Human Breast Milk and Infant Formulas Differentially Modify the Intestinal Microbiota in Human Infants and Host Physiology in Rats

Zhenmin Liu,1 Nicole C. Roy,5,6 Yanhong Guo,4 Hongxin Jia,4 Leigh Ryan,5 Linda Samuelsson,5 Ancy Thomas,7 Jeff Plowman,7 Stefan Clerens,8 Li Day,5 and Wayne Young5*

1State Key Laboratory of Dairy Biotechnology, Dairy Research Institute, Bright Dairy and Food Co. Ltd., Shanghai, China; 2Food Nutrition and Health Team, Food and Bio-Based Products Group, AgResearch Ltd., Palmerston North, New Zealand; 3Riddet Institute, Massey University, Palmerston North, New Zealand; 4Proteins and Biomaterials Team, Food and Bio-Based Products Group, AgResearch Ltd., Christchurch, New Zealand; and 5Biomolecular Interaction Centre, University of Canterbury, Christchurch, New Zealand

Abstract

Background: In the absence of human breast milk, infant and follow-on formulas can still promote efficient growth and development. However, infant formulas can differ in their nutritional value.

Objective: The objective of this study was to compare the effects of human milk (HM) and infant formulas in human infants and a weanling rat model.

Methods: In a 3 wk clinical randomized controlled trial, babies (7- to 90-d-old, male-to-female ratio 1:1) were exclusively breastfed (BF), exclusively fed Synlait Pure Canterbury Stage 1 infant formula (SPCF), or fed assorted standard formulas (SFs) purchased by their parents. We also compared feeding HM or SPCF in weanling male Sprague-Dawley rats for 28 d. We examined the effects of HM and infant formulas on fecal short chain fatty acids (SCFAs) and bacterial composition in human infants, and intestinal SCFAs, the microbiota, and host physiology in weanling rats.

Results: Fecal Bifidobacterium concentrations (mean log copy number ± SEM) were higher (P = 0.003) in BF (8.17 ± 0.3) and SPCF-fed infants (8.29 ± 0.3) compared with those fed the SFs (6.94 ± 0.3). Fecal acetic acid (mean ± SEM) was also higher (P = 0.007) in the BF (5.5 ± 0.2 mg/g) and SPCF (6.3 ± 2.4 mg/g) groups compared with SF-fed babies (4.3 ± 0.2 mg/g). Colonic SCFAs did not differ between HM- and SPCF-fed rats. However, cecal acetic acid concentrations were higher (P = 0.001) in rats fed HM (42.6 ± 2.6 mg/g) than in those fed SPCF (30.6 ± 0.8 mg/g). Cecal transcriptome, proteome, and plasma metabolite analyses indicated that the growth and maturation of intestinal tissue was more highly promoted by HM than SPCF.

Conclusions: Fecal bacterial composition and SCFA concentrations were similar in babies fed SPCF or HM. However, results from the rat study showed substantial differences in host physiology between rats fed HM and SPCF. This trial was registered at Shanghai Jiao tong University School of Medicine as XHEC-C-2012-024. J Nutr 2016;146:191-9.

Keywords: breast milk, infant formula, metabolomics, microbiota, transcriptomics

Introduction

Human milk (HM)9 contains a wide range of components that makes it the optimal source of nutrients for growing infants.

1 This study was supported by funding from Bright Dairy and Food Co. Ltd., Shanghai, China.
2 Author disclosures: Z Liu, Y Guo, and H Jia are employees of Bright Dairy and Food Co. Ltd.; NC Roy, L Ryan, L Samuelsson, A Thomas, J Plowman, S Clerens, L Day, and W Young, no conflicts of interest.
3 Supplemental Figure 1 is available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
4 Abbreviations used: BF, breastfed; FDR, false discovery rate; GSEA, gene set enrichment analysis; HM, human milk; IDF, International Dairy Federation; ISO, International Organization for Standardization; SF, standard formula; SPCF, Synlait Pure Canterbury Stage 1 infant formula.
5 To whom correspondence should be addressed. E-mail: wayne.young@agresearch.co.nz.

However, many circumstances can arise that preclude breast feeding, leading to the use of ruminant milk–based infant formula to meet nutritional requirements. In these situations, the formulation and composition of infant formula have important consequences for the growing young; therefore, maximization of the nutritional properties of ruminant milk–based formula could provide important health benefits. An ideal infant formula would closely match the composition of HM. However, to develop new and improved formulations that can better mimic the benefits of HM, it is critical to better understand the differences in physiologic responses to HM and infant formula.

HM is thought to have superior effects on the growth and barrier integrity of the intestinal tract, and on the development of mucosal defenses (1, 2), compared with infant formulas (3). Breast feeding has also been shown to drive the intestinal
microbiota toward a more beneficial community composition dominated by *Bifidobacterium* and *Lactobacillus* (4). These microbiota changes are mediated, at least in part, by milk oligosaccharides that cannot be digested by the infant, but are instead used by select members of the large bowel microbiota (5, 6). Other milk components that shape the infant microbiota include secreted IgA, antimicrobial proteins such as CD14, cytokines, and FAs (7). In humans, the microbial composition at birth is characterized by low diversity and rapid changes (4). However, a more complex and adult-like community is usually established by 1–2 y of age (8), the composition of which is believed to influence the risk and severity of allergy (7, 9) and obesity (10).

To gain a better understanding of how different infant formulas affect the large bowel intestinal microbial community and metabolism, we conducted a study examining fecal SCFA concentrations and fecal bacterial composition in babies assigned to 1 of 3 groups: 1) a group fed exclusively HM from breastfeeding, 2) a group fed exclusively Synlait Pure Canterbury Stage 1 infant formula (SPCF), and 3) a group fed exclusively a variety of standard formulas (SFs) sourced at the discretion of the parents. Considering the increasing evidence showing that the intestinal microbiota plays a key role in human health, we used a weaning rat model to study the effects of feeding either HM or a cow milk–based SPCF on cecal and colonic microbiota composition, SCFA concentrations, and mucosal architecture. We also assessed the differences in cecal gene and protein expression and plasma metabolomics profiles.

**Methods**

**Collection of breast milk.** HM samples for nutritional profiling and rat studies were collected from 900 volunteer Chinese mothers concurrent with the postnatal check at a number of hospitals within the Shanghai city area. The procedure for HM collection and the human clinical study was approved by the Xinhua Hospital Ethics Committee Affiliated to Shanghai Jiao tong University School of Medicine (approval no. XHEC-C-2012–024).

The samples were collected in 4 batches, from between 60 and 92 volunteers per batch, and freeze-dried. The pooled freeze-dried HM was packaged and transferred to the AgResearch laboratories in New Zealand for analyses and rat studies. The samples were stored at −20°C upon their arrival. Before use for composition analysis and the rat feeding study, samples of the pooled HM were prepared by reconstitution of the freeze-dried milk (13 g) in a volume of water (100 mL) consistent with the starting volume of fresh milk (wt:vol).

**Infant formula.** The infant formula used for the controlled infant formula cohort of the human study and the rat feeding study was SPCF (Synlait), constituted as per the manufacturer’s recommended guidelines (7.5 g/50 mL) before use. Before reconstitution, the SPCF was stored at 4°C.

**Human clinical study.** A total of 461 volunteers from the Shanghai city area were recruited between December 2013 and April 2014, of which 120 babies (male-to-female ratio: 1:1) between 7 and 90 d of age were selected for the study. The study protocol was explained in detail to the mothers, and informed written consent was obtained before study enrollment. The babies were randomly assigned by the study doctor with the use of computer-generated random numbers to 1 of 3 groups, with 40 babies in each group (Figure 1). Babies were excluded if they were one of multiple births; had a low or high birth weight; had a gestational age <37 wk; suffered from a serious disease such as neonatal sepsis, pneumonia, heart failure, or other disease; had a mother with pregnancy complications; or had a risk of potential metabolic diseases, chronic diseases, congenital malformations, central nervous system disorders, neuromuscular disease, or bone metabolism disease. The first group of babies was breastfed (BF), the second group was fed SPCF, and the third group was fed a variety of SFs purchased at the discretion of the parents. Fecal samples were collected from all participants at day 1 (baseline) and day 21, with a maximum of 30 g from each baby, which were kept in an anerobic container, sealed and frozen before analyses for SCFAs (acetic, propionic, and butyric acid) and detection of bacteria by qPCR.

