

## Effect of salmon consumption during pregnancy on maternal and infant faecal microbiota, secretory IgA and calprotectin

Heidi J. Urwin<sup>1</sup>, Elizabeth A. Miles<sup>2</sup>, Paul S. Noakes<sup>2</sup>, Lefkothea-Stella Kremmyda<sup>2</sup>, Maria Vlachava<sup>2</sup>, Norma D. Diaper<sup>2</sup>, Keith M. Godfrey<sup>2,3,4</sup>, Philip C. Calder<sup>2,4</sup>, Jelena Vulevic<sup>5</sup> and Parveen Yaqoob<sup>1\*</sup>

<sup>1</sup>Hugh Sinclair Unit of Human Nutrition, Department of Food and Nutritional Sciences, Whiteknights Campus, PO Box 226, The University of Reading, Reading RG6 6AP, UK

<sup>2</sup>Human Development and Health Academic Unit, Faculty of Medicine, University of Southampton, Southampton SO16 6YD, UK

<sup>3</sup>Medical Research Council Lifecourse Epidemiology Unit, University of Southampton, Southampton SO16 6YD, UK

<sup>4</sup>NIHR Southampton Biomedical Research Centre, University of Southampton, University Hospital Southampton NHS Foundation Trust, Southampton SO16 6YD, UK

<sup>5</sup>Clasado Research Services Limited, Science and Technology Centre, The University of Reading, Reading RG6 6BZ, UK

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### Abstract

The gut microbiota plays an important role in the development of the immune and gastrointestinal systems of infants. In the present study, we investigated whether increased salmon consumption during pregnancy, maternal weight gain during pregnancy or mode of infant feeding alter the markers of gut immune defence and inflammation. Women ( $n$  123) who rarely ate oily fish were randomly assigned to continue consuming their habitual diet or to consume two 150 g portions of farmed salmon per week from 20 weeks of pregnancy to delivery. Faecal samples were collected from the mothers ( $n$  75) at 38 weeks of gestation and from their infants ( $n$  38) on days 7, 14, 28 and 84 post-partum. Fluorescence *in situ* hybridisation was used to determine faecal microbiota composition and ELISA to measure faecal secretory IgA (sIgA) and calprotectin concentrations. There was no effect of salmon consumption on maternal faecal microbiota or on maternal or infant faecal sIgA and calprotectin concentrations. The degree of weight gain influenced maternal faecal microbiota, and the mode of infant feeding influenced infant faecal microbiota. Faecal samples collected from infants in the salmon group tended to have lower bacterial counts of the *Atopobium* cluster compared with those collected from infants in the control group ( $P=0.097$ ). This difference was significant in the formula-fed infants ( $P<0.05$ ), but not in the exclusively breast-fed infants. In conclusion, the impact of oily fish consumption during pregnancy on maternal and infant gut microbiota composition is limited, but significant differences are associated with maternal weight gain during pregnancy and mode of infant feeding.

**Key words:** Gut microbiota: Infants: Long-chain PUFA: Pregnancy: Salmon: Oily fish

From birth onwards, there is rapid microbial colonisation of the gastrointestinal tract of infants<sup>(1)</sup>. The gut microbiota plays an important role in the priming and maturation of the immune system of infants<sup>(2,3)</sup>. It is influenced by many factors including the duration of gestation, mode of delivery, mode of infant feeding, hospitalisation, antibiotic treatment, geographical location<sup>(4–7)</sup> and, according to recent evidence, maternal pregnancy-associated weight gain and BMI<sup>(8)</sup>. Perturbations in the composition of the gut microbiota have been reported to be associated with inflammation and disease, including, but not limited to, allergies<sup>(9)</sup>, metabolic disorders<sup>(10)</sup>, CVD<sup>(11)</sup>, obesity<sup>(11)</sup> and Crohn's disease<sup>(12)</sup>. The consumption

of probiotics by women during pregnancy and/or early lactation has been reported to influence the microbiota of their infants<sup>(13,14)</sup>, which in some cases has lasting effects beyond the duration of the intervention<sup>(13,15)</sup>. Some studies have suggested that PUFA have an effect on faecal microbiota in animals<sup>(16–19)</sup>, in infants aged 9–18 months<sup>(20,21)</sup> and in adult humans<sup>(22)</sup>. To the best of our knowledge, there are no studies that have investigated the faecal microbiota within a population of pregnant women with increased oily fish consumption. The Salmon in Pregnancy Study (SiPS) is a randomised, controlled intervention investigating the effects of the consumption of two portions of salmon per week,

**Abbreviations:** BF, breast-fed; DAPI, 4,6-diamidino-2-phenylindole; FF, formula-fed; LC, long-chain; sIgA, secretory IgA; SiPS, Salmon in Pregnancy Study.

\* **Corresponding author:** Professor P. Yaqoob, email p.yaqoob@reading.ac.uk

providing 3.45 g/week EPA and DHA, from 20 weeks of pregnancy to delivery<sup>(23)</sup>. We have demonstrated higher levels of EPA and DHA in maternal and cord blood plasma<sup>(23)</sup> and in breast milk<sup>(24)</sup> in the salmon group in the SiPS. The primary focus of the study is the effect of long-chain (LC) *n*-3 PUFA from the salmon, although, as a whole food, salmon is a good source of vitamin D and Se. In the present study, we tested the hypotheses that increased consumption of salmon by pregnant women would affect the composition of their faecal microbiota and that of the faecal microbiota of their infants.

## Materials and methods

### Study design and subject characteristics

The SiPS is a single-blind, randomised, controlled intervention with salmon during pregnancy; the full study design and baseline subject characteristics have been reported previously<sup>(23)</sup>. In brief, women (*n* 123) who rarely ate oily fish and were at a risk of having a child with atopy because of a family history were randomly assigned to either continue consuming their habitual diet (control group; *n* 61) or consume farmed salmon and requested to consume two 150 g portions of salmon per week from 20 weeks of pregnancy to delivery (salmon group; *n* 62). The complete details of the farmed salmon have been reported previously<sup>(23)</sup>; the two portions of salmon typically provided 3.45 g of EPA plus DHA per week, equivalent to approximately 0.48 g/d EPA + DHA. The two portions of salmon also provided 28 µg of vitamin D and 86 µg of Se; contaminant levels were below the provisional weekly tolerable intake levels<sup>(23)</sup>. The study was conducted according to the guidelines laid down in the Declaration of Helsinki. All procedures involving human subjects were approved by the Southampton and South West Hampshire Research Ethics Committee (07/Q1704/43), and written informed consent was obtained from all the women. The SiPS is registered at <http://www.clinicaltrials.gov> (clinical trials identifier NCT00801502).

### Assessment of maternal and infant diets

Maternal diet was assessed using an administered 100-item FFQ at 20 and 34 weeks of gestation and 3 months post-partum. Women reported their frequency of consumption of the listed foods during the preceding 3 months. This FFQ has been validated previously in a group of pregnant women and was used to assess the diets of women in the Southampton Women's Survey<sup>(25)</sup>. For the SiPS, a section on the intakes of functional foods was added to record any regular consumption of prebiotics and/or probiotics. A daily record of the mode of infant feeding, i.e. breast-, formula- or mixed-fed, was made for the first 6 months post-partum, including details on the type of infant formula, if used, and any illnesses that the infants may have suffered<sup>(26)</sup>. Infant formula was described as standard or supplemented depending on whether prebiotics (fructo-oligosaccharides/galacto-oligosaccharides) had been added. All infant formulae contained DHA (5.8–7.0 mg/100 ml).

### Faecal sample collection and preparation

During the clinic visit at 38 weeks of gestation, freshly voided faecal samples were collected from the mothers. Faecal samples from infants were collected at their homes on days 7, 14, 28 and 84 post-partum from their nappies as soon as possible after defaecation, taking care to avoid collecting any faeces that had been mixed with urine. All the samples were immediately stored at  $-20^{\circ}\text{C}$  until processing. The samples were defrosted on ice, then diluted 1:10 (w/w) with PBS (0.1 M; pH 7.0) and mixed in a Stomacher400 (Seward) for 2 min at normal speed (460 paddle beats per min). Into a 50 ml sterile plastic centrifuge tube containing 2 g of glass beads (5 mm diameter), 5 ml of faecal slurry were transferred and vortexed for 30 s to further homogenise the samples. The samples collected from the mothers were subjected to centrifugation at 13 000 **g** for 2 min. The resulting suspensions from samples collected from infants and supernatants of the faecal samples collected from the mothers were immediately processed for the microbial analysis. For the determination of secretory IgA (sIgA) and calprotectin concentrations, two 1 ml aliquots of homogenates were frozen at  $-20^{\circ}\text{C}$ .

### Culture-independent enumeration of faecal bacteria using 16S ribosomal RNA probes labelled with cyanine 3 and fluorescence in situ hybridisation

Fluorescence *in situ* hybridisation was carried out as described previously<sup>(27)</sup> with some modifications. Briefly, faecal homogenate samples (375 µl) were fixed with ice-cold 4% filtered paraformaldehyde (pH 7.2; Sigma-Aldrich) at a ratio of 1:3 (v/v) at 4°C overnight. They were then centrifuged at 13 000 **g** for 5 min and washed twice with filtered PBS (1 ml). The resultant pellet was then re-suspended in 300 µl of filter-sterilised PBS–ethanol (1:1, v/v) and stored at  $-20^{\circ}\text{C}$  until analysis. For the hybridisations, 20 µl of each sample were pipetted into a well (10 mm diameter) of Teflon and poly-L-lysine-coated six-well slides (Tekdon, Inc.). The samples were dried on a desktop plate incubator (Amersham International) at 46°C for 15 min. To permeabilise the cells for use with probes Bif164, Lab158 and Ato291, the samples were treated with 20 µl of lysozyme (1 mg/ml in 100 mM-HCl, pH 8.0) for 15 min at 37°C and then briefly washed with distilled water (2–3 s). All the samples were dehydrated in a series of ethanol solutions (50, 80 and 96% ethanol in distilled water v/v) for 3 min each. The slides were dried for a further 2 min on a desktop plate incubator. The nucleic acid stain 4,6-diamidino-2-phenylindole (DAPI; Sigma Aldrich) was used for total cell counts. The group- or genus-specific probes used are listed out in Table 1: Bif164, specific for the *Bifidobacterium* genus; Lab158, for the *Lactobacillus*–*Enterococcus* group; Ato291, for the *Atopobium* cluster, including most Coriobacteriaceae species; Erec482, for the *Eubacterium rectale*–*Clostridium coccoides* group (*Clostridium* cluster XIVa); Bac303, for the *Bacteroides* and *Prevotella* group; CHis150, for the *Clostridium histolyticum* subgroup (*Clostridium* clusters I and II); EC1531, for *Escherichia coli*; FPr655, for the *Faecalibacterium prausnitzii* group

**Table 1.** 16S ribosomal RNA probes used in the fluorescence *in situ* hybridisation analysis

Targets*	Accession no.	Probe sequence (5'–3')
Bif164	pB-00037	CATCCGGCATTACCACCC
Lab158	†	GGTATTAGCAYCTGTTTCCA
Ato291	pB-00943	GGTCGGTCTCTCAACCC
Erec482	pB-00963	GCTTCTTAGTCARGTACCG
Bac303	pB-00031	CCAATGTGGGGGACCTT
Chis150	pB-00962	TTATGCGGTATTAATCTYCCTTT
Ec1531	†	CACCGTAGTGCCTCGTCATC
Fpra655	pB-00734	CGCTACCTCTGCACTAC

\* Probe designation according to probeBase (<http://www.microbial-ecology.net/probebase>). All the probes were used at a concentration of 50 ng/ml.

† No information related to these probes has been deposited in probeBase.

(*Clostridium* cluster IV). All the probes were labelled at the 5' end with the fluorescent dye cyanine 3 (Cy3) (Sigma Aldrich). The compositions of the hybridisation and wash buffers were as reported in probeBase<sup>(28)</sup> depending on the ribosomal RNA-targeted oligonucleotide probe used. The probe (5 µl of a 5 ng/µl stock) in hybridisation buffer (45 µl) was applied to the surface of each well, and hybridisation was carried out for 4 h using an ISO20 oven (Grant Boekel). The slides were washed with wash buffer (50 ml) containing DAPI (20 µl, 50 ng/µl) for 15 min, then dipped briefly in ice-cold water (2–3 s) and dried with compressed air. Prolong Gold Antifade (Invitrogen) (5 µl) was added to each well, and the well was covered with a coverslip. The slides were stored in the dark at room temperature for 24 h and then at 4°C until cell count using a SP460F fluorescence microscope (Brunel Microscopes Limited). An average count from thirty random fields of view was taken for the enumeration of bacterial cells<sup>(29)</sup>. Microbial counts are expressed as log<sub>10</sub> bacterial cells per g faeces (wet weight) or as a percentage of total bacteria (DAPI). The limit of detection was about log<sub>10</sub> 5.06.

#### Determination of secretory IgA concentrations in faeces

A commercially available ELISA kit was used for the determination of sIgA concentrations in faeces (Immundiagnostik AG). A faecal suspension (500 µl; 1:10, v/v faeces in 0.1 M-PBS) was centrifuged at 13 000 g for 5 min, and the resulting supernatant was used in the assay. The manufacturer's guidelines were followed. The plates were read at 450 nm with 620 nm as the reference wavelength on a GENios SpectraFLUOR plus (Tecan UK Limited). The limit of detection was 13.4 ng/ml, and the intra- and inter-assay CV were 3.0 and 14%, respectively.

