Comparison of stool microbiota compositions, stool alpha1-antitrypsin and calprotectin concentrations, and diarrhoeal morbidity of Indonesian infants fed breast milk or probiotic/prebiotic-supplemented formula

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Aim: The composition of faecal microbiota of babies is known to be influenced by diet. Faecal calprotectin and alpha1-antitrypsin concentrations may be associated with mucosal permeability and inflammation. We aimed to assess whether there was any difference after consumption of a probiotic/prebiotic formula on faecal microbiota composition, calprotectin and alpha1-antitrypsin levels, and diarrhoea in comparison with breast milk-fed Indonesian infants.

Methods: One hundred sixty infants, 2 to 6 weeks old, were recruited to the study. They were either breastfed or formula fed (80 per group). Faecal samples were collected at recruitment and 3 months later. Bacterial groups characteristic of the human faecal microbiota were quantified in faeces by quantitative polymerase chain reaction. Calprotectin and alpha1-antitrypsin concentrations were measured using commercial kits. Details of diarrhoeal morbidity were documented and rated for severity.

Results: The compositions of the faecal microbiota of formula-fed compared with breast milk-fed children were similar except that the probiotic strain Bifidobacterium animalis subsp. lactis DR10 was more abundant after 3 months consumption of the formula. Alpha1-antitrypsin levels were higher in breastfed compared with formula-fed infants. The occurrence of diarrhoea did not differ between the groups of babies.

Conclusion: Feeding Indonesian babies with a probiotic/prebiotic formula did not produce marked differences in the composition of the faecal microbiota in comparison with breast milk. Detrimental effects of formula feeding on biomarkers of mucosal health were not observed.

Key words: alpha1-antitrypsin; Bifidobacterium animalis subsp. lactis; calprotectin; Indonesia; prebiotic; probiotic.

What is already known on this topic
1. Members of the bacterial genus Bifidobacterium have been found to predominate in the relatively simple faecal microbiota of infants in early life in studies of western babies.
2. Calprotectin and alpha1-antitrypsin levels in faeces may reflect intestinal permeability and inflammatory status of the bowel mucosa.
3. Probiotic/prebiotic formulas are generally considered safe.

What this paper adds
1. Bifidobacteria predominated in the faecal microbiota of both breast milk-fed and probiotic/prebiotic formula-fed infants in Indonesia.
2. Calprotectin and alpha1-antitrypsin levels did not indicate adverse effects of formula feeding.
3. Diarrhoeal morbidity of the infants was unaffected by formula feeding compared with breast milk feeding.

The large bowel of adult humans contains a natural microbial assemblage, mostly composed of bacteria, that has considerable phylogenetic diversity. This community, often referred to as the gut microbiota, uses non-digestible dietary components and other digestive tract waste products for growth. In so doing, additional energy, mostly in the form of short chain fatty acids, is captured for use by the human host. Both the metabolic activities and antigenicity of the microbiota have important physiological and immunological repercussions for the host.

A period of considerable instability in bowel microbiota composition occurs after birth but soon a predictable biological succession proceeds in which, by 3 months of age, members of the genus Bifidobacterium become the most abundant population in the infant faeces. Bifidobacterial abundance is generally found to be greater in the faeces of breast milk-fed babies and has been considered the bacteriological gold standard for the infant bowel for more than 100 years. Probiotic and prebiotic...
supplementation of cow milk-based formula is aimed at boosting the numbers of bifidobacteria in the infant bowel and is generally considered to be a safe practice in normal babies. A small number of reports have noted the incidence of diarrhoea (increase/decrease/no difference) in relation to the use of probiotic formulas. Therefore, we recorded the occurrence of diarrhoeal episodes in the infants in our study.

Alpha1-antitrypsin (AAT) is a serum protein that is resistant to enzymatic proteolysis in the gastrointestinal tract and is thus excreted in the faeces. As it is not present in the diet, faecal AAT (f-AAT) can reflect protein entering the intestine from the intravascular space. Determination of f-AAT has been considered as a reliable and inexpensive method for estimation of enteric protein loss.

Calprotectin is a calcium and zinc binding protein that makes up about 60% of neutrophil cytosolic protein, and is present in monocytes, macrophages and epithelial cells. Calprotectin is thought to regulate inflammatory processes and to have antimicrobial and anti-proliferative properties. Calprotectin is resistant to proteolysis and stable even at room temperature. Its concentration in stool is about six times that of plasma. High faecal calprotectin levels correlate with an increased turnover of leukocytes in the intestinal mucosa and granulocyte migration to the intestinal lumen. Faecal calprotectin levels have been reported to be much higher during the first few weeks of life both in healthy full-term and preterm infants than in healthy adults and older children.

There is still much to learn about the impact of formulas containing probiotic bacteria and prebiotics on the composition of the bowel microbiota, and the impact of the diet on mucosal barrier integrity and inflammation. Differences in the compositions of stool microflora of infants occur between geographical regions, including between babies in developed and developing countries. We hypothesized that, due to the incorporation of probiotic and prebiotic in the formula, bifidobacterial abundance in the faeces of the babies would resemble that of breastfed infants but that this would not result in adverse alterations to mucosal properties. Therefore, we compared the faecal microflora, faecal calprotectin, f-AAT concentrations and diarrhoeal morbidity of Indonesian children fed breast milk or probiotic/prebiotic formula.

### Methods

#### Study design

A prospective cohort study of 160 healthy infants (80 infants from Group 1 and 80 infants from Group 2) was undertaken in Jati Padang, Pasar Minggu district, a suburban area of South Jakarta, Indonesia (USA Clinical Trail Registry NCT01721512). Infants were recruited at 2–6 weeks of age and were divided into groups according to diet. Group 1 babies were formula fed, and Group 2 babies were breast fed. Infants were eligible for inclusion as breastfed if the mother intended to exclusively breastfeed her child from birth to at least 6 months. Exclusively breastfed infants were included in the formula-fed group if the following criteria were satisfied: the mother had been exclusively feeding her infant with formula milk before being enrolled to the study protocol and that the mother consented to her infant receiving infant formula for 12 months. In both groups, the infant was a healthy full-term infant with gestational age of 37–42 weeks, and birthweight ≥2.5 kg and ≤4.75 kg. Exclusion criteria were: severe congenital or metabolic disease likely to affect infant feeding or infant growth, or multiple birth.

Infants in the formula-fed group received an infant formula (Annum Infacare 1 infant formula; Table 1) supplied by Fonterra Brands (Singapore) Pte Ltd (Singapore). This formula contained probiotic bacteria (Bifidobacterium animalis subspecies lactis HN019; DR10), gangliosides, prebiotic (fructosyl oligosaccharides) and long-chain polyunsaturated fatty acids (LCPUFA).

### Table 1 Annum Infacare formula

<table>
<thead>
<tr>
<th>Nutritional information</th>
<th>Unit</th>
<th>Codex</th>
<th>Annum Infacare formula</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Min (100 kcal)</td>
<td>Max (100 kcal)</td>
</tr>
<tr>
<td>Protein</td>
<td>g</td>
<td>1.8</td>
<td>3</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>g</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>Fructo-oligosaccharide</td>
<td>mg</td>
<td>Permitted but levels not defined by Codex Standard</td>
<td>98</td>
</tr>
<tr>
<td>Fat</td>
<td>g</td>
<td>4.4</td>
<td>6</td>
</tr>
<tr>
<td>Linoleic acid (LA)</td>
<td>mg</td>
<td>300</td>
<td>GUL = 1400</td>
</tr>
<tr>
<td>α-linolenic acid (ALA)</td>
<td>mg</td>
<td>70</td>
<td>N/S</td>
</tr>
<tr>
<td>LA/ALA ratio</td>
<td></td>
<td>5 to 1</td>
<td>15 to 1</td>
</tr>
<tr>
<td>Docosahexaenoic acid (DHA)</td>
<td>mg</td>
<td>N/S</td>
<td>GUL = 0.5% of total fatty acid</td>
</tr>
<tr>
<td>Arachidonic acid (AA)</td>
<td>mg</td>
<td>N/S</td>
<td>If DHA is added AA must be added at least the same amount</td>
</tr>
<tr>
<td>Milk fat (Gangliosides)</td>
<td>mg</td>
<td>Codex permits the addition of milk fat to infant formula</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Energy: Codex states; Infant formula prepared ready for consumption in accordance with instructions of the manufacturer shall contain per 100 ml not less than 60 kcal (250 kJ) and not more than 70 kcal (295kJ) of energy. Codex Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants (Codex Stan 72–1981). GUL, Guidance Upper Limit; N/S, not specified.
The babies in this group received infant formula exclusively from within 6 weeks of birth and continued to at least 6 months of age. The infant formula contained 3.1 × 10⁷ bacteria/100 g (6.1 × 10⁷/100 kcal; 4.5 × 10⁷/100 mL) of probiotic strain DR10. Intake of strain DR10 per day was estimated to range from 2.17 × 10⁶ to 4.88 × 10⁶ probiotic bacteria during this period. The formula is sold in Indonesia, priced similarly to other brands, and complies with regulatory requirements for products aimed at supporting the growth and development of normal infants that are not breastfed. In the breastfed group, mothers were encouraged to continue breastfeeding until 6 months. Exclusive milk feeding for both groups (breast milk and formula) was defined as the infant receiving only milk and no other liquids or solids with the exception of drops or syrups consisting of vitamins, mineral supplements or medicines.

The complete study encompassed eight visits (6 weeks; 2, 3, 4, 6, 8, 10, 12 months) during the course of 12 months. Birthweight, length and head circumference, mode of delivery, gestational age at birth, feeding history at study entry, the parents’ characteristics (age, education, occupation, smoking status), family size, water supply, toilet facilities, number of families sharing the house, and socio-economic status were recorded for all babies. All episodes of hospitalisation, symptoms of respiratory infection or gastrointestinal infection, days of fever, vaccination history, and antibiotic usage were documented and rated for severity. Details of the growth and development of the babies during the complete 12 months study period will be reported separately.

The focus of this report is on assays conducted with faeces that were collected at recruitment and 3 months later. Bifidobacteria are generally predominant in the microbiota at this latter time, and stool frequency and consistency were reported by mothers for the 3 days prior to each visit up to the fourth month. For these observations, mothers were provided with the Bristol Stool Form Scale to compare and score stools. Stool consistency was defined as hard, formed-soft or liquid. Stool colour was designated as either brown, green, yellow or black. Diarrhoea was defined as the passage of three or more loose or watery stools in 24 h. Diarrhoea severity was scored as: 1) mild (no more than 4 stools, not excessively thin or watery), 2) moderate (4 or more stools, thin or watery), and 3) severe (at least 4 stools, and profound in character). Two stool samples were collected at the time of enrollment (V0) and 3 months later (V3). Samples were obtained from diapers and put in sterile plastic tubes, then stored at –80°C until analysis. A 100-mg faecal sample was added to a tube containing 300 mg of zirconium beads (diameter, 0.1 mm) and 1 mL of sterile TN150 buffer (10 mM Tris-HCl, 150 mM NaCl (pH 8)). The faecal material was mixed by vortexing and bacterial cells were sedimented by centrifugation (14 600 × g, 5 min, 4°C). The supernatant was discarded and the pellet was suspended in 1 mL of TN150 buffer. The tubes were placed in a mini-bead beater (Biospec Products, Bartlesville, OK, USA), shaken at 5000 rpm for 3 min, cooled on ice for 1 min and centrifuged at 14 600 × g (5 min, 5°C). Five hundred microlitres of the supernatant was extracted sequentially with 500 µL of TE buffer (10 mM Tris, 1 mM EDTA (pH 8.5))-saturated phenol and with chloroform-isoamyl alcohol (24:1). The sequential phenol and chloroform extractions were repeated a further two times. The cleaned DNA was precipitated overnight by the addition of two volumes of cold ethanol and a 0.1 volume of 3 M sodium acetate, at −20°C. The preparations were centrifuged at 14 600 × g (20 min, −5°C) and the pellets were dried at room temperature and then dissolved in 50 µL of TE buffer. The DNA solution was diluted 1/20 with water prior to polymerase chain reaction (PCR).

Real-time quantitative PCR

The aim was to compare the compositions of faecal microbiotas, with respect to commonly occurring phylogenetic groups of bacteria, from infants fed breast milk or the formula feed. Real-time quantitative PCR (qPCR) was carried out using an ABI 7500 Fast system in MicroAmp Fast Optical 96-Well plates with optical adhesive film (Applied Biosystems, Foster City, CA, USA). Primers targeting the 16S rRNA genes of all bacteria, selected bacterial groups common in the human faecal microbiota, and primers specific for B. animalis subsp. lactis, were purchased from Invitrogen (Life Technologies NZ Ltd, Auckland, New Zealand) and are listed in Table 2. All reactions were carried out in a final volume of 20 µL containing 1× Fast SYBR® Green PCR Mastermix (Applied Biosystems), 300 nM of each primer and 2 µL of template DNA. The thermocycling programme consisted of an initial activation of the polymerase at 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Fluorescence levels were measured after the 60°C annealing/extension step. A melt curve was generated to analyse product specificity. Standard curves were generated using genomic DNA extracted from B. animalis subsp. lactis HN019 (DR10) using the Qiagen DNeasy blood and tissue kit (Valencia, CA, USA) and following the gram-positive bacteria protocol. The standard DNA was quantified spectrophotometrically using a Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA) and diluted in 10-fold steps from 5 × 10¹ to 5 × 10¹⁰ genomes/reaction, calculated using target gene copies per genome obtained from genome sequence information (NCBI). All reactions were carried out in duplicate and were run twice on separate plates. No-template controls were also included on each plate. The qPCR results were normalised according to the total 16S rRNA gene target abundance, as measured by qPCR using primers targeting all bacteria.

Analysis of f-AAT and calprotectin in stools

The aim was to determine the relationship of infant-feeding practices to the faecal calprotectin and f-AAT concentrations in infants younger than 6 months of age. Calprotectin and f-AAT were measured in duplicate faecal aliquots using a commercial enzyme-linked immunoassay (Phical, Immunodiagnostik, Ben- sheim, Germany).
Statistical analysis

Categorical data were recorded as counts and percentages. Depending on the numbers for each combination, \( \chi^2 \) test, Fisher’s exact test or Mantel–Haenszel test was used to test for independence of factors (SPSS 17.0, SPSS Inc., Chicago, IL, USA). Effects of treatment group and visit number on faecal microbiota composition, calprotectin and AAT concentrations were tested using a mixed model approach to repeated measures analysis of variance (SAS 9.1, Cary, NC, USA), followed by Tukey’s test for pairwise comparisons. Raw data were log\(_{10}\) transformed to achieve homogeneity of variance, if required, and results are presented as least-squares means and 95% confidence interval.

Results

Subjects

The infants in both groups were similar with respect to age, weight, body length, head circumference and gender at recruitment. They were mostly from middle-class families (per capita income USD 1026–12 475). The birthweight (\( P = 0.022 \)) and weight at recruitment (\( V_0; P = 0.003 \)) were significantly higher in the breastfed group compared with the formula-fed group (Table 3). There was no significant difference between groups according to route of delivery (Table 3). Dropout rate was 6.25% (5/80; family relocated) for the breastfed group and 13.75% (11/80) for the formula-fed group. Dropout from the formula-fed group was due to family relocation (5 babies) and non-compliance (6 babies). Non-compliance was due to refusal of formula (4 babies), mixed formula and breast milk (1 baby), and no stool sample at \( V_3 \) (1 baby).

Faecal microbiota compositions

The primer sets that we used targeted the taxonomic groups of bacteria commonly present in human faeces.\(^{20}\) These groups have often been quantified using fluorescent DNA probes in both adult and infant microbiotas, but qPCR has also been used.\(^{18,21}\) Coverage of the members of the microbiota was approximately 60% in our study, similar to that reported by others in Asian infants.\(^{21}\) A recent phylogenetic study of the faecal microbiotas of formula-fed infants using pyrosequencing of 16S rRNA genes showed that, on average, 10 bacterial families represented 94% of the bacterial community. Numerous other bacterial groups at very low abundance (<1%) were present and these were not measured in our study.\(^{22}\) Bifidobacterial populations predominated in the faecal microbiotas at both sampling times of both breast milk-fed and formula-fed infants (average 44%). The proportions of bifidobacteria in the microbiotas did not differ between sampling times or between groups (Fig. 1a, \( P > 0.05 \)). \( B. \) animalis subsp. lactis was present in very low abundance in the infant groups except for formula-fed infants sampled at \( V_3 \). This greatly increased abundance would be due to the consumption of the probiotic strain DR10 which belongs to this species (Fig. 1b, \( P \leq 0.05 \)). \( B. \) animalis subsp. lactis comprised about 42% of total bifidobacterial targets at \( V_3 \) and was detected in 97% of the babies.

Other bacterial groups did not differ between breast milk-fed or formula-fed groups at \( V_0 \) (Fig. 2a, \( P > 0.05 \)). Enterococci were more abundant in the faeces of formula-fed babies compared with breast milk-fed babies at \( V_1 \) but were nevertheless present as a very small proportion of the microbiota (Fig. 2b, \( P \leq 0.05 \)). Lactobacilli had decreased in abundance at \( V_3 \) compared with \( V_0 \) in both dietary groups (Fig. 2a,b, \( P \leq 0.05 \)).
Diarrhoeal morbidity

The incidence of diarrhoea did not differ between groups (Table 3).

Faecal calprotectin and f-AAT

One hundred forty-four faecal samples were analysed for both calprotectin and AAT levels. The breast milk-fed infants showed an increase ($P = 0.002$) in AAT levels from V0 to V3 and these levels were greater than for formula-fed infants (Table 4). In contrast, AAT levels did not increase between V0 and V3 in formula-fed babies. Faecal calprotectin values did not differ between groups or sampling times (Table 4).

Discussion

Bifidobacterial abundance is generally found to be greater in the faeces of breast milk-fed babies compared with formula-fed babies. However, this was not observed in our study of Indonesian infants in which bifidobacterial abundance did not differ between dietary groups. A study of faecal microbiota compositions of Asian babies showed similar abundances of bifidobacteria in the stool of Indonesian infants to those recorded in our study. The abundance of bifidobacteria does appear to differ between babies in different geographical regions although they are always predominant. This bifidobacterial predominance occurs regardless of diet. The kinds of bifidobacteria that are detected in faeces, as shown in this study, can be influenced by the consumption of probiotic/prebiotic formula as B. animalis subsp. lactis abundance was greatest in babies that had been fed formula for 3 months. Whether the effect was due entirely to the consumption of probiotic strain DR10 could not be determined because the prebiotic component of the formula might have stimulated the growth of other strains of B. animalis subsp. lactis which, as reported by Wickens et al. and confirmed by our study, is present at low abundance in the faeces of babies.

Other populations were not affected by the nature of the diet except that enterococci had greater abundance in babies fed formula. The abundance of these bacteria in the formula-fed infants was, even so, still low (5%). Increased enterococcal populations have been recorded in association with the administration of probiotic lactobacilli but this phenomenon has not been investigated further and so is of unknown significance.

Determination of f-AAT has been considered as a suitable, reliable and cheap method for estimation of enteric protein loss and hence a marker for possible increased intestinal leakiness. Our study provided an interesting result because the f-AAT levels in the breast milk-fed infants were greater than the formula-fed group at both sampling times. Levels also increased from V0 to V3 within the breast milk-fed group itself.

Using endoscopic techniques to obtain biopsies from healthy infants, Thompson et al. compared intestinal morphometry in infants of 2–6 months of age who were entirely breast milk-fed or formula fed, and found that crypt depth was increased by 30% in formula-fed infants. This increased depth was accompanied by an increase of mitotic count per crypt of nearly 200%. Therefore, it seemed that formula feeding induced gut hypertrophy and possibly rendered the intestinal barrier less permeable to larger proteins and other potential antigenic molecules. This might explain our f-AAT findings in the breast milk-fed infants who continued to have more permeable intestinal barriers without evidence of gut hypertrophy. Similarly, breast milk contains a number of factors (including secretory IgA, cytokines and fatty acids) that can modulate and promote the development of both the immune system and intestine in infants and young children which could also account for these f-AAT results and is deserving of further study and investigation.
Faecal calprotectin levels have been reported to be higher during the first few weeks of life both in healthy full-term and preterm infants than in healthy adults and children, suggesting that the intestinal mucosa in young infants is potentially at risk for inflammatory events. This may have a longer-term impact upon the health and well-being of the child later in life. In contrast to our findings with f-AAT, neither breastfeeding nor formula feeding appeared to have any negative impact upon the calprotectin levels measured in the stools of infants at times V0 and V3. This suggested that there was no gross evidence of intestinal inflammation.

**Fig. 1** (a) Bifidobacterial abundance shown as proportion of the total microbiota in breast milk-fed and formula-fed infants at enrollment (V0) and at 3 months of age (V3). Least squares means and 95% confidence intervals are shown. (b) B. animalis subsp. lactis abundance shown as proportion of the total microbiota in breast milk-fed and formula-fed infants at enrollment (V0) and 3 months later (V3). Least squares means and 95% confidence intervals are shown. Abundance at V3 of formula-fed group was greater (\( P \leq 0.05 \)) than at V0.

**Fig. 2** (a) Abundances of various bacterial groups shown as proportion of the total microbiota in breast milk-fed and formula-fed infants at enrollment (V0). Least squares means and 95% confidence intervals are shown. (b) Abundances of various bacterial groups shown as proportion of the total microbiota in breast milk-fed and formula-fed infants 3 months later (V3). Least squares means and 95% confidence intervals are shown. Enterococci had greater abundance in faeces of formula-fed infants at V3 (\( P \leq 0.05 \)) than at V0.

**Conclusions**

Overall, feeding Indonesian babies with a probiotic/prebiotic formula did not produce marked differences in the composition of the faecal microbiota in comparison with the breast milk-fed ‘gold standard’. Moreover, detrimental effects of formula feeding on biomarkers of mucosal health were not observed.
Therefore, the data that we obtained supported our hypothesis that bifidobacterial abundance in the faeces of the babies would resemble that of breast-fed infants but that this would not result in adverse alterations to mucosal properties. *B. animalis* subsp. *lactis* abundance in faeces was boosted by consumption of the probiotic/prebiotic formula and was detected in the majority of the formula-fed infants after 3 months administration.

### References


### Table 4  Faecal calprotectin and α1-antitrypsin concentrations

<table>
<thead>
<tr>
<th>Visit</th>
<th>Breastfed</th>
<th>Formula-fed</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calprotectin mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V0</td>
<td>136.34 (113.13–159.55)</td>
<td>141.15 (117.16–165.14)</td>
<td>0.393</td>
</tr>
<tr>
<td>VS</td>
<td>134.67 (110.86–158.48)</td>
<td>158.18 (133.53–182.83)</td>
<td></td>
</tr>
<tr>
<td>α1-Antitrypsin mg/dL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V0</td>
<td>97.66 (60.56–134.77)</td>
<td>38.04 (–0.32–76.39)</td>
<td>0.019</td>
</tr>
<tr>
<td>VS</td>
<td>195.19 (157.35–233.03)</td>
<td>61.70 (22.25–101.15)</td>
<td></td>
</tr>
</tbody>
</table>

Results are least-squares means and 95% confidence interval; P-value refers to the interaction between group and visit.