebo and probiotics+placebo and, IFN $\alpha 2$  in the fish oil+probiotics group as compared to the fish oil+placebo group. Placebo+placebo

group did not differ significantly with any other groups in these immune markers. However, these differences did not retain significance after correction for multiple testing. No significant differences in any of the other analyzed immune cell derived compounds were detected between the intervention groups.

### 3.3. The Associations between Clinical Characteristics and Colostrum Immune Mediator Concentrations

To evaluate the associations between the clinical characteristics (Supplement 4, Supporting Information, p < 0.05) and the concentrations of immune mediators in all colostrum samples (n = 187), a multivariable linear model (including intervention groups) was created (Table 3).

Use of antibiotics and pain relief medication during labor and puerperium influenced the levels of immune mediators in the colostrum, even when accounting for confounding factors (Table 3). The use of antibiotics during labor correlated with higher concentrations of TNF $\alpha$  (p = 0.029) or during puerperium higher concentrations of IL-6 (p = 0.028). Pain relief during labor increased the concentrations of IL-8 (p =0.039) and it also elevated concentrations of IL-3 (p = 0.005) when provided during puerperium. The provision of epidural anesthesia during labor was related to lower concentrations of IL-3 (p = 0.001) and IFN $\gamma$  (p = 0.004) (Table 3). Administration of opioids decreased the IL-10 concentrations (p = 0.017) in colostrum.

Higher concentrations of CCL2 were associated with "Unhealthy diet" patterns in early pregnancy (p = 0.026), while lower concentrations of CCL2 were measured when the newborn's birth weight was over 4 kg as compared to smaller babies (p = 0.033). The level of IFN $\gamma$  was negatively associated with a higher body fat mass measured in late pregnancy (p = 0.036). A lower concentration of IL-12p70 was associated with maternal allergy (p = 0.038).

An overview of the study population, immune mediators, and clinical characteristics is presented in Figure 3 as a heatmap. The study population could be clustered into six cluster groups (vertical rows) and clinical characteristics and immune mediators into seven subclusters (horizontal rows). We highlighted five different sections (named a–e), which included distinguishable associations between participants and clinical characteristics.

The heatmap demonstrates that participants in section a included all mothers with children born small for gestational age (in cluster I, subcluster 5). Participant in section b (in cluster IV, subclusters 4 and 5) seemed to be associated with fat mass change and weight gain during pregnancy and intervention (section b). In addition, 19/48 of infants in section b had macrosomy, and 32/48 of mothers in this section were primiparous. Section c was linked to the IL-8, IL-10, IL-1 $\beta$ , IL-1RA, IL-6, and TNF $\alpha$  (in cluster V, subcluster 3). Cluster V contains only two participants, who had quite similar background variables: both of them were from placebo+placebo group, were overweight, non-smokers, did not have GDM, and had vaginal delivery. Similarly, section d was linked to higher concentrations of the IL-10, IL-1 $\beta$ , IL-1RA, IL-6, and TNF $\alpha$  in cluster VI (subcluster 3). Interestingly, higher concentrations of IL-3, IL-2, IL-12p70, IFN $\gamma$ , FLT-3L, and IFN $\alpha$ 2 were linked to section e (cluster V and VI, subcluster 6) possibly indicative of increased immune cell activity. Higher concentrations of IL-6, IL-10, IL-1 $\beta$ , IL-1RA, and TNF $\alpha$  (section d) were associated with this cluster VI. There were no clinical parameters highlighted in this cluster VI.

#### 4. Discussion

We hypothesized that the combined use of probiotics and n-3 LC-PUFA supplements from early pregnancy onwards could modify the levels of inflammatory components present in colostrum. Indeed, we did not find an effect in immune markers towards placebo+placebo group. Although higher concentrations of three out of 16 studied immune mediators in colostrum were observed in the fish oil+probiotics group compared to the probiotics+placebo group or fish oil+placebo group, these effects were not statistically significant after correction for multiple testing. Our hypothesis that the levels of the adipose tissue derived immune mediators would increase with obesity was not fully supported by our results, as only IFN $\gamma$  displayed a statistically significant correlation with body fat mass in this group of overweight and obese mothers. The most important finding is that certain clinical characteristics, including smoking during pregnancy, and particularly administration of medication including opioids, epidural anesthesia, and antibiotics during labor influenced the concentrations of immune mediators in colostrum. Based on our results, drug therapy during the perinatal period appears to be the strongest external determinant of the colostrum immune mediators.