Nutritional composition analysis was carried out by Eurofins NZ Laboratory Services (International Accreditation New Zealand-accredited) with the use of standardized methods for measuring total solids [International Organization for Standardization (ISO) 6731/International Dairy Federation (IDF) 22:2008], protein (ISO 8968/IDF 20–2:2001), carbohydrate (1.2.8.FSANZ [Food Standards Australia/New Zealand] calculation), energy [1.2.8.FSANZ (Food Standards Australia/New Zealand) calculation], and sugar profile [Association of Official Analytical Chemists (AOAC) 980.13/2012/1992].

**Rat study.** At the start of the study, male Sprague Dawley rats sourced from the AgResearch Ruakura Research Centre breeding colony, were weaned and individually caged at 21 d of age. The rats were divided randomly between 2 treatment groups (n = 12), which were fed HM or SPCF. The milk samples were prepared freshly 2 times/d at a concentration of 130 g milk solid/L H₂O, with the rats consuming them ad libitum for 28 d in place of drinking water. During this period, all rats were fed a modified AIN-93M (11) rodent maintenance diet containing beef protein instead of milk proteins. Body weight and milk and food intake were monitored throughout the study period. All rats were kept under 12 h light cycles with a constant environmental temperature of
21–22°C. After the study period of 28 d, the rats were killed by CO2 inhalation and cervical dislocation, after which plasma and intestinal tissue and contents from the cecum and colon were collected for later analysis. This study was conducted under the oversight of the AgResearch Grasslands Animal Ethics Committee (approval number AEC13099) according to the New Zealand Animal Welfare Act of 1999.

**SCFA analysis.** SCFA concentrations in rat cecal and colonic content samples and baby fecal samples were measured with the use of a previously described capillary GC method (12).

**Baby fecal bacteria analysis.** Baby fecal DNA was extracted with the use of TIANamp Stool DNA kits according to the manufacturer’s instructions. Bacterial counts of *Bifidobacterium, Lactobacillus*, and *Clostridium perfringens* were determined by qPCR with the use of Toyobo SYBR Realtime PCR Master Mix and the following primer sets, respectively: BFID-F (5’-CTCTGTGAAACGGTG-3’) and BFID-R (5’-GGTTTCTTCCGATATCTACA-3’); LAC-F (5’-GGATTTTCTCACAATGGAAGC-3’) and LAC-R (5’-CGCTTTTAGCGCCGATAAATCCGGG-3’); and CPF-F (5’-CGGTATGAGATGGACCGGACG-3’) and CP-R (5’-GGCTTTCACCACGTACAC-3’). Amplification was carried out with the use of the following thermocycling conditions: 95°C for 3 min; 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min each cycle, followed by a final extension step of 10 min.

**Rat cecal and colonic microbiota composition.** DNA was extracted from cecal and colon content samples with the use of NucleoSpin Soil kits (Macherey Nagel) for bacterial composition analysis. The V456 region of the bacterial 16S ribosomal RNA gene was amplified with the use of forward and reverse primers with the GS FLX B adapter sequence and template-specific sequence (CTATGCCCTTCCTGCGGCACTCA TNNNNNNNN ANGGGCACCAGCCGGGTTAA, with “N” indicating barcode nucleotides), and reverse primers with the GS FLX A adapter sequence and template-specific sequence (CTATGCCCTTCCTGCGGCACTCAC GCRRC ACGAGCTGACGAC) according to the New Zealand Animal Welfare Act of 1999. Baby fecal DNA was extracted with the use of the extraction method described by Knoch et al. (16). RNA from 8 randomly selected rats from each of the HM and SPCF groups and each tissue (cecum and colon) were used as templates for the synthesis of Cy3- and Cy5-labeled complementary RNA probes with the use of Low RNA Input Linear Amplification kits (Agilent Technologies). The labeled probes were hybridized to 4 × 44K Agilent Whole Rat Genome Oligo Microarrays (Agilent Design ID 028282) with the use of a loop design with dye swap. Intensity ratios for all microarray spots were normalized with the use of a global loess algorithm in Bioconductor (17) with the limma package (18). Transcripts were analyzed by gene set enrichment analysis (GSEA), which is the analysis of changes in groups of genes treated as a unit rather than as single genes. The advantage of GSEA over traditional per gene analyses is that rather than treating each gene as an independent agent (which in a biological sense is unlikely to be true), GSEA aggregates the per gene statistics across genes within a gene set, making it possible to detect circumstances in which all genes in a predefined set change in a small but coordinated way (19, 20). In this study, the predefined gene sets used were Reactome *Rattus norvegicus* pathways (21).

**Rat cecal and colon tissue gene expression.** Gene expression analysis of cecal tissue was analyzed by microarray as follows. Total RNA for microarray analysis was extracted from tissue samples with the use of the extraction method described by Knoch et al. (16). RNA from 8 randomly selected rats from each of the HM and SPCF groups and each tissue (cecum and colon) were used as templates for the synthesis of Cy3- and Cy5-labeled complementary RNA probes with the use of Low RNA Input Linear Amplification kits (Agilent Technologies). The labeled probes were hybridized to 4 × 44K Agilent Whole Rat Genome Oligo Microarrays (Agilent Design ID 028282) with the use of a loop design with dye swap. Intensity ratios for all microarray spots were normalized with the use of a global loess algorithm in Bioconductor (17) with the limma package (18). Transcripts were analyzed by gene set enrichment analysis (GSEA), which is the analysis of changes in groups of genes treated as a unit rather than as single genes. The advantage of GSEA over traditional per gene analyses is that rather than treating each gene as an independent agent (which in a biological sense is unlikely to be true), GSEA aggregates the per gene statistics across genes within a gene set, making it possible to detect circumstances in which all genes in a predefined set change in a small but coordinated way (19, 20). In this study, the predefined gene sets used were Reactome *Rattus norvegicus* pathways (21).

**Rat cecum and colon tissue proteomics analysis.** Samples of the cecum and colon from rats fed HM or SPCF were pooled within their respective treatment groups. Proteins were extracted by homogenizing the samples in 5 mL lysis buffer (7 mol/L urea, 2 mol/L thiourea, and 1% dithiothreitol; pH 8.5) and the supernatant was collected for 2D-differential in-gel electrophoresis analysis. The protein samples were labeled with Cy3 or Cy5 cyanine dye and combined with a Cy2-labeled internal standard before being applied to immobilized pH gradient strips (pH 3–11 nonlinear; 24 cm) for separation along 2 dimensions by isoelectric focusing. Gels were scanned with the use of a Typhoon FLA 9500 scanner (GE Healthcare) at excitation/emission wavelengths for each of the CyDyes (Cy3, 523/580 nm; Cy5, 633/670 nm; and Cy2, 488/520 nm) at 100 μm resolution.

Gel images were analyzed with the use of Delta2D v4.5 (Decodon). The differentially expressed spots were excised from the gel for in-gel digestion and analyzed by LC-tandem MS. This was performed on a nano-Advance ultrahigh-pressure LC coupled to a Bruker Daltonik amaZon speed ETD ion trap mass spectrometer equipped with a CaptiveSpray ion source (Bruker Daltonik) operated at 1400 V. Samples were measured in data-dependent tandem MS mode. Four precursors were selected from each respective MS spectrum over the range 310–1400 m/z followed by 3 tandem MS spectra acquired during each acquisition cycle.

**TABLE 1** Macronutrient composition of single samples of pooled HM collected from 900 volunteers and SPCF1

<table>
<thead>
<tr>
<th>Macronutrient</th>
<th>HM</th>
<th>SPCF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total solids, %/100 g</td>
<td>12.7</td>
<td>12.9</td>
</tr>
<tr>
<td>Fat, %/100 g</td>
<td>3.32</td>
<td>3.67</td>
</tr>
<tr>
<td>Protein, %/100 g</td>
<td>1.10</td>
<td>1.46</td>
</tr>
<tr>
<td>Lactose, %/100 g</td>
<td>8.10</td>
<td>7.37</td>
</tr>
<tr>
<td>Calculated energy, kJ/100 g</td>
<td>282</td>
<td>287</td>
</tr>
</tbody>
</table>

1. HM, human milk; SPCF, Synlait Pure Canterbury Stage 1 infant formula.
6. Calculated by 1.2.8.FSANZ calculation.

**TABLE 2** SCFA concentrations in infants who were BF or fed SPCF or SFs for 3 wk

<table>
<thead>
<tr>
<th>Acid</th>
<th>BF</th>
<th>SPCF</th>
<th>SF</th>
<th>P2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic, mg/g</td>
<td>5.5 ± 0.2a</td>
<td>5.3 ± 2.4a</td>
<td>4.3 ± 0.2b</td>
<td>0.007</td>
</tr>
<tr>
<td>Propionic, mg/g</td>
<td>1.4 ± 0.1</td>
<td>1.4 ± 0.6</td>
<td>1.4 ± 0.1</td>
<td>0.93</td>
</tr>
<tr>
<td>Butyric, mg/g</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.5</td>
<td>0.8 ± 0.1</td>
<td>0.53</td>
</tr>
</tbody>
</table>

1. Values are means ± SEMs, n = 35–38. Labeled means in a row without a common letter differ, P < 0.05. BF, breastfed; SF, standard formula; SPCF, Synlait Pure Canterbury Stage 1 infant formula.
2. Determined by Kruskal-Wallis ANOVA.
and plasma metabolites were analyzed by Kruskal-Wallis ANOVA.

Milk nutritional composition. The total solids, fat, protein, and ash contents of the reconstituted HM and SPCF samples are summarized in Table 1, together with the sugar profile and total carbohydrates and energy by calculation. The total solids, protein, and fat in the SPCF were within the range reported for infant formula (29).

Human study fecal microbial composition. Reflecting the changes seen in fecal SCFA concentrations, fecal Bifidobacterium copy numbers were significantly higher (P = 0.001) in the ceca of rats fed HM than in rats fed SPCF (Table 4). However, no significant differences in SCFA concentrations were detected in colon contents between the HM and SPCF groups.

Cecal and colonic microbiota composition in rats fed HM and SPCF. Consumption of the different diets led to noticeable differences in the cecal and colonic microbiota composition of rats fed HM or SPCF as shown by the principal coordinate analysis scores of the Unifrac phylogenetic distances (Figure 2), and the differences were largely driven by differences in relative abundance of Prevotella. At the genus level, Prevotella, Dorea, Mucispirillum, unclassified Clostridales, unclassified Porphyromonadaceae, unclassified Enterobacteriaceae, and unclassified Bacteroidales showed significantly different mean relative abundances in the cecum (Table 5). Among these taxa, Dorea, unclassified Enterobacteriaceae, and unclassified Clostridales were assessed with the use of a Student’s t test. GSEA was implemented with the use of the mroast function in the limma package, which uses a rotation, a Monte Carlo based approach for multivariate regression (28).

Differences between treatments were deemed significant when P values or FDR were < 0.05.

Results

Table 4 SCFA concentrations in the cecal and colon contents of rats fed HM or SPCF for 28 d

<table>
<thead>
<tr>
<th>Region and diet</th>
<th>Acetic acid</th>
<th>Propionic acid</th>
<th>Butyric acid</th>
<th>Lactic acid</th>
<th>Succinic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HM</td>
<td>18.1 ± 1.1</td>
<td>5.2 ± 0.5</td>
<td>2.2 ± 0.2</td>
<td>1.8 ± 1.4</td>
<td>0.8 ± 0.5</td>
</tr>
<tr>
<td>SPCF</td>
<td>16.8 ± 1.2</td>
<td>5.6 ± 0.5</td>
<td>2.4 ± 0.3</td>
<td>1.8 ± 1.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Cecum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HM</td>
<td>42.6 ± 2.6*</td>
<td>10.8 ± 0.6</td>
<td>6.2 ± 0.6</td>
<td>2.4 ± 0.9</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>SPCF</td>
<td>30.6 ± 0.8</td>
<td>9.7 ± 0.5</td>
<td>5.2 ± 0.8</td>
<td>3.4 ± 1.6</td>
<td>0.6 ± 0.2</td>
</tr>
</tbody>
</table>

*Values are means ± SEMs, n = 12. *Different from SPCF (Kruskal-Wallis ANOVA, P = 0.001). HM, human milk; SPCF, Synlait Pure Canterbury Stage 1 infant formula.
TABLE 5  Relative abundance of bacterial groups at the lowest identified taxonomic level in the cecum and colon of rats fed HM or SPCF for 28 d

<table>
<thead>
<tr>
<th>Region and phylum</th>
<th>Identification 2</th>
<th>HM, %</th>
<th>SPCF, %</th>
<th>p 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cecum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Dorea [g]</td>
<td>1.04 ± 0.28</td>
<td>0.24 ± 0.06</td>
<td>0.005</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Clostridiales [o]</td>
<td>14.1 ± 1.34</td>
<td>5.10 ± 1.20</td>
<td>0.005</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Enterobacteriaceae [f]</td>
<td>0.08 ± 0.06</td>
<td>ND</td>
<td>0.005</td>
</tr>
<tr>
<td>Definibacteriae</td>
<td>Mucispirillum [g]</td>
<td>0.03 ± 0.02</td>
<td>ND</td>
<td>0.009</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Bacteroidales [a]</td>
<td>0.34 ± 0.06</td>
<td>0.86 ± 0.22</td>
<td>0.015</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Prevotella [g]</td>
<td>4.76 ± 2.32</td>
<td>29.7 ± 8.61</td>
<td>0.020</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Porphyromonadaceae [a]</td>
<td>13.8 ± 1.94</td>
<td>7.69 ± 2.00</td>
<td>0.044</td>
</tr>
<tr>
<td>Colon</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Clostridiales [o]</td>
<td>11.7 ± 1.39</td>
<td>4.45 ± 1.01</td>
<td>0.005</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Coprobacillus [g]</td>
<td>0.54 ± 0.14</td>
<td>0.17 ± 0.04</td>
<td>0.015</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>Collinsella [g]</td>
<td>7.04 ± 1.71</td>
<td>2.76 ± 0.05</td>
<td>0.020</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Anaerotrunuc [g]</td>
<td>0.04 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>0.020</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Enterobacteriaceae [f]</td>
<td>0.03 ± 0.01</td>
<td>ND</td>
<td>0.020</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Eubacterium [g]</td>
<td>0.07 ± 0.02</td>
<td>0.18 ± 0.04</td>
<td>0.034</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Dorea [g]</td>
<td>0.51 ± 0.004</td>
<td>0.22 ± 0.09</td>
<td>0.049</td>
</tr>
</tbody>
</table>

1 Values are means ± SEMs, n = 12. f, family; g, genus; HM, human milk; ND, not detected at a sampling depth of 1349 sequences; o, order; SPCF, Synlait Pure Canterbury Stage 1 infant formula.
2 Lowest taxonomic classification identified.
3 Determined by permutation ANOVA.

were also significantly different in the colon (Table 5). Chao1 α diversity analysis showed that the cecal and colonic microbial communities in rats fed HM were more diverse than those in rats fed SPCF (HM, 190 ± 44.2; SPCF, 158 ± 17.1; mean Chao1 index ± SEM, P = 0.049).

Rat body weight and milk and food intake. All rats remained healthy and gained weight normally throughout the study. As expected, all rats showed a significant weight gain over the course of the study within the range expected of growing rats (P < 0.001), but no significant difference in weight gain was observed between the 2 groups after 28 d (P = 0.18). Daily milk intake (Figure 3A) was significantly higher in rats fed SPCF (P < 0.001). However, solid food consumption (Figure 3B) was higher in rats fed HM (P < 0.001).

Rat cecum and colon histology. All rats had cecal and colonic mucosal architecture typical of young weaning rats. No significant differences were observed in scores for cellular infiltration by immune cells, mucosal hyperplasia, goblet cell frequency, or crypt depth.

Rat cecal and colonic tissue gene and protein expression. Microarray analysis of gene expression by GSEA showed the different effects of HM and SPCF on pathways in the cecum and colon (Supplemental Figure 1). GSEA involves the analysis of overall expression of groups of genes, in this instance the Reactome pathways, treated as a unit rather than as individual genes. Pathways more highly expressed in the cecal tissue of rats fed HM included those implicated in gut-brain axis signaling (e.g., Axon guidance and γ-minobutyric acid receptor activation) and cell surface glycan metabolism and signaling (e.g., L1 cell adhesion molecule interactions, mucopolysaccharides, and glycosaminoglycan metabolism). Pathways more highly expressed in rats fed SPCF included numerous immune system- and cell cycle–related pathways, such as the downregulation of TGF-β receptor signaling, IL receptor Src homology 2 domain containing transforming protein 1 signaling, anaphase promoting complex: cell-division cycle protein 20-mediated degradation of mitotic proteins, and cell cycle checkpoints. Differences in pathways were more pronounced in the cecum than in the colon (Supplemental Figure 1).

Proteomics analysis of cecal tissue identified 7 proteins that differed in expression levels between rats fed HM or SPCF, whereas expression of 8 proteins were found to differ in the colon (Table 6). In the cecum, the structural protein keratin K8 was more highly expressed in SPCF-fed rats, whereas hemoglobin subunit a2 protein was more highly expressed in HM-fed rats. In the colon, hemoglobin subunit a2, serum albumin precursor, and actin cytoplasmic 2 were more highly expressed in rats fed SPCF.

Rat plasma metabolomics. LC-MS analysis of plasma metabolites resolved 5290 and 5050 mass features in negative and positive ionization mode, respectively. Sparse partial least squares discriminant analysis scores showed that the plasma metabolome profiles of HM- and SPCF-fed rats were distinct (Figure 4). Six mass features showed significantly different signal of these (FDR < 0.05), 2 were putatively characterized by accurate mass: C16 sphingosine-1-phosphate and PGE2-p-acetamidophenyl ester, both of which had significantly higher signal intensities (FDR = 0.02 and 0.03, respectively) in rats fed SPCF (Figure 5).

Discussion
This study shows that exclusive breastfeeding or SPCF feeding had similar effects on babies’ fecal SCFA concentrations (an indication of microbial metabolism) and on abundance of 3 groups of bacteria: Bifidobacterium, Lactobacillus, and Clostridium perfringens. In contrast, concentrations of acetic acid...
and Bifidobacterium in feces were lower in babies from the SF group (infant formulas purchased at the discretion of the parents). Previous reports have shown that fecal concentrations of acetic acid in BF babies are higher than those in formula-fed babies (30, 31). However, the similarity of the SPCF used in our study to HM, at least in the variables examined here, shows the potential for infant formula, in general, to be made more similar to HM. In the rat study, colonic SCFA concentrations were also similar between HM- and SPCF-fed rats, which mirrored the results seen in the baby fecal SCFA concentrations. However, it must be noted that luminal SCFA concentrations only represent the balance between SCFA production and epithelial absorption; it is conceivable that 2 systems with very different rates of both could have the same net luminal SCFA accumulation. Therefore, despite these similarities between HM and SPCF, more detailed examination of their effects in weaning rats highlighted many differences in other biological variables examined, ranging from cecal and colonic tissue gene expression to microbiota composition.

In the rat cecum, acetic acid concentrations were higher in the group fed HM than in those fed SPCF. Acetic acid in the large intestine is an end product of bacterial fermentation, which is an important source of energy for enterocytes (32). Activation of the SCFA receptor G protein-coupled receptor 4 can also stimulate the release of leptin from adipose tissue (33) and inhibit insulin-mediated fat deposition (34). These reports point to an important role for SCFAs and the microbiota in controlling energy utilization and metabolic homeostasis. The higher cecal acetic acid concentrations observed in rats fed HM suggests that HM and infant formula can have different effects on host metabolism. The differences in cecal SCFA concentrations may be mediated by differences in oligosaccharide composition between HM and infant formula (35), although it cannot be ruled out that differences in milk lipid and protein content, or indeed solid food consumption, may also contribute to the difference in SCFA concentrations. Indeed, differences observed in solid food intake and milk consumed by rats may indicate differences in nutrient conversion or bioavailability between HM and SPCF.

Reflecting the differences seen in SCFA concentrations, the cecal and colonic microbiota compositions also differed between rats fed HM or SPCF. The microbial diversity of the colon and cecum was significantly higher in rats fed HM. A more diverse microbiota is generally considered to be beneficial, and, indeed, a lower intestinal microbial diversity in infants has been associated with increased prevalence of obesity and allergy later
It is possible that a more diverse microbiota is better able to maintain homeostasis of the microbial ecosystem, because the loss of one species occupying an ecological niche is more likely to be replaced by another species when biodiversity is higher. The loss of diversity is also evident in inflammatory disorders (39–42), further supporting the importance of having a diverse microbiota.

In the cecum, the relative abundance of unclassified Clostridiales and unclassified Porphyromonadaceae was higher in rats fed HM, whereas Prevotella were more prominent in rats fed SPCF. The elevated acetic acid concentrations in HM-fed rats are most likely to be caused by the expansion in Clostridiales, because these are prominent producers of hydrogen, which is then converted to acetic acid by other members of the microbial community through the reduction of carbon dioxide (43).

Although the fermentative role of Porphyromonadaceae in the cecum is unclear, the expansion of this taxa in association with increased acetic acid concentrations has also been observed in other studies examining the effects of feeding resistant starch to rats (12, 44). A possible explanation for the increased Porphyromonadaceae in rats fed HM may be related to an increase in endogenous substrates such as mucin (44), because Porphyromonadaceae are proficient degraders of mucin (45). In support of this hypothesis, expression of mucopolysaccharide pathways were significantly elevated in the cecal tissue of rats fed HM. Another mucin-degrading bacterium, Mucispirillum (46), was also substantially more relatively abundant in rats fed HM. Surprisingly, Prevotella were substantially enriched in the cecum of rats fed SPCF, despite the fact that SPCF has a slightly higher fat and protein content than does HM. Other studies have shown that increased Prevotella abundance is associated with diets lower in fat and protein and high in carbohydrates (47, 48). Although the reason this disparity is unclear, the microbiota is complex, and varied trophic interactions between the many members of the community make predicting diet-mediated changes difficult. For example, although Prevotella are associated with diets high in complex carbohydrates (49), they are also proficient fermenters of protein (50), which may explain their expansion in rats fed SPCF. Furthermore, the addition of galacto-oligosaccharides to an in vitro model of the human colon has been shown to decrease the relative abundance of Prevotella (51), which may explain our results, because of the higher concentrations of free oligosaccharides found in HM compared with bovine milk (52).

Although the cecal and colonic morphology of rats fed HM and SPCF showed no differences under light microscopy, GSEA of Reactome pathways showed that feeding SPCF generally increased the expression of pathways involved in cell cycle regulation in cecal tissue. In contrast, pathways involved in glycoprotein synthesis and metabolism were more highly expressed in the cecal tissue of rats fed HM. This suggests that HM may improve barrier function, because mucin, an endogenous glycoprotein, is a critical component of the intestinal barrier system (53). However, differences in pathway expression in the...
Acknowledgments

We thank Stacey Burton, Kelly Armstrong, and Marita Broadhurst for their technical assistance, and Matthew Barnett and Rex Munday for their critical review of the manuscript. We also thank Brendan Haigh and Jolon Dyer for discussion over the course of the study. ZL, NCR, YG, HJ, LR, LS, AT, JP, SC, LD, and WY designed the study; WY wrote the manuscript with input from all authors; ZL, YG, HJ, LR, LS, AT, JP, and WY conducted the research and analyzed the data; and NCR, LD, and WY had primary responsibility for the final content. All authors read and approved the final manuscript.

References