#### Determination of calprotectin concentrations in faeces

A commercially available ELISA kit (Nova Tec Immundiagnostica GmbH) was used for the determination of calprotectin concentrations in faeces. A faecal suspension (500 µl; 1:10, v/v faeces in 0.1 M-PBS) was mixed at a ratio of 1:5 with the extraction buffer. In a sealed Sterilin tube, the sample was shaken on a Vibrax-VXR (IKA-Werke GmbH & Co. KG) for 30 min at 1000 rpm, and then the sample (1 ml) was

centrifuged at 10 000 g for 20 min at 4°C. The resulting supernatant was used in the assay. The manufacturer's guidelines were followed. The plates were read at 405 nm on a GENios SpectraFLUOR plus (Tecan UK Limited). The intra- and inter-assay CV were 3.5 and 13%, respectively.

#### Statistical analysis

Data were checked for normality with histograms and the Kolmogorov–Smirnov test; data were log-transformed to establish normality as required. Continuous data are expressed as means and their standard errors or medians and 25th–75th percentiles as appropriate. In the analysis of the composition of the faecal microbiota using fluorescence *in situ* hybridisation, when a bacterial group was not detected, the minimum detection value (log<sub>10</sub> 5.06 cells/g wet faeces) was applied for the statistical analysis. Infant feeding was categorised as exclusively breast-fed (BF) or exclusively formula-fed (FF) from day 2 post-partum. Infants who were mixed-fed were excluded from the analysis. The generalised linear mixed model was used to test the main effects of intervention, time post-partum and infant feeding and any interaction effects. Categorical data are expressed as number and proportion with comparisons using the  $\chi^2$  test or Fisher's exact test as appropriate. Correlations were carried out with Spearman's rank correlation or partial correlations on transformed data, adjusting for intervention, time post-partum or mode of infant feeding. The Statistical Package for Social Sciences version 20 (IBM Corporation) was used for all the statistical tests. In all cases, a *P* value < 0.05, after Bonferroni adjustment for multiple comparisons, was considered statistically significant.

## Results

### Subject characteristics

The characteristics of women who provided a faecal sample at 38 weeks of gestation are given in Table 2. Weights at the screening appointment (13 weeks of gestation) compared with those at the clinic appointment during the third trimester (34 weeks of gestation) were used to calculate weight gain during pregnancy. Normal weight gain was considered to be 11.4–15.9 kg for normal-weight women (BMI 18.5–24.9 kg/m<sup>2</sup>), 6.8–11.4 kg for overweight women (BMI 25.0–29.9 kg/m<sup>2</sup>) and 5.0–9.1 kg for obese women (BMI > 30.0 kg/m<sup>2</sup>)<sup>(30)</sup>. Total weight gains above these values were considered excessive. Age, parity, self-reported atopy (report of asthma or allergy in a first-degree relative of the unborn child), weight, weight gain and BMI did not differ significantly between the two groups (Table 2). There were no differences in the intake of total energy, protein, fat, SFA, MUFA, carbohydrates, dietary fibre and functional foods between the groups (Table 2), but there was an increased intake of oily fish (salmon), EPA and DHA<sup>(23)</sup> and two other oily fish-derived nutrients, vitamin D and Se in the salmon group (Table 2)<sup>(23)</sup>.

**Effect of salmon intervention and habitual diet on maternal microbiota composition**

There were no significant effects of increased oily fish consumption on any of the bacteria enumerated in maternal faecal samples, sampled at 38 weeks, expressed as either log<sub>10</sub> cells/g of wet faeces (Table 3) or as a percentage of total bacteria (data not shown).

**Influence of weight gain on maternal microbiota composition**

Women who had a normal weight gain had a median weight gain of 9.3 kg (7.3–11.2 kg; 25th–75th percentiles), which was lower ( $P < 0.001$ ) than that observed in women who had an excessive weight gain (median 13.8 kg (12.3–15.9 kg;

25th–75th percentiles)). The BMI values of women with normal and excessive weight gains were significantly different at both 13 weeks of gestation (median 22.8 *v.* 27.7 kg/m<sup>2</sup>;  $P < 0.001$ ) and 34 weeks of gestation (median 26.6 *v.* 33.1 kg/m<sup>2</sup>;  $P < 0.001$ ). The numbers of total bacteria ( $P = 0.045$ ; ratio 1.23) and bacteria of the *F. prausnitzii* group ( $P = 0.039$ ; ratio 1.91) in samples collected from women with a normal weight gain were higher than those present in samples collected from women with an excessive weight gain (Table 4). There were also trends towards higher numbers (log<sub>10</sub> cells/g wet faeces) of bacteria of the *Bifidobacterium* genus ( $P = 0.094$ ) and *Atopobium* cluster ( $P = 0.057$ ) in women with a normal weight gain (Table 4). There was a difference in the faecal microbiota of women with a normal weight gain compared with that of women with an excessive weight gain.

**Table 2.** Characteristics of the women who provided a faecal sample at 38 weeks of pregnancy (Mean values with their standard errors; median values with their 25th and 75th percentiles; number of participants and percentages)

	Control (n 32)		Salmon (n 43)*		P
	Median	25th–75th percentile	Median	25th–75th percentile	
Age (years)†					0.243
Mean		28.7		29.9	
SEM		0.9		0.6	
Weight at 13 weeks of gestation (kg)‡	66.9	60.5–82.0	64.1	60.2–75.4	0.208
Weight at 34 weeks of gestation (kg)‡	78.8	71.1–88.1	74.3	68.8–84.8	0.202
Weight gain during pregnancy (kg)‡§	10.4	7.0–12.7	10.0	7.9–12.3	0.711
Normal weight gain§					0.974
n		26		34	
%		81		81	
BMI at 13 weeks of gestation (kg/m <sup>2</sup> )‡	24.4	21.6–31.5	23.0	21.0–26.9	0.218
BMI at 34 weeks of gestation (kg/m <sup>2</sup> )‡	27.8	25.9–33.3	27.4	24.6–31.3	0.186
First pregnancy					0.423
n		13		21	
%		41		50	
Self-reported atopy					0.248
n		19		31	
%		59		72	
Total energy (kJ/d)‡	9451.5	7984.8–11686.3	8954.5	7436.0–9948.0	0.126
Protein (g/d)‡	84.0	68.4–98.8	78.9	65.5–89.9	0.416
Total fat (g/d)‡	88.1	71.6–88.1	84.5	66.3–99.7	0.428
SFA (g/d)‡	35.5	26.5–41.7	29.4	25.1–38.3	0.234
MUFA (g/d)‡	30.8	26.0–38.8	31.0	24.9–37.2	0.740
Carbohydrates (g/d)‡	285.5	252.2–370.7	272.1	224.7–301.0	0.075
Dietary fibre (g/d)‡	17.1	14.4–21.3	18.2	14.1–21.0	0.923
Vitamin D (µg/d)†					< 0.001
Mean		2.5		5.3	
SEM		0.1		0.1	
Se (µg/d)†					0.021
Mean		46.8		54.1	
SEM		2.6		1.8	
Probiotics – yes					0.476
n		4		8	
%		13		19	
Prebiotics – yes					1.000
n		2		2	
%		6		5	
Synbiotics – yes					1.000
n		1		2	
%		3		5	

\* Weight measurement and parity data missing for one woman in the salmon group.

† Values were compared with Student's *t* test.

‡ Values were compared with the Mann–Whitney *U*-test.

§ Weight gain between week 13 and week 34 of pregnancy.

|| Values were compared with the  $\chi^2$  test.

**Table 3.** Effect of salmon consumption on bacterial numbers (log<sub>10</sub> cells/g wet faeces) and secretory IgA (sIgA) and calprotectin concentrations (mg/kg wet faeces) in maternal faecal samples at 38 weeks of pregnancy (Median values with their 25th and 75th percentiles)

	Control (n 32)		Salmon (n 43)		P*
	Median	25th–75th percentile	Median	25th–75th percentile	
Total bacteria (DAPI)	10.87	10.75–11.01	10.85	10.74–11.01	0.568
Bif164	9.63	9.38–9.84	9.64	9.43–9.99	0.493
Lab158	8.25	7.87–9.01	8.27	7.82–8.94	0.772
Ato291	9.64	9.15–9.87	9.54	9.28–9.87	0.716
Erec482	9.89	9.64–10.15	9.92	9.70–10.16	0.549
Chis150	7.24	6.72–7.77	7.28	7.02–7.68	0.643
Bac303	9.13	8.93–9.41	9.23	8.92–9.44	0.427
Ec1531	7.09	6.54–7.46	7.24	6.78–7.69	0.364
Fpra655	9.34	9.17–9.69	9.39	9.16–9.61	0.972
Firmicutes:Bacteroidetes†	7.16	4.37–13.27	8.46	4.72–13.45	0.636
sIgA (mg/kg wet faeces)	981	452–1509	940	456–1831	0.820
Calprotectin (mg/kg wet faeces)	4.7	1.8–19.1	5.7	4.3–13.1	0.432

DAPI, 4,6-diamidino-2-phenylindole.

\* Mann–Whitney U-test.

† Firmicutes = Lab158 + Erec482 + Chis150 + Fpra655; Bacteroidetes = Bac303.

*Effect of salmon consumption on maternal faecal calprotectin and secretory IgA concentrations*

There were no effects of salmon intervention on maternal faecal calprotectin or sIgA concentrations (Table 3). There was also no influence of weight gain (normal *v.* excessive) on either calprotectin or sIgA concentrations (Table 4), and there was no correlation between breast milk sIgA concentrations<sup>(24)</sup> and maternal faecal sIgA concentrations ( $P=0.630$ ).

*Influence of mode of infant feeding on faecal microbiota composition*

The numbers of bacteria of the *Clostridium* cluster XIVa, *Atopobium* cluster, *C. histolyticum* subgroup and *Bacteroides*–*Prevotella* group in the faeces of the exclusively BF infants

were lower than those in the faeces of the exclusively FF infants ( $P=0.005$ ,  $0.050$ ,  $0.016$  and  $0.037$ , respectively). After stratification of the data for intervention, the numbers of bacteria of the *Atopobium* cluster in the faeces of the BF infants were significantly lower than those in the faeces of the FF infants in the control group ( $P=0.012$ ,  $P<0.05$  after adjustment for multiple testing), but this was not the case for those in the salmon group ( $P=0.671$ ). The numbers of bacteria of the *C. histolyticum* subgroup in the faeces of the BF infants were lower than those in the faeces of the FF infants in the salmon group ( $P=0.008$ ,  $P<0.05$  after adjusting for multiple testing), but this was not significant in the control group ( $P=0.647$ ). The numbers of bacteria of the *Clostridium* cluster XIVa in the faeces of the BF infants were lower than those in the faeces of the FF infants in the control group ( $P=0.006$ ,

**Table 4.** Effect of weight gain during pregnancy on bacterial numbers (log<sub>10</sub> cells/g wet faeces) and secretory IgA (sIgA) and calprotectin concentrations (mg/g wet faeces) in maternal faecal samples at 38 weeks of pregnancy (Median values with their 25th and 75th percentiles)

	Normal weight gain* (n 60)		Excessive weight gain* (n 14)		Ratio of normal:excessive weight gain	P†
	Median	25th–75th percentile	Median	25th–75th percentile		
Total bacteria (DAPI)	10.88	10.76–11.02	10.79	10.54–10.90	1.23	0.045
Bif164	9.65	9.47–9.88	9.47	8.86–10.10	1.51	0.094
Lab158	8.27	7.84–8.94	8.10	7.74–8.75	1.48	0.512
Ato291	9.60	9.30–9.88	9.25	8.91–9.69	2.24	0.057
Erec482	9.91	9.71–10.12	9.70	9.43–10.23	1.62	0.381
Chis150	7.28	6.84–7.73	7.24	7.12–7.42	1.10	0.767
Bac303	9.17	8.93–9.41	9.21	8.84–9.35	0.91	0.654
EC1531	7.14	6.54–7.56	7.19	6.54–7.84	0.89	0.755
Fpra655	9.47	9.17–9.69	9.19	8.13–9.48	1.91	0.039
Firmicutes:Bacteroidetes‡	8.46	4.94–13.45	5.52	3.81–10.93	1.53	0.177
sIgA (mg/g wet faeces)	995	564–1856	863	387–1278	1.15	0.349
Calprotectin (mg/g wet faeces)	5.8	3.4–17.8	5.1	2.9–9.3	1.14	0.693

DAPI, 4,6-diamidino-2-phenylindole.

\* Normal weight gains over pregnancy according to the Institute of Medicine<sup>(30)</sup>: 11.4–15.9 kg (BMI 18.5–24.9 kg/m<sup>2</sup>); 6.8–11.4 kg (BMI 25.0–29.9 kg/m<sup>2</sup>); 5.0–9.1 kg (BMI > 30.0 kg/m<sup>2</sup>).

† Mann–Whitney U-test.

‡ Firmicutes = Lab158 + Erec482 + Chis150 + Fpra655; Bacteroidetes = Bac303.

$P < 0.05$  after adjusting for multiple testing) but not in the faeces of those in the salmon group ( $P = 0.580$ ).

### Effect of salmon intervention on infant microbiota composition

There were no effects of maternal salmon consumption on the duration of gestation, birth weight or mode of delivery (Table 5), and there was no statistically significant difference in mode of infant feeding and/or formula type used between the control and intervention groups (Table 5). There was an increase over time in the number of total bacteria and of the individual bacterial groups enumerated, with the exception of *E. coli*, the bacterial counts of which decreased, and the *Atopobium* cluster, the bacterial counts of which did not change significantly during the sampling period (Table 6). In a primary analysis of infants who were exclusively BF or exclusively FF during the first 84 d post-partum, a trend towards lower numbers of bacteria of the *Atopobium* cluster ( $P = 0.097$ ) in the faeces of infants in the salmon group was observed. In a *post hoc* analysis, maternal salmon consumption during pregnancy was found to be associated with significantly lower numbers of bacteria of the

*Atopobium* cluster in the faeces of the exclusively FF infants ( $P < 0.05$ ), but not with those in the exclusively BF infants (Fig. 1).

### Effect of salmon intervention on infant faecal secretory IgA and calprotectin concentrations

Infant faecal sIgA concentrations were not significantly affected by maternal salmon consumption during pregnancy (data not shown). However, there was a main effect of mode of infant feeding and a mode of infant feeding  $\times$  time interaction ( $P < 0.001$ , for both) (Fig. 2); compared with those in the faeces of the FF infants, sIgA concentrations were higher in the faeces of the BF infants at all post-partum sampling times ( $P < 0.05$ , for all), and with time, faecal sIgA concentrations decreased in the BF infants but increased in the FF infants (Fig. 2). However, in the exclusively BF infants, there was no correlation between breast milk sIgA concentrations<sup>(24)</sup> and infant faecal sIgA concentrations ( $P = 0.579$ ; data not shown). There were no significant effects of salmon intervention or mode of infant feeding on faecal calprotectin concentrations (Fig. 2).

## Discussion

Herein, we report that increased maternal oily fish consumption during pregnancy is associated with a trend towards lower numbers of bacteria of the *Atopobium* cluster in the faeces of infants during the first 84 d post-partum. Exclusive breast-feeding was associated with lower numbers of bacteria of the *Clostridium* cluster XIVa, *Atopobium* cluster, *C. histolyticum* subgroup and *Bacteroides-Prevotella* group compared with exclusive formula feeding, in agreement with the findings of other studies<sup>(7)</sup>. However, in the salmon group, the numbers of bacteria of the *Atopobium* cluster in the faeces of the BF infants compared with those in the faeces of the FF infants were not significantly different, in contrast to previous findings<sup>(7)</sup>. It appears from the present study that the mode of infant feeding also had an impact on the effect of intervention, since the faeces of the FF infants in the salmon group had significantly lower numbers of bacteria of the *Atopobium* cluster than those of the FF infants in the control group, but this was not significant for the BF infants. In a study<sup>(20)</sup> where infants aged 9–18 months were supplemented with fish oil, the magnitude of the effect of intervention on the composition of the microbiota was also found to be affected by the mode of infant feeding. In that study, the composition of the faecal microbiota was determined using terminal restriction fragment length polymorphism. Terminal restriction fragments Bp100 and Bp102, both presumed to be related to Bacteroidetes, exhibited larger and smaller increases, respectively, during the course of intervention, in the faeces of the fish oil-supplemented group compared with those in the faeces of the sunflower oil-supplemented group, but this was only significant for those infants who had ceased breast-feeding before intervention<sup>(20)</sup>. In the present study, we did not find a significant difference in numbers of bacteria of the *Bacteroides-Prevotella* group between the groups. Furthermore, in a recent

**Table 5.** Characteristics of the infants from whom faecal samples were collected at days 7, 14, 28 and 84 post-partum

(Mean values with their standard errors; median values with their 25th and 75th percentiles; number of participants and percentages)

	Control (n 19)		Salmon (n 19)		P
	n*	%	n*	%	
Gestation duration (d)†					0.396
Mean	280		282		
SEM	2.3		1.8		
Birth weight (kg)†					0.985
Mean	3.61		3.61		
SEM	0.1		0.1		
Vaginal delivery	17	90	18	95	1.000
Mode of infant feeding					0.737
Breast-fed	13	68	11	58	
Formula-fed	6	32	8	42	
Breast milk DHA (mg/100 ml)					<0.001
Day 1 PP‡					
Median	4.6		6.6		
25th–75th percentile	2.6–6.9		4.6–11.7		
Day 5 PP‡					
Median	4.8		10.8		
25th–75th percentile	4.0–6.9		6.1–17.8		
Day 14 PP‡					
Median	5.9		8.9		
25th–75th percentile	3.6–8.5		6.4–17.2		
Day 28 PP‡					
Median	6.2		7.9		
25th–75th percentile	4.6–7.7		5.3–14.4		
Formula with prebiotics					0.627
Yes	4	67	4	50	
No	2	33	4	50	

PP, post partum.

\* Values were compared with the  $\chi^2$  test.

† Values were compared with Student's *t* test.

‡ Values were compared with the generalised linear mixed model.



**Table 6.** Effect of maternal salmon consumption on infant faecal bacterial numbers (log<sub>10</sub> cells/g wet faeces) for those infants who were exclusively breast-fed (BF) or exclusively formula-fed (FF)\* (Median values with their 25th and 75th percentiles)