### 4.1. Dietary Intervention has a Modest Effect on Colostrum Immune Mediator Concentrations

Previously the individual role of both probiotics and n-3 LC-PUFA supplements in reducing inflammation has been reported.<sup>[20–22]</sup> Our findings suggest that the combined use of n-3 LC-PUFA and probiotics supplementation may result in increased concentrations of IL-12p70, Flt-3L, and IFN $\alpha$ 2 in the colostrum of obese mothers, as compared to fish oil+placebo and/or probiotics+placebo groups. However, here these effects did not retain statistical significance after correction for multiple testing, and thus further studies are called for to clarify the matter. As far as we are aware, the combined effect of these two supplements on breast milk immune mediators has not been investigated before.

In one study, maternal consumption of probiotics (Lactobacillus casei LC5, Bifidobacterium longum BG7, and Bacillus coagulans SANK70258) was related to a higher concentration of IL-12p70 in breast milk samples collected at 1 and 2 months' postpartum.<sup>[33]</sup> In another trial, good adherence to a Western diet, typically high in saturated fatty acids, was found to be related to lower levels of IL-12p70 in the breast milk<sup>[6]</sup> suggesting that the quality of dietary fat contributes to the concentration of IL-12p70 in breast milk. The increase in the level of IL-12p70 found in our study after the combined intervention was minor; this can be explained by its low concentrations in breast milk as reported also previously.<sup>[34]</sup> Thus, it is unlikely that combined intervention induced more intense T-helper (Th) 1 type responses, a conclusion supported by the fact that other proinflammatory cytokines, i.e., IL-1 $\beta$ , IL-6, and IFN $\gamma$  were mostly unaffected by the intervention. In conclusion, the previous limited data indicates that minor IL-12p70 changes in breast milk could be associated with diet,<sup>[6]</sup> but we could not confirm the finding in our study. The specific biological role of IL-12p70 in breast milk is yet to be determined but it might be of some significance as IL-12 could benefit the child's early maturation of the immune system.[35]

The levels of IFN $\alpha$ 2 were higher in the fish oil+probiotics group when compared to the fish oil+placebo group. Similarly to IL-12p70, its levels were negatively correlated to consumption of a Western diet in a previous study.<sup>[6]</sup> but not to an "Unhealthy diet" or to a "Healthy diet" pattern as identified here. It is known that IFN $\alpha$ 2 is typically present in colostrum<sup>[36]</sup> and it is an important component of a protective immune response,<sup>[37]</sup> but its exact role in colostrum remains to be revealed.

We observed that the combined use of fish oil+probiotics was associated with higher levels of Flt-3L. The presence of Flt-3L in breast milk has only recently been discovered.<sup>[38]</sup> In the published literature, no data were found on whether food supplements can alter the amount of Flt-3L in breast milk. However, an association has been demonstrated between consumption of a Western diet and lower levels of Flt-3L,<sup>[6]</sup> although we did not observe any link between Flt-3L and an "Unhealthy diet" pattern. In addition, Flt-3L has been associated with factors such as BMI and primiparity.<sup>[6]</sup> A possible explanation for this might be that food supplementation could reduce the effects of a Western diet on these immune mediators in early breast milk. We found that macrophage derived CCL2 levels were not affected by the interventions. Breast milk is rich in maternal viable macrophages, but their actual role remains to be clarified.<sup>[39]</sup> Nevertheless, in our study, those mothers with an "Unhealthy diet" pattern had more colostrum CCL2, which supports the previously detected link between a less healthy diet and low-grade inflammation in white adipose tissue.<sup>[40]</sup>

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#### 4.2. Clinical Characteristics Modulate Colostrum Immune Mediator Concentrations

The results of this study indicate that the provision of pain relief during perinatal period is associated with higher concentrations of IL-3 and IL-8 in colostrum. IL-3 is a multipotent hematopoietic growth factor<sup>[41]</sup> whereas IL-8 is an inflammatory cytokine. The concentration of IL-8 is usually increased in several tissues in the presence of infection, inflammation, ischemia, and trauma.<sup>[42]</sup> Thus, a possible explanation for our finding might be that higher IL-8 concentrations reflect higher levels of pain and therefore an increased need for pain relief. Very little was found in the literature on the question of potential impact of neuro-immune interactions in breast milk composition. However, it has previously been observed that epidural morphine reduced IL-10 concentration in plasma during labor but in breast milk it requires further research.<sup>[43]</sup> Another interesting finding is that epidural anesthesia during labor led to lower concentrations of IFNy and IL-3 in colostrum. IFN $\gamma$  has been shown to mediate inflammatory Th1 type reactions and may suppress the type Th2 response related to allergy.<sup>[44]</sup> We also found that the amount of IL-12p70, a cytokine which activates Th1 type responses, was significantly lower in colostrum in mothers with allergies. Deficiencies in the IL-12/IFN $\gamma$  axis have been previously suggested to pose an increased risk for allergies. Recently it was shown that exposure to anti-TNF $\alpha$  may induce hypersensitivity and compromise the infant's immune system.<sup>[45,46]</sup> It is possible that both the IL-12 and IFN $\gamma$  present in breast milk play a role in developing allergies; this should be clarified also in view of the findings from other studies that have highlighted the relationship between maternal allergies and breast milk immune cell derived compounds.<sup>[21,47,48]</sup>

It is somewhat surprising that the use of opioids during labor was associated with lower concentrations of IL-10 in colostrum. One implication of this result is that opioids may suppress the anti-inflammatory effect of IL-10. This finding may be of significance as IL-10 for is known to be important for the development of the neonate's immune system and the inflammatory response.<sup>[44,49,50]</sup> Previous research has also suggested that a lower concentration of IL-10 in breast milk may increase the risk of the infant developing necrotizing enterocolitis.<sup>[51]</sup>

The results of this study also indicate that the use of antibiotics during the perinatal period is associated with higher immune mediator concentrations (Table 3). This was demonstrated in two ways. First, TNF $\alpha$  concentrations in colostrum were higher

in those cases when the mother had received antibiotics during labor. TNF $\alpha$  is an inflammatory cytokine and, for example, higher concentrations have previously been linked to mastitis.<sup>[52]</sup> Secondly, the use of antibiotics during puerperium resulted in higher concentrations of IL-6 which have also been linked with mastitis.<sup>[53]</sup> Antibiotic treatment is initiated either due to diagnosed infection or prophylactically, thus the higher concentrations of TNF $\alpha$  and IL-6 are likely linked to maternal bacterial infections.

Interestingly, our results suggest that smoking during pregnancy can result in higher concentrations of IL-1RA being present in colostrum. The effect was evident even though there were only a few women who smoked during pregnancy (n = 12). IL-1RA binds to the IL-1 receptor due to the homology with IL-1 $\alpha$ and IL-1 $\beta$ .<sup>[54]</sup> A previous study has noted an association between smoking during pregnancy and higher concentrations of IL-1 $\alpha$ <sup>[55]</sup> but an opposite association was described in another report.<sup>[56]</sup> Therefore, these results imply that smoking during pregnancy may be altering the properties of the IL-1 receptor family, but further research is needed.

Our hypothesis was that each food supplement separately and further the combined use of probiotics and fish oil would decrease the markers of low-grade inflammation of colostrum in overweight and obese mothers. However, we did not find an association between weight status (overweight/obese) and most of breast milk immune mediator levels. This result is consistent with a recent study which detected no association between maternal BMI and breast milk levels of IL-8, IL-6, and IL-1 $\beta$ .<sup>[58]</sup> Therefore, our failure to detect either a reduction of pro- inflammatory compounds or a shift toward an anti-inflammatory cytokine milieu after the diet interventions may be explained by the strong homeostasis surrounding the process of the secretion of breast milk.

#### 4.3. Strengths and Limitations

The strengths of this study include the prospective, randomized placebo-controlled design, detailed data collection from pregnancy onwards in a clinical trial setting and the fact that the effect of combined use of n-3 LC-PUFA and probiotics supplementation was investigated. One limitation of this study is the absence of a normal weight control group. However, we have a wide range in the values of BMI, and the analysis was also based on the measured body fat mass. We chose to study an at-risk group of overweight and obese pregnant women, since obesity is a common challenge worldwide with known health concerns for both mother and child. Also, it is possible that chance findings play a role. A second limitation of this study is that the breast milk collection process was not entirely under our control as the colostrum collection was performed in the maternity ward. We conducted a stability test (pilot study) to assess colostrum immune mediator concentrations during storage. The pilot study was performed for IL-6, IL-8, CCL2, and TNFa. Based on our pilot study, the time between sample collection and freezing was considered in the selection of samples for analysis to avoid potential impacts on immune mediators. Also, one limitation is that the participants who provided a sample differ somewhat from those who did not and this should be taken into consideration when

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accessing the generalizability of the results. We report low concentrations of TGF- $\beta$  in colostrum, which is puzzling as we followed appropriate analytical methods, including activation and neutralization of the sample.<sup>[58]</sup> However, due to the inclusion of diluting steps in this process, it is possible that the assay was not sufficiently adapted for breast milk samples, which may have limited the relevant detection of TGF- $\beta$ . Another limitation of this study is that we did not study other immune components, e.g., the presence of immune cells in breast milk. This could have provided further insights into the association between immune cells and their mediators and thus it remains a topic for further research.

#### 4.4. Concluding Remarks

In order to characterize the regulation of immune mediator levels in colostrum, we have studied the roles of maternal, environmental, and birth characteristics as well as the effect of a maternal food supplement intervention. We found that the immune mediators present in colostrum are surprisingly resistant towards external modulation. Factors such as maternal obesity, diet, and allergies, which are often associated with changes in immune cell number and activity, had only a modest or no effect on the types and levels of cytokines in the colostrum. These results suggest a high robustness of breast milk composition in front of various clinical factors, suggesting that it may be difficult to modify breast milk composition by diet related intervention. Nevertheless, administration of different kinds of medications during the labor significantly affected the levels of specific compounds produced by immune cells. Perinatal medications may overflow some of the changes in colostrum, nevertheless, the interventions may still induce a detectable effect on transition/mature milk (i.e., away from the perinatal medication). Further research should be carried out to investigate whether these changes possess a clinical significance for the newborn.

The potential benefits of the changed immune mediators due to the combined consumption of fish oils and probiotics will need to be confirmed. In addition, the recognition of subpopulations among lactating mothers may help to plan personalized recommendations and medication in the future. The increased knowledge about which biologically active constituents are present in breast milk may also promote the development of more natural infant-friendly formulas.

#### **Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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#### **Conflict of Interest**

The authors declare no conflict of interest.

#### **Author Contributions**

J.S. investigation. J.S., L.P., L.S., K.L., methodology. L.S. data curation. J.S., T.D.L., and T.V. formal analysis. J.S., L.P., M.K., T.D.L., T.V., D.M.T., and K.L. data interpretation. T.D.L. visualization. J.S., writing - original draft. L.P., M.K., L.S., T.D.L., T.V., D.M.T., and K.L. writing - review and editing. K.L. and L.P. supervision. K.L. conceptualization, resources, funding acquisition, and project administration. All authors have read and agreed the final version of the article.

#### **Data Availability Statement**

The datasets are not available due to their containing information that could compromise participant privacy and consent.

#### Keywords

colostrum, cytokines, fish oil, infant, probiotics

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