Dietary nucleotides and fecal microbiota in formula-fed infants: a randomized controlled trial\textsuperscript{1–3}

Atul Singhal, George Macfarlane, Sandra Macfarlane, Julie Lanigan, Kathy Kennedy, Alun Elias-Jones, Terence Stephenson, Peter Dudek, and Alan Lucas

\textbf{ABSTRACT}

\textbf{Background:} Dietary nucleotides are nonprotein nitrogenous compounds that are thought to be important for growth, repair, and differentiation of the gastrointestinal tract. A higher nucleotide intake may also have favorable effects on the fecal microbial composition and incidence of diarrhea in infancy. However, few studies have tested this hypothesis with an experimental study design.

\textbf{Objective:} We tested the hypothesis that nucleotide supplementation of infant formula has beneficial effects on fecal bacteriology.

\textbf{Design:} Oligonucleotide probes were used to measure bacterial genus-specific 16S ribosomal RNA in stools of a subset of infants (mean age: 20.4 wk) who were randomly assigned to nucleotide-supplemented (31 mg/L; \( n = 35 \)) or control formula (\( n = 37 \)) from birth until age 20 wk or were breastfed (reference group; \( n = 44 \)). The microbial pattern was assessed as the ratio of \textit{Bacteroides-Porphyromonas-Prevotella} group (BPP) to \textit{Bifidobacterium} species.

\textbf{Results:} The ratio of BPP to \textit{Bifidobacterium} spp. rRNA in infants randomly assigned to the nucleotide-supplemented formula was lower than in infants receiving the control formula (mean difference: \(-118\%; 95\% \text{ CI: } -203\% \text{ to } -34\%; \ P = 0.007\)), but it did not differ in infants who were breastfed. The difference between randomized formula-fed groups was independent of potential confounding factors (\( P = 0.003\)).

\textbf{Conclusions:} Our data support the hypothesis that nucleotide supplementation improves the composition of the gut microbiota in formula-fed infants. Because this effect could contribute to previously described benefits of nucleotide supplementation for gastrointestinal tract and immune function, these findings have important implications for optimizing the diet of formula-fed infants. \textit{Am J Clin Nutr} 2008;87:1785–92.

\textbf{INTRODUCTION}

Breast milk was shown to have a number of short- and long-term benefits, but the most frequently cited benefit is for a lower incidence of gastroenteritis in infants breastfed rather than formula-fed (1, 2). Several factors were suggested to contribute to this protective effect, including, eg, a higher concentration of nucleotides in human milk compared with cow-milk-based formula (3–6). Nucleotides are nonprotein nitrogenous compounds suggested to be essential nutrients in certain clinical conditions, to modulate immune function, and to be important for growth, repair, and differentiation of the gastrointestinal tract (7–12).

However, although nucleotides have been added to some infant formulas for many years, relatively few data from large-scale randomized studies support their benefits and use in humans.

In formula-fed infants, dietary nucleotide supplementation was shown to reduce the incidence of diarrhea (3–6), possibly by a favorable effect on the gastrointestinal microbiota. For instance, in vitro studies suggest that nucleotides enhance the growth of bifidobacteria (13–15), which by reducing stool pH could reduce the growth of pathogenic bacteria and hence the incidence of infectious diarrhea (7, 8, 10, 16). \textit{Bifidobacterium} spp. are found in higher proportions in the stools of breastfed compared with formula-fed infants (16–19), but whether this effect is related to the higher concentration of nucleotides in breast milk compared with formula is uncertain.

Data to support an effect of dietary nucleotides on the gut microbiota are conflicting (20, 21). One study concluded that the addition of nucleotides to infant formula resulted in a microbial pattern in the stool that was more like that of breastfed infants (20), whereas, in contrast, another investigation suggested that dietary nucleotides discouraged the growth of bifidobacteria (21). However, those previous studies were small and nonrandomized and used bacterial culture rather than more robust molecular techniques to assess fecal microbiology (20, 21). Here, we have investigated the effects of nucleotide supplementation of infant formulas on fecal microbiota, incidence of diarrhea, and stool characteristics with the use of an experimental study design. We used genus-specific 16S ribosomal RNA (rRNA)–targeted, oligonucleotide probes to test the hypothesis that a higher dietary intake of nucleotides has beneficial effects on the fecal microbiota of formula-fed infants. Specifically, we assessed the ratio of

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Bacteroides-Porphyromonas-Prevotella group (BPP) to Bifidobacterium spp. rRNA because the relative abundance of these bacteria are known to differ between breastfed and formula-fed infants (19, 22), are known to be affected by dietary factors in infancy (16, 18), and are suggested to influence neonatal health (22).

SUBJECTS AND METHODS

Study population

Participants were recruited prospectively from 4 hospitals (2 each in Leicester and Nottingham) in the United Kingdom between 1999 and 2002. All mothers of healthy singletons > 37 wk of gestation and without congenital abnormalities were eligible to participate. For the formula-fed arm, only infants of mothers who had decided not to breastfeed and had commenced formula feeding were eligible (n = 200). A reference group of breastfed infants was identified at birth, and the infants were eligible for the study if they were still breastfeeding at 8 wk of age (n = 101). Informed and written consent was obtained from all participants, and the study was approved by both national and local ethics committees in each center.

Study design

Formula-fed infants were randomly assigned within a few days of birth to a nucleotide-supplemented infant formula (31 mg/L; n = 100) or a control formula with <5 mg/L nucleotides (n = 100). A random permuted block design, stratified by center (Nottingham or Leicester), allocated by an independent statistician, and concealed by sealed opaque envelopes was used. Infants not withdrawn from the study continued to receive the assigned formula until 5 mo of age. All mothers and research staff members were blinded to the identity of the formula.

The nucleotide composition of supplemented formula was based on the concentration and composition of free nucleotides and nucleosides in human milk as reflected in current European regulations (23). The supplemented formula contained cytidine monophosphate (15 mg/L), uridine monophosphate (5 mg/L), adenosine monophosphate (6 mg/L), guanosine monophosphate (2 mg/L), and inosine monophosphate (3 mg/L), whereas the control formula (Farley’s First Milk) had <3 mg/L of measurable CMP. The study formulas, manufactured by HJ Heinz Company Ltd, Hayes Middlesex, United Kingdom, met European guidelines for the composition of infant formula and were the same except for their nucleotide concentration. Their composition is given in Table 1.

Demographic, social, clinical (including details of the method of delivery, birth weight, and gestational age), and anthropometric data were collected at random assignment in formula-fed infants and at age 8 wk in breastfed infants. Weight was recorded with the use of electronic scales accurate to 1 g (Seca, Hamburg, Germany), length was recorded with the use of a Rollametre (Raven, Dunmow, United Kingdom) accurate to 1 mm, and head circumference was measured with the use of a nonstretchable tape accurate to 1 mm. Social class was based on the occupation of the parent providing the main financial support for the family (or if both parents worked, the father’s occupation), according to the registrar general’s classification and coded into 6 categories. Mothers were asked to record clinical information on their infants, including episodes of illness, any treatment given, use of any medication (eg, antibiotics), and consultations with health care professionals. Research nurses followed the study participants in their homes at 8, 16, and 20 wk of age and verified the recorded information. Mothers also recorded their infant’s volume of milk intake (in formula-fed infants only), food intake, bowel function, and stool characteristics for a 3-d period before each follow-up visit and the age at which foods other than milk were first introduced.

Tolerance to the formulas was monitored throughout the study. Mothers were asked to record crying time per 24-h period, the number of nighttime waking periods, pacