

Prebiotic consumption in pregnant and lactating women increases IL-27 expression in human milk

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Abstract

The consumption of probiotics by pregnant and lactating women may prevent the onset of allergic disorders in their children by increasing the concentrations of immunoactive agents such as cytokines in breast milk. Prebiotics such as fructo-oligosaccharides (FOS) increase the number of beneficial organisms such as bifidobacteria. Thus, prebiotics may have an effect similar to that of probiotics. The objective of the present study was to carry out a comprehensive analysis of mRNA expression in human milk cells to identify changes in the concentrations of cytokines in breast milk after the consumption of FOS (4 g × 2 times/d) by pregnant and lactating women. The microarray analysis of human milk cells demonstrated that the expression levels of five genes in colostrum samples and fourteen genes in 1-month breast milk samples differed more than 3-fold between the FOS and control groups (sucrose group). The mRNA expression level of *IL-27*, a cytokine associated with immunoregulatory function, was significantly higher in 1-month breast milk samples obtained from the FOS group than in those obtained from the control group. In addition, the protein concentrations of IL-27 in colostrum and 1-month breast milk samples were significantly higher in the FOS group than in the control group. In conclusion, the consumption of FOS by pregnant and lactating women increases the production of IL-27 in breast milk. Future studies will address the association of this phenomenon with the onset of allergic disorders in children.

Key words: Prebiotics: Breast milk: Pregnancy: IL-27

Breast milk is the most important source of nutrients for infants, significantly influencing growth and development. Previously, breast-feeding had been reported to prevent allergy in children⁽¹⁾, but recent observational studies of breast milk components have revealed various immunoactive agents with positive and negative roles in the onset of allergic disorders^(2–5). Intervention studies of food products have revealed that the consumption of fish oil and probiotics by pregnant and lactating women increases the concentrations of anti-allergic immunoactive agents such as soluble cluster of differentiation 14 (CD14), IgA and transforming growth factor (TGF)- β in breast milk^(6,7). The consumption of probiotics during pregnancy and lactation may help prevent atopic dermatitis, suggesting that probiotic administration in mothers induces the production of immunoactive agents in breast milk.

Prebiotics are defined as ‘selectively fermented ingredients that allow specific changes, in both the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health’; these include dietary fibre, resistant starch and oligosaccharides⁽⁸⁾. Typical indigestible oligosaccharide prebiotics such as fructo-oligosaccharides (FOS) and galacto-oligosaccharides increase the number of beneficial organisms such as bifidobacteria^(9,10). Thus, prebiotics may have an effect similar to that of probiotics. Recent human studies have shown that the incidence of atopic dermatitis decreases in low- and high-risk infants when fed prebiotic-supplemented formula *v.* placebo-supplemented formula^(11,12), although its effect is not well established because of problems such as high dropout rates. However, there have been no studies about the effect of prebiotics on

Abbreviations: FOS, fructo-oligosaccharides; TGF, transforming growth factor; Th, T helper.

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immunoactive agents in breast milk when given to pregnant and lactating women. In one study of atopic dermatitis in NC/Nga mice, FOS administration in mothers has been shown to control the onset of atopic dermatitis in mouse pups⁽¹³⁾; however, the effect of FOS administration on cytokine levels in breast milk has not been studied.

Although there have been a few studies on the effect of food products on specific cytokines in breast milk, a comprehensive analysis has not been reported. Immunoactive agents in breast milk are mostly derived from serum or mammary gland epithelial cells⁽¹⁴⁾, but are potentially produced by immune cells in breast milk⁽¹⁵⁾. Thus, a comprehensive analysis of the gene expression of human milk cells can serve as an effective method for analysing immunoactive agents in breast milk. In the present study, we carried out a comprehensive analysis of gene expression in human milk cells to characterise the effect of FOS administration in pregnant and lactating women on the production of immunoactive agents in breast milk. We also evaluated the concentrations of candidate immune-related proteins in breast milk.

Subjects and methods

Subjects and study protocol

The present randomised, double-blind trial was registered in the UMIN Clinical Trial Registry (UMIN 000008142) (<http://www.umin.ac.jp/>). This trial was conducted at Masuda Maternity Clinic in Chiba prefecture, where healthy pregnant woman were enrolled with written informed consent. Pregnant women with complications such as pregnancy-induced hypertension were excluded. However, subjects with a history of allergic disease were not excluded.

The study was conducted according to the guidelines of the Declaration of Helsinki, and all procedures were approved by the Ethics Committee of Chiba University. Subjects (eighty-four individuals) were randomly assigned to the FOS (Meiologo-P[®], obtained from Meiji Food Materia Company Limited) (FOS, *n* 41) or sucrose (placebo, *n* 43) intake groups. Trial compound (4 g) was administered after breakfast and supper daily from 26 weeks of gestation to 1 month after delivery (Fig. 1).

Questionnaire evaluations and follow-up

The subjects were given diaries to record each dose of trial compound taken during the intervention period to characterise eating habits. A FFQ (Microsoft Excel Eiyou-Kun, Kenpakusha) was also given upon the initiation of enrolment (26 weeks of gestation) and 10 weeks later (36 weeks of gestation) (Fig. 1). Study compound compliance, consumption of foods similar to the study compound, parturition style and dietary habits were evaluated based on the diaries and FFQ results and medical records.

Selection of subjects

Due to ethical reasons, there was no control over the daily consumption of oligosaccharides by the study subjects. Instead, based on their diaries and FFQ results, from the placebo group, we excluded subjects who consumed marketed oligosaccharide products for more than 2 d a week. In addition, from both the groups, subjects with less than 80% test compound intake were excluded due to insufficient intervention (Fig. 1).

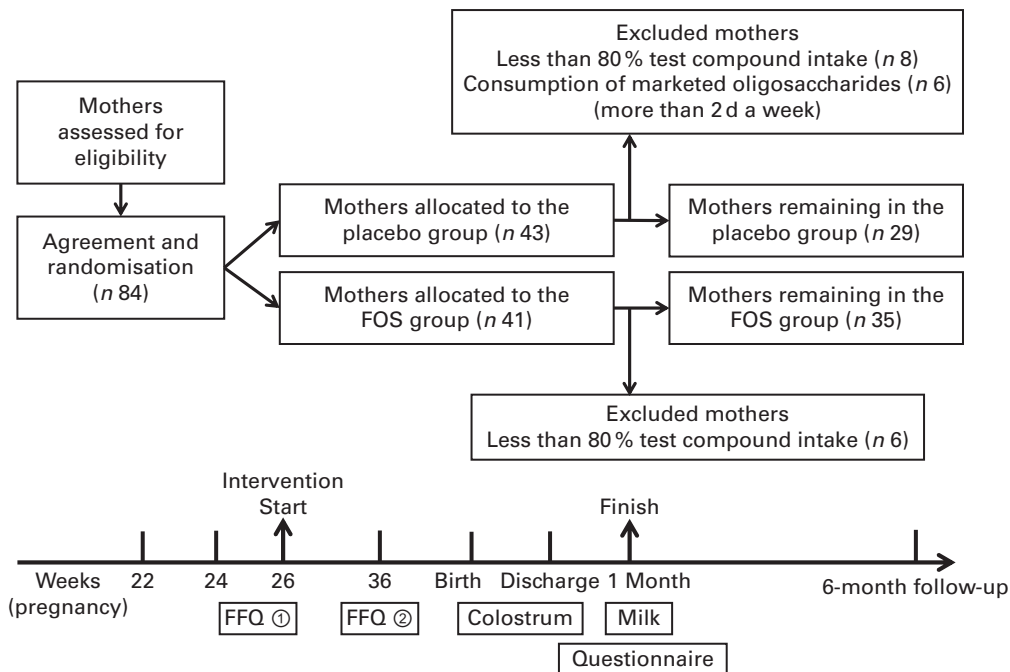


Fig. 1. Experimental design. Trial compound (4 g) was administered twice daily from 26 weeks of gestation to 1 month after delivery. FOS, fructo-oligosaccharides.

Table 1. Comparison of dietary habits between the groups

	Placebo group	FOS group	P*
	Median (n 29)	Median (n 35)	
Energy value (kJ/d)	7370	7801	0.777
Protein/energy value (%)	13.0	13.4	0.670
Fat/energy value (%)	31.0	31.8	0.627
Carbohydrate/energy value (%)	55.7	55.4	0.838
Grain energy ratio (%)	34.9	35.6	0.443
Animal protein ratio (%)	52.7	51.7	0.306
Brightly coloured vegetable ratio (%)	35.4	38.2	0.241
Dietary fibre (g/d)	10.9	11.7	0.423
Fatty acid (g/d)	57.3	56.2	0.718
n-6 Fatty acid:n-3 fatty acid ratio	5.1	5.1	0.770
Milk constituents (g/d)	212	208	0.854

FOS, fructo-oligosaccharides.

* Mann–Whitney U-test.

Breast milk sampling

Colostrum samples were collected during hospitalisation after childbirth and breast milk samples were acquired 1 month after childbirth. The samples were collected in sterile plastic tubes and processed within 24 h. The breast milk samples were separated into whey and cellular elements by centrifugation (2500 rpm for 5 min) at room temperature. To remove fat from the whey, centrifugation was carried out at 10 000 rpm for 10 min at 4°C. The whey was used to measure the concentrations of immune-related proteins. Total RNA was extracted from the cellular fraction using the RNeasy Mini Kit (QIAGEN); samples with low RNA yields were concentrated using the RNeasy Micro Kit (QIAGEN). The quantity and quality of RNA were determined using BioSpec-nano (Shimadzu-Biotech) and the Agilent 2100 BioAnalyzer (Agilent Technologies).

Maternal blood sampling

Maternal blood samples were collected during hospitalisation after childbirth. The samples were collected in sterile plastic tubes and allowed to clot for serum analysis. Serum was separated from the samples by centrifugation (2500 rpm for 10 min) at 4°C and used to measure the concentrations of IL-27.

Comprehensive microarray analysis

Cy3-labelled complementary RNA was synthesised from 100 ng of total RNA using the Low Input Quick Amp Labeling Kit, One-Color (Agilent Technologies). After checking the quality and quantity of complementary RNA using BioSpec-nano (Shimadzu-Biotech) and the Agilent BioAnalyzer (Agilent Technologies), the complementary RNA was hybridised at 65°C for 17 h to Agilent Whole Human Genome Microarrays 4 × 44K (Design ID: 014850; Agilent Technologies). After washing, the microarrays were scanned using an Agilent DNA microarray scanner (Agilent Technologies). All procedures from labelling to scanning were carried out according to the manufacturer's protocols. The intensity value of each scanned feature was

quantified using the Agilent Feature Extraction Software (Agilent Technologies), which performs background subtractions. Normalisation was carried out using Agilent GeneSpring GX version 11.0.2 (per-chip normalisation: 75 percentile shift; per-gene normalisation: none; Agilent Technologies). Data were analysed using the statistical language R (CRAN, R version 2.7.2; <http://www.r-project.org/>). Gene ontology and pathway analyses were carried out using the Database for Annotation, Visualization, and Integrated Discovery (<http://david.abcc.ncifcrf.gov/>), National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). High-quality RNA, such as those with optical density (OD)_{260/280} > 1.8, were subjected to microarray analysis.

ELISA for the measurement of IL-27, soluble CD14, IgA and transforming growth factor-β1 concentrations

The Human IL-27 DuoSet ELISA kit (R&D Systems) was used to detect IL-27 in whey and serum samples. The lower limit of IL-27 detection is 156.25 pg/ml. The concentrations of IL-27 were measured according to the manufacturer's protocol in duplicate assays. The Human CD14 DuoSet ELISA kit (R&D Systems) and Human TGF-β1 DuoSet ELISA kit (R&D Systems) were also used to detect soluble CD14 and TGF-β1 in whey, respectively. The concentrations of IgA in whey were measured using an immunoturbidimetric assay.

Statistical analysis

The experimental data were not normally distributed and therefore were evaluated by the Mann–Whitney U-test or Pearson's χ^2 test using the software Dr. SPSS II 11.0 (SPSS Japan, Inc.). Differences were considered significant at $P < 0.05$.

Table 2. Comparison of the characteristics of subjects between the groups (Median values and number of participants)

	Placebo group (n 29)	FOS group (n 35)	P
	n	n	
Mothers' characteristics			
Age (years)			0.276*
Median	33	30	
Primipara	13	16	0.943†
Pollen disease	8	10	0.930†
History of atopic diseases	1	6	0.255†
History of other allergic diseases	10	20	0.054†
Smoking during pregnancy	6	3	0.113†
BMI during prepregnancy			0.370*
Median	21.2	20.3	
BMI during pregnancy			0.243*
Median	25.3	24.6	
Birth history			
Caesarean section	2	3	0.590†
Fetus week number (weeks)			0.861*
Median	39.1	39.3	
Weight of the baby (g)			0.345*
Median	2990	3165	

FOS, fructo-oligosaccharides.

* Mann–Whitney U-test.

† Pearson's χ^2 test.

Table 3. List of genes that exhibited a significant difference in the microarray analysis of colostrum cells (Median values and ranges)

Probe identity*	Gene symbol	Fold change	Expression values (log ₂ ratio)				P †
			Placebo group		FOS group		
			Median	Range	Median	Range	
A_24_P694738	<i>LOC388796</i>	+4.40	-5.99	-6.42 to -1.46	-3.85	-5.73 to -1.08	0.046
A_32_P29551	No name	+3.77	-5.23	-7.32 to -2.14	-3.32	-5.08 to -1.94	0.027
A_23_P143748	<i>TTL12</i>	-4.41	1.47	-0.74 to 1.99	-0.67	-1.30 to 0.85	0.016
A_24_P212021	<i>GSTM5</i>	-5.18	-3.03	-6.46 to -0.93	-5.40	-7.44 to -2.87	0.046
A_32_P163089	<i>C12orf75</i>	-9.82	0.46	-2.29 to 2.41	-2.83	-3.99 to 0.16	0.009

FOS, fructo-oligosaccharides; *TTL12*, tubulin tyrosine ligase-like family member 12; *GSTM5*, glutathione S-transferase mu 5; *C12orf75*, chromosome 12 open reading frame 75.

* Genes in colostrum cells with ≥3-fold difference in expression between the FOS and placebo groups.

† Mann-Whitney U-test.

Results

Analysed subjects

After excluding subjects with insufficient test compound intake, the number of subjects included in the analysis was reduced. However, we found no differences between the placebo (*n* 29) and FOS (*n* 35) groups, suggesting that the comparison of these groups was appropriate. Comparison of dietary habits demonstrated no significant difference between the groups (Table 1). There was no difference in the frequency of yogurt intake between the two groups as most mothers reported eating yogurt (data not shown). There

were also no significant differences among the characteristics of the mothers (Table 2). Although there were more mothers with a history of other allergic diseases in the FOS group than in the control group, this did not reach statistical significance. Subjects in both the groups consumed 8 g/d of the test compound; there were no side effects or subject withdrawals from the study (data not shown).

Comprehensive microarray analysis of human milk cells

Microarray of colostrum-derived samples. Randomly selected high-quality cellular RNA extracted from eight colostrum

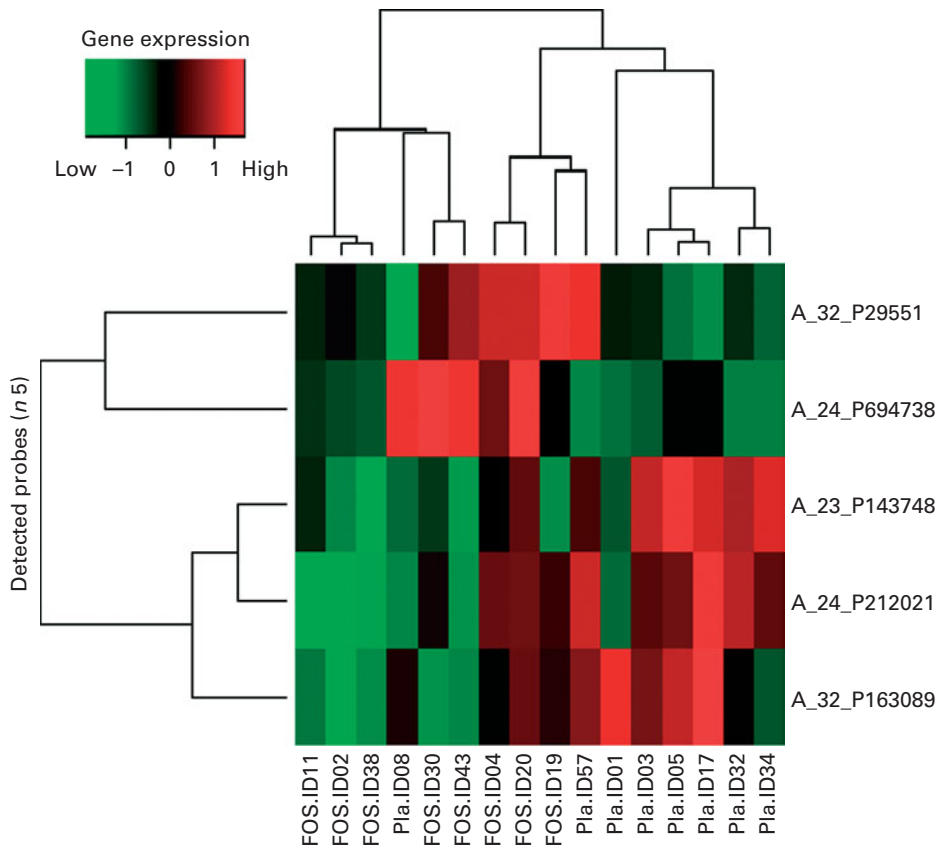


Fig. 2. Heat map of colostrum samples. The heat map was based on five probes with significantly altered expression in the fructo-oligosaccharides (FOS) group. Pla, placebo.

samples from each group were subjected to a microarray analysis and then compared. There were no significant differences in the characteristics of mothers from whom samples were collected (data not shown). Of the 41 000 probes on the microarray chip, the total number of probes extracted from the sixteen samples was 23 008. Comparison of the placebo and FOS groups yielded statistically significant differences, in which five probes exhibited a ≥ 3 -fold change when a mean signal value of 1 was observed in the placebo group ($P < 0.05$) (Table 3).

A heat map was created for the five probes that exhibited a significant difference in the microarray analysis. A hierarchical cluster analysis was carried out to classify the type of gene expression in each sample into groups: six samples on the left side and ten samples on the right side of the vertical axis, showing the clustering of the placebo and FOS groups. However, two samples from the placebo group were located on the right side, suggesting that the expression of these genes was close to that in the FOS group (Fig. 2).

The highest, most differentially expressed gene in the FOS group was *LOC388796* (+4.40-fold; complementary DNA *DKFZp686B0328* from clone *DKFZp686B0328*). The lowest, most differentially expressed gene was *C12orf75* (-9.82-fold; chromosome 12 open reading frame 75). Glutathione *S*-transferase mu 5 (*GSTM5*) was also highly differentially expressed, with a F_c value of -5.18 (Table 3).

Microarray of 1-month breast milk-derived samples. Randomly selected high-quality cellular RNA extracted from three breast milk samples from each group were subjected to a microarray analysis and then compared. There was no significant difference in the characteristics of the collected samples (data not shown). As shown in the subject identity in Figs. 2 and 3, the colostrum and 1-month breast milk samples were not collected from the same subjects. Similar to the microarray analysis of colostrum samples, the number of probes extracted from these six samples was 19 354. Significant differences were found, with fourteen probes exhibiting a difference of more than 3-fold ($P < 0.05$); these probes were organised in a heat map. A hierarchical cluster analysis was used to classify gene expression in each sample into groups: three samples on the left side and three samples on the right side of the vertical axis, showing a clustering of the placebo and FOS groups (Fig. 3).

Among the genes with high signal values in the FOS group, *ABCA13* (ATP-binding cassette, subfamily A (ABC1), member 13) was the most differentially expressed (+6.72-fold), followed by *DNAH12* (+4.46-fold; dynein, axonemal, heavy chain 12, transcript variant 1), *THRSP* (+3.96-fold; thyroid hormone responsive (SPOT14 homologue, rat)), *IL-27* (+3.58-fold) and *THRSP* (+3.01-fold). Genes in the FOS group with low signal values included *UCA1* (-6.88-fold; urothelial cancer associated 1), *LOC100289410* (-4.27-fold; similar to *bCG1774568*), *GPX7* (-3.92-fold; glutathione

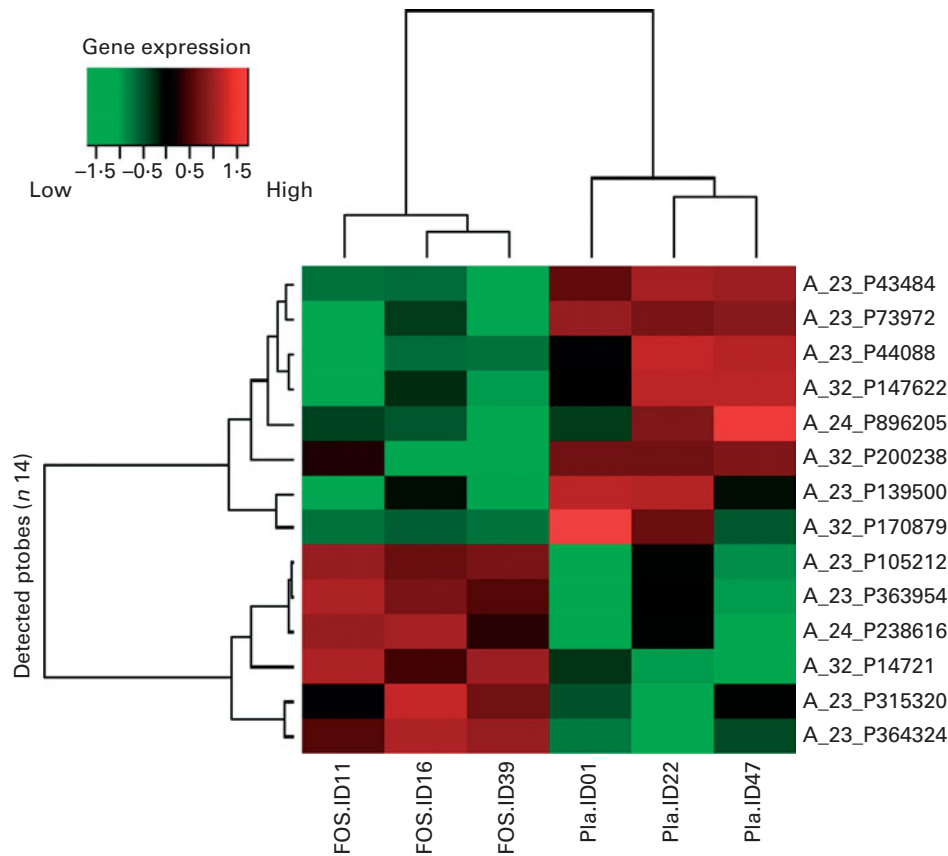


Fig. 3. Heat map of 1-month milk samples. The heat map was based on fourteen probes with significantly altered expression in the fructo-oligosaccharides (FOS) group. Pla, placebo.

Table 4. List of genes that exhibited a significant difference in the microarray analysis of 1-month milk cells

Probe identity*	Gene symbol	Fold change	Expression values (log ₂ ratio)				P†
			Placebo group		FOS group		
			Median	Range	Median	Range	
A_23_P364324	<i>ABCA13</i>	+6.72	-3.75	-4.89 to -3.24	-1.00	-1.69 to -0.77	0.049
A_32_P14721	<i>DNAH12</i>	+4.46	-2.85	-3.16 to -2.10	-0.69	-1.30 to -0.55	0.049
A_23_P363954	<i>THRSP</i>	+3.96	0.79	0.27 to 1.96	2.77	2.52 to 3.13	0.049
A_23_P315320	<i>IL-27</i>	+3.58	2.00	0.09 to 2.85	3.84	2.94 to 4.62	0.049
A_24_P238616	No name	+3.33	1.87	1.65 to 2.84	3.60	3.08 to 3.70	0.049
A_23_P105212	<i>THRSP</i>	+3.01	5.59	4.93 to 6.43	7.18	7.10 to 7.36	0.049
A_23_P44088	<i>hCG_1774568</i>	-3.24	-2.36	-3.27 to -2.26	-4.06	-4.48 to -4.00	0.049
A_24_P896205	<i>LOC645722</i>	-3.34	-2.97	-4.46 to -1.94	-4.71	-5.51 to -4.48	0.049
A_23_P43484	<i>CDKN2A</i>	-3.56	-0.95	-1.36 to -0.92	-2.78	-3.43 to -2.72	0.049
A_23_P139500	<i>BHLHE41</i>	-3.63	-1.75	-2.83 to -1.69	-3.61	-3.72 to -2.84	0.049
A_23_P73972	<i>GPX7</i>	-3.92	1.27	1.19 to 1.35	-0.70	-0.71 to 0.08	0.049
A_32_P147622	<i>LOC100289410</i>	-4.27	1.01	-0.11 to 1.01	-1.08	-1.27 to -0.35	0.049
A_32_P200238	<i>UCA1</i>	-6.88	1.57	1.51 to 1.65	-1.22	-1.62 to 0.89	0.049
A_32_P170879	No name	-7.45	-4.22	-6.77 to -1.88	-7.11	-7.16 to -6.83	0.049

FOS, fructo-oligosaccharides; *ABCA13*, ATP-binding cassette, subfamily A (ABC1), member 13; *DNAH12*, dynein, axonemal, heavy chain 12; *THRSP*, thyroid hormone responsive (SPOT14 homologue, rat); *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *BHLHE41*, basic helix-loop-helix family, member e41; *GPX7*, glutathione peroxidase 7; *UCA1*, urothelial cancer associated 1.

* Genes in cells in milk 1 month after delivery with ≥ 3 -fold difference in expression between the FOS and placebo groups.

† Mann-Whitney *U*-test.

peroxidase 7), *BHLHE41* (-3.63-fold; basic helix-loop-helix family, member e41), *CDKN2A* (-3.56-fold; cyclin-dependent kinase inhibitor 2A), *LOC645722* (-3.34-fold; similar to *bCG2037003*) and *bCG_1774568* (-3.24-fold; complementary DNA clone IMAGE:2959727) (Table 4).

Concentrations of IL-27, soluble CD14, IgA and transforming growth factor- β 1 in whey

Based on the microarray analysis results, samples obtained from the FOS group with high gene expression levels were scanned for cytokine IL-27, and then the protein concentrations of IL-27 in colostrum and breast milk samples were measured. The concentrations of IL-27 in colostrum samples were significantly higher in the FOS group (n 35; range: <0.156–46.6 ng/ml; median: 2.4 ng/ml) than in the placebo group (n 29; range: <0.156–95.8 ng/ml; median: 0.2 ng/ml) ($P = 0.027$, Fig. 4(a)). Comparison of 1-month breast milk samples revealed that the FOS group had significantly higher concentrations of IL-27 (n 35; range: <0.156–32.6 ng/ml; median: 1.7 ng/ml) than the placebo group (n 29; range: <0.156–23.4 ng/ml; median: 0.2 ng/ml) ($P = 0.040$, Fig. 4(b)). Conversely, there was no significant difference between the FOS and placebo groups with regard to the concentrations of soluble CD14 (median: 54.7 *v.* 56.8 μ g/ml; range: 21.3–91.1 *v.* 29.8–126.2 μ g/ml; $P = 0.36$), IgA (median: 1.07 *v.* 0.76 mg/ml; range: 0.03–0.33 *v.* 0.03–0.45 mg/ml; $P = 0.91$) and TGF- β 1 (median: 680 *v.* 753 pg/ml; range: 204–1700 *v.* 270–2777 pg/ml; $P = 0.14$) in colostrum samples. These results refer to the same sixty-four subjects.

Serum concentrations of IL-27

The serum concentrations of IL-27 tended to be higher in the FOS group than in the placebo group (median: 5.18 *v.*

1.69 ng/ml; range: 0.73–40.19 *v.* 0.33–65.74 ng/ml), but this difference was not statistically significant ($P = 0.084$).

Discussion

In the present study, it has been demonstrated for the first time that the consumption of FOS by pregnant and lactating

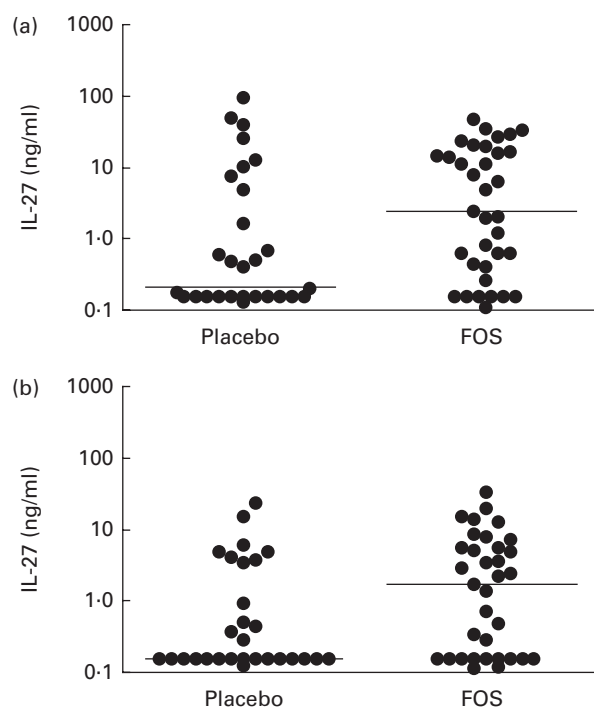


Fig. 4. Comparison of the concentrations of IL-27 in (a) colostrum samples and (b) 1-month milk samples obtained from the placebo and fructo-oligosaccharide (FOS) groups. The Mann-Whitney *U*-test was used to analyse differences between the experimental groups (bar, median) ((a) $P = 0.027$; (b) $P = 0.040$).

women increases the expression of various molecules in cells present in breast milk (five in colostrum samples and fourteen in 1-month breast milk samples). The function of most of these genes has not been well elucidated, except that of the genes related to oxidative stress (*GSTM5* and *GPX7*). Thus, differentially expressed genes were not directly associated with immune response; however, enhanced expression of the immunoregulatory cytokine IL-27 was observed at the transcript and protein levels.

IL-27 is produced by activated dendritic cells and macrophages⁽¹⁶⁾. The present study did not analyse the human milk cell phenotype, but because many macrophages are found in breast milk⁽¹⁷⁾, it is thought that macrophages are the main IL-27-producing cells in human milk.

The measurement of IL-27 concentrations in colostrum and maternal blood samples after delivery revealed no correlation (data not shown), suggesting that IL-27 in breast milk does not derive from serum, but is mainly produced by macrophages in breast milk. The protein concentrations of IL-27 were high in colostrum and 1-month breast milk samples obtained from the FOS group, whereas the microarray analysis of human milk cells revealed a significant difference in IL-27 expression only in 1-month breast milk samples. The reason for this discrepancy is unclear; however, one possible reason is that the colostrum and mature milk samples used for the microarray analysis were different, as only high-quality RNA was subjected to this analysis. Moreover, there is a high chance of inter-individual variations in the macrophage population of colostrum. The composition of colostrum varies widely between individuals, and there have been reports of large differences in cell type and proportion^(17–19). In contrast, 1-month breast milk production is more stable and subject to less inter-individual variation.

IL-27, together with IL-12, IL-23 and IL-35, belongs to the family of cytokines associated with T-cell differentiation^(20,21). IL-27 induces the differentiation of T helper (Th)1 cells, regulates the induction of Th17 cells, increases the production of IL-10^(22,23), and induces type 1 regulatory T cells (Tr1)^(24,25). In the absence of IL-27, the Th2 response is increased, and because the Th17 and Th2 immune responses are controlled by the administration of IL-27, a strong involvement in allergy and inflammation control is likely. In the immune system of the digestive tract, IL-27 is responsible for maintaining epithelial barrier function and is involved in anti-inflammatory and antibacterial activities⁽²⁶⁾. Enteritis has been shown to resolve in experimental mice given parenteral IL-27⁽²⁷⁾. Whether the oral administration of IL-27 is resistant to digestion is not well understood; therefore, it is important to analyse the biological activity of breast milk IL-27 in the infant digestive tract.

Some previous reports have indicated that the concentrations of soluble CD14, IgA, and TGF- β are increased in breast milk^(6,7). In contrast to the demonstrated effects of probiotics, no significant difference in colostrum TGF- β , total IgA and soluble CD14 concentrations between the FOS and control groups was observed in the present study. These data suggest that prebiotics may act differently when compared with probiotics.

In conclusion, the present study demonstrated the effect of the consumption of FOS by pregnant and lactating women on gene expression in cells present in breast milk, particularly the enhanced expression of the immunoregulatory cytokine IL-27. Furthermore, the increased concentration of IL-27 in whey suggests a significant biological effect. Future studies should investigate the correlation between IL-27 concentration in breast milk and allergic disorders in infants and the effect of the consumption of prebiotics by pregnant and lactating women on the prevention of allergic disorders.

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None of the authors has any conflicts of interest.

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