		No. of days post-partum												Group	Time	Feed
		7 d			14 d			28 d			84 d					
		<i>n</i>	Median	25th–75th percentile	<i>n</i>	Median	25th–75th percentile	<i>n</i>	Median	25th–75th percentile	<i>n</i>	Median	25th–75th percentile			
<b>DAPI</b>																
Control	BF	7	10.26	10.11–10.47	10	10.71	10.49–10.90	9	10.84	10.33–10.89	8	10.67	10.16–10.99	0.516	0.002	0.039
Salmon	BF	6	10.13	9.85–10.69	8	10.55	10.35–11.14	7	10.62	10.35–11.05	7	10.87	10.38–11.09			
Control	FF	6	10.58 <sup>a</sup>	10.19–11.02	6	10.91 <sup>a</sup>	10.09–11.18	4	10.82 <sup>a</sup>	10.40–11.17	5	11.09 <sup>b</sup>	10.97–11.62			
Salmon	FF	7	10.69	10.21–10.91	6	10.80	10.63–11.05	8	10.66	10.57–11.02	7	10.74	10.25–10.93			
<b>Bif164</b>																
Control	BF	7	9.87	8.93–9.99	10	10.12	9.66–10.54	9	10.35	9.24–10.77	8	10.25	9.14–10.43	0.397	0.066	0.384
Salmon	BF	6	9.44	8.99–10.08	8	10.06	9.68–10.40	7	10.19	10.05–10.74	7	10.05	9.70–10.43			
Control	FF	6	9.95	5.06–10.75	6	10.46	9.41–10.85	4	10.58	9.90–10.86	5	10.85	10.54–10.99			
Salmon	FF	7	10.42	9.62–10.74	6	10.49	10.20–10.83	8	10.23	10.06–10.77	7	10.26	9.83–10.52			
<b>Lab158</b>																
Control	BF	7	8.10	6.97–8.71	10	7.67	7.17–8.52	9	7.54	6.54–9.08	8	8.09	7.50–8.38	0.765	0.041	0.340
Salmon	BF	6	7.28	6.43–8.79	8	7.32	5.06–8.68	7	8.14	6.84–9.05	7	8.25	7.94–8.86			
Control	FF	6	7.78	7.56–8.18	6	7.93	7.81–8.76	4	8.38	7.46–8.71	5	8.27	7.87–9.02			
Salmon	FF	7	7.72	5.06–8.93	6	8.89	8.24–9.09	8	8.45	7.52–8.89	7	7.62	7.54–8.79			
<b>Ato291</b>																
Control	BF‡	7	7.32	5.06–8.10	10	7.54	5.06–8.18	9	7.14	5.06–9.20	8	7.54	6.25–9.11	0.097	0.297	0.050
Salmon	BF	6	6.84	5.98–7.43	8	7.76	6.85–8.60	7	7.02	6.09–8.39	7	7.58	6.54–8.59			
Control	FF‡	6	9.22	8.00–10.07	6	9.52	6.72–9.96	4	9.62	9.16–10.09	5	8.62	7.72–10.14			
Salmon	FF	7	7.02	6.54–8.03	6	6.94	6.42–8.86	8	7.47	6.72–9.37	7	7.32	6.84–8.96			
<b>Erec482</b>																
Control	BF‡	7	6.84	5.06–7.39	10	7.54	5.06–8.28	9	6.37	5.06–7.97	8	7.90	7.28–8.46	0.234	<0.001	0.005
Salmon	BF	6	6.09	5.06–7.05	8	7.14	6.38–7.37	7	5.06	5.06–7.84	7	7.14	5.06–8.36			
Control	FF‡	6	7.77	7.50–8.06	6	8.11	7.85–8.84	4	8.18	7.70–9.35	5	9.09	8.48–10.09			
Salmon	FF	7	7.21	5.06–7.64	6	7.06	5.06–8.14	8	8.67	6.48–9.03	7	8.57	6.67–9.32			
<b>Chis150</b>																
Control	BF	7	5.06	5.06–6.84	10	6.72	6.25–7.09	9	7.02	5.06–7.18	8	7.66	6.09–8.12	0.556	0.002	0.016
Salmon	BF‡	6	5.06	5.06–6.42	8	5.06	5.06–6.25	7	6.55	5.06–7.47	7	7.14	7.02–7.58			
Control	FF	6	5.06	5.06–7.24	6	6.97	5.06–7.28	4	7.16	6.78–7.75	5	7.54	7.21–7.93			
Salmon	FF‡	7	7.77	5.06–8.28	6	6.77	5.06–7.95	8	7.56	7.02–8.31	7	7.14	6.54–7.39			
<b>Ec1531</b>																
Control	BF	7	8.16	7.54–8.35	10	7.19	5.06–8.78	9	7.02	6.54–8.32	8	7.39	7.02–8.50	0.181	0.033	0.280
Salmon	BF	6	7.28	6.54–8.41	8	6.84	6.25–7.62	7	6.84	6.72–8.26	7	6.54	7.02–7.24			
Control	FF	6	8.38	7.65–9.18	6	7.78	6.72–8.53	4	7.72	7.02–8.19	5	8.13	7.32–8.56			
Salmon	FF	7	7.75	7.02–9.09	6	8.41	7.09–9.01	8	7.80	7.14–8.83	7	5.06	5.06–8.53			
<b>Bac303</b>																
Control	BF	7	7.39	5.06–8.19	10	7.93	7.20–8.09	9	7.72	6.84–8.51	8	8.04	7.35–9.01	0.557	0.014	0.037
Salmon	BF	6	7.73	6.64–8.22	8	7.82	7.35–7.96	7	7.50	5.06–8.00	7	7.44	6.84–8.51			
Control	FF	6	7.28	5.06–8.56	6	7.97	6.94–8.51	4	7.14	6.26–8.16	5	8.19	7.51–8.70			
Salmon	FF	7	7.75 <sup>a</sup>	7.58–7.96	6	7.92 <sup>a,b</sup>	7.69–8.60	8	7.81 <sup>a,b</sup>	7.60–8.42	7	8.42 <sup>b</sup>	8.05–8.48			

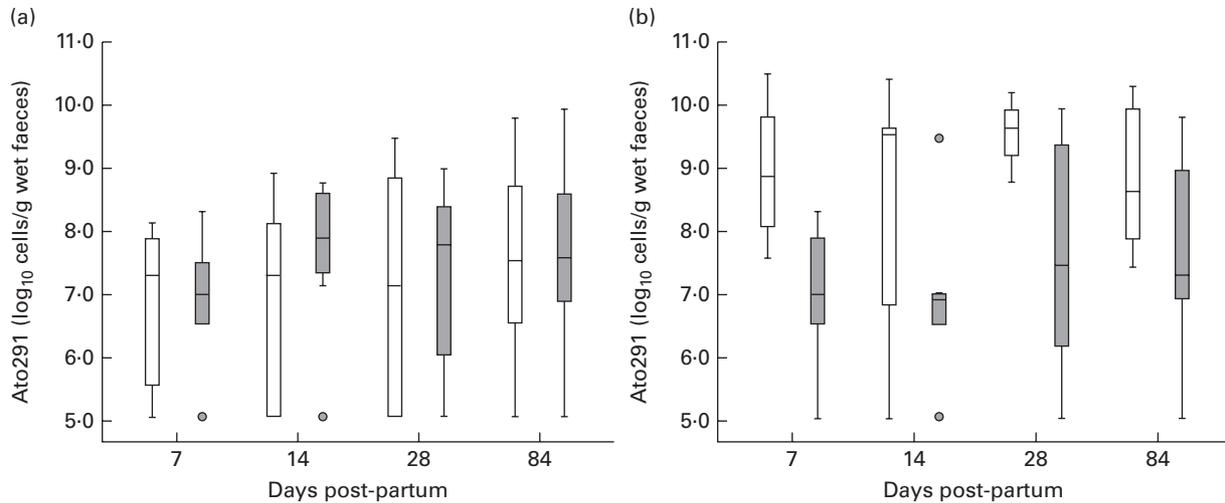
Maternal oily fish intake and the infant gut

<sup>a,b</sup> Values within a row with unlike superscript letters were significantly different ( $P < 0.05$ ).

\* Control ( $n = 19$ ) and salmon ( $n = 19$ ); samples were not available at all time points from all the infants. There were no significant group  $\times$  time interactions. Although there were significant effects of time and mode of infant feeding, there were a limited number of significant *post hoc* tests.

† Generalised linear mixed model.

‡ Significant difference between modes of infant feeding ( $P < 0.05$ ).



**Fig. 1.** Influence of maternal salmon consumption during pregnancy and mode of infant feeding on the numbers of bacteria of the *Atopobium* cluster (Ato291) in the faeces of infants. Statistical significance of intervention, time and mode of infant feeding was  $P=0.097$ ,  $0.297$  and  $0.050$ , respectively. In the *post hoc* analysis, the numbers of Ato291 in the (a) breast-fed infants were found to be not significantly affected by salmon consumption, but they were significantly affected in the (b) formula-fed infants (intervention:  $P<0.05$ ). □, Control group; ■, salmon group. Circles represent outliers.

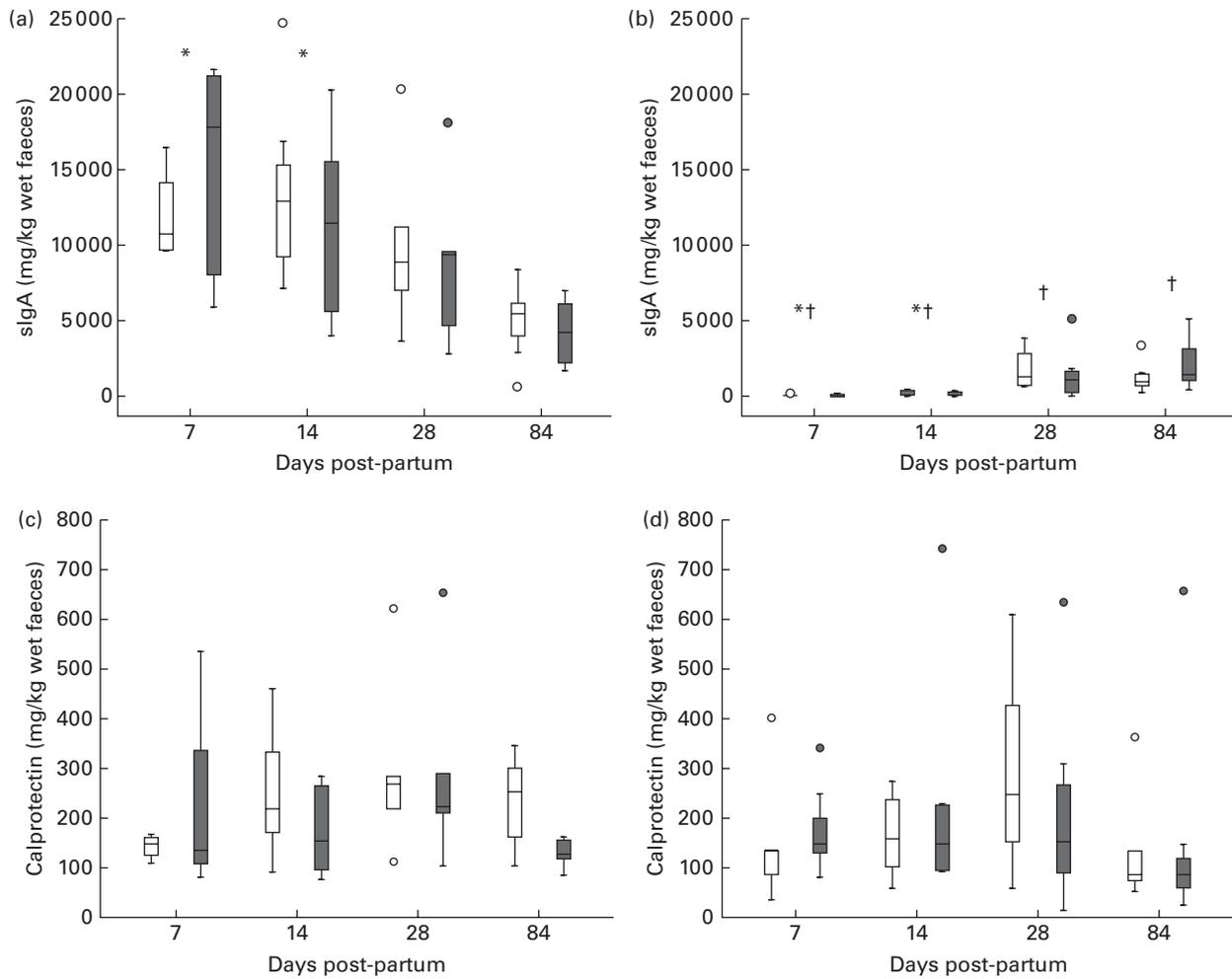
study in mice, *n-3* PUFA supplementation has been found to decrease the number of Bacteroidetes, but not to the same extent as SFA did, which had a greater overall influence on the shift in the composition of the faecal microbiota than PUFA did<sup>(17)</sup>. The effect of PUFA supplementation has also been investigated in piglet caeca<sup>(16)</sup>, where fish oil was found to be associated with higher numbers of bacterial types assumed to be Actinobacteria and Proteobacteria, while sunflower oil was found to be associated with a higher abundance of bacterial types assumed to be Bacteroidetes. It is important to acknowledge that in the previous studies, the provision of *n-3* PUFA was postnatal (i.e. directly to the infant), whereas in the present study, that of salmon, a source of *n-3* PUFA, was prenatal (i.e. indirectly to the fetus), although breast milk content of *n-3* PUFA was higher in the salmon group in the present study<sup>(24)</sup>. It is also important to note that although *n-3* PUFA might be mediating the effect of salmon feeding, the salmon used was also a good source of some other nutrients including vitamin D and Se, and these nutrients may have a role in any effect of the salmon.

The influence of the difference in the numbers of bacteria of the *Atopobium* group, as has been observed in the present study, and any effect that they may have on the maturation of the immune system and the longer-term health of infants are unknown, although higher bacterial counts of the *Atopobium* group have been found in children with active coeliac disease<sup>(31)</sup>. Further development and research on the bacterial communities and their effect on the host, such as the Microbiome Project<sup>(32)</sup>, will provide valuable information in the future.

The BF infants in the salmon group consumed milk enriched with LC *n-3* PUFA and lower in MUFA during the first month post-partum compared with controls<sup>(24)</sup>. The exclusively FF infants consumed milk supplemented with DHA, but at a concentration lower than that in the breast

milk of the salmon group. Since the exclusively FF infants were not exposed to breast milk, any possible effect of intervention in this group must have originated *in utero*. The mothers of infants in the salmon group had a higher intake of LC *n-3* PUFA, vitamin D and Se during pregnancy, and infants in the salmon group were born with a higher LC *n-3* PUFA status<sup>(25)</sup> and with some differences in cord blood immune cell phenotypes and cord blood immune response to endotoxins<sup>(33)</sup>. The increased LC *n-3* PUFA status<sup>(25)</sup> and altered immune response<sup>(33)</sup> may have had an impact on the success and viability of the initial colonisers of the gut, having made the environment more favourable for some species of bacteria. It has been found that when PUFA are added to intestinal epithelial cells, they are incorporated into the membrane phospholipid fraction and improve barrier integrity<sup>(34)</sup>, and higher concentrations of PUFA have been reported to alter bacterial adhesion sites<sup>(35)</sup>. There is recent evidence to suggest that microbiological exposure can take place *in utero*<sup>(14,36,37)</sup>. Since the effect of intervention is significant in the exclusively FF infants, this may, in part, be because breast-feeding *per se* and the associated predominant bifidus microbiota are influenced to a lesser degree by salmon intervention and to a greater degree by formula feeding. There is also the possibility that the differences between the FF infants in the control and salmon groups were due to differences in the types of infant formulae consumed, since formulae with and without supplementation of prebiotics (galacto-oligosaccharide and fructo-oligosaccharide) can influence the microbiota<sup>(38)</sup>. However, the records of infant feeding practices during the first 6 months post-partum show that there was no significant difference in their usage between the control and intervention groups<sup>(26)</sup>.

Faecal sIgA has a role in mucosal immunity, protecting the gastrointestinal tract from pathogenic invasion. The production of sIgA in infants is limited during the first month



**Fig. 2.** Influence of mode of infant feeding on (a, b) infant faecal secretory IgA (sIgA) concentrations (mg/kg faeces) and (c, d) infant faecal calprotectin concentrations (mg/kg faeces). (a) Breast-fed infants (time:  $P < 0.001$ ); (b) formula-fed infants (time:  $P < 0.001$ ); (c) breast-fed infants (time:  $P = 0.167$ ); (d) formula-fed infants (time:  $P = 0.068$ ). Statistical significance for the main effects of mode of infant feeding and time for sIgA was  $P < 0.001$  and  $0.012$ , respectively. \* Values were significantly different from day 84 post-partum ( $P < 0.05$ ). † Values were significantly different from breast-fed ( $P < 0.05$ ). Statistical significance of mode of infant feeding and time for calprotectin was  $P = 0.474$  and  $0.010$ , respectively. □, Control group; ■, salmon group. Circles represent outliers.

post-partum<sup>(39)</sup>, but the BF infants benefit from the supply in their mothers' milk<sup>(40)</sup>. Breast milk also contains prebiotic oligosaccharides that pass undigested into the lower gastrointestinal tract<sup>(41)</sup> and may also provide protection against potential pathogens<sup>(42)</sup>. Infants fed a formula supplemented with a blend of galacto-oligosaccharide and fructo-oligosaccharide have been found to have higher concentrations of sIgA in their faeces than those consuming an unsupplemented formula<sup>(43,44)</sup>. In the present study, sIgA concentrations in the faeces of the exclusively BF infants were higher than those in the faeces of the exclusively FF infants throughout the study period. However, the concentrations declined over time in the BF infants, perhaps reflecting the reduction in breast milk sIgA concentrations<sup>(24)</sup>. In contrast, in FF infants, faecal sIgA concentrations increased with time, perhaps reflecting the initiation of the infants' own production. Faecal calprotectin is a non-specific biomarker indicating the presence of intestinal inflammation<sup>(45)</sup>. Concentrations in healthy adults

and school-aged children are typically  $< 50$  mg/kg faeces, whereas those in infants are much higher<sup>(46)</sup>. The median faecal calprotectin concentration of the infants in the present study during the first 84 d post-partum was 146 mg/kg, range 13–743 mg/kg (data not shown), which is in agreement with previous findings<sup>(46)</sup>. We found no significant effect of mode of infant feeding on faecal calprotectin concentrations, which is in agreement with the findings of some studies<sup>(47)</sup>, but not with those of others, where elevated concentrations have been reported in exclusively BF infants<sup>(48)</sup>. The faecal calprotectin concentrations of the BF infants were higher than those of the FF infants, but large inter-individual variation and limited sample size possibly limited our ability to detect a significant difference. There was also no effect of salmon intervention on faecal calprotectin. A pilot study of patients with inflammatory bowel disease supplemented with seal oil, a source of *n-3* PUFA, found a decrease in disease activity, but faecal calprotectin concentrations were found to be unchanged<sup>(49)</sup>.

Salmon consumption during pregnancy did not have a significant effect on maternal faecal microbiota composition. There was no baseline sample before intervention, and therefore, it was not possible to determine any longitudinal effects due to pregnancy itself, but only possible to compare the control and intervention groups at 38 weeks of gestation. There have been recent reports on maternal faecal microbiota during pregnancy and, in particular, the effects of maternal BMI and weight gain<sup>(50,51)</sup>. It is, therefore, possible that the effects of pregnancy combined with the large inter-individual variation outweighed any effect of increased salmon consumption. When the women were divided according to pregnancy weight gain, there were higher numbers of total bacteria and bacteria of the *F. prausnitzii* group in those with a normal weight gain than in those with an excessive weight gain, irrespective of the intervention group. There was also a trend towards higher numbers of bacteria of the *Bifidobacterium* genus and the *Atopobium* cluster in women showing a normal weight gain. These findings concur with those of previous studies, which also reported trends towards higher numbers of *Bifidobacterium* in the faeces of women with a normal weight gain in pregnancy<sup>(50)</sup>. Salmon also had no effect on maternal faecal sIgA or calprotectin concentrations. The faecal calprotectin concentrations of all the mothers were below the upper limit for healthy adults (<50 mg/kg faeces).

In conclusion, salmon consumption during pregnancy had no significant effect on the maternal microbiota, but it is possible that the effect of dietary intervention was obscured by changes associated with pregnancy *per se*, including the effect of maternal weight gain. Salmon consumption was associated with a trend towards lower bacterial counts of the *Atopobium* cluster in the faeces of infants during the first 84 d post-partum, especially those who were not exclusively BF. Although this group of bacteria comprises a small proportion of the total microbiota during the early stages post-partum, this is a critical time for the development and maturation of the immune system of infants, and the study demonstrates the potential of maternal diet during both pregnancy and lactation to influence the infant gut microbiota. This should be explored further.

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The authors' contributions were as follows: E. A. M., K. M. G. and P. C. C. were responsible for designing the study and P. C. C. had overall responsibility for all aspects of the study; P. S. N., M. V., L.-S. K. and N. D. D. recruited and screened the volunteers, carried out the intervention and collected all the samples; H. J. U. carried out the laboratory analyses, supervised by P. Y. and J. V.; H. J. U. conducted the statistical analyses and drafted the manuscript; all authors contributed to and approved the final version of the manuscript.

P. C. C. serves on the Danone Scientific Advisory Board on Immunity and Allergy and on the Syral Scientific Advisory Board; acts as a consultant to the Danone Research Centre for Specialised Nutrition, Mead Johnson Nutritionals, Christian Hansen and Beneo-Orafti; has received speaking honoraria from Abbott Nutrition and Nestlé; and is currently receiving research funding from Abbott Nutrition and Beghin-Meiji. K. M. G. has acted as a consultant to Abbott Nutrition and Nestlé Nutrition and has received reimbursement for speaking at an Abbott Nutrition Conference on Pregnancy Nutrition and Later Health Outcomes and at a Nestlé Nutrition Institute Workshop. He is part of an academic consortium that has received research funding from Abbott Nutrition, Nestlé and Danone. J. V. is an employee of Clasado Research Services. P. Y. has received research funding from Cereal Partners UK. None of the other authors has any potential conflicts of interest.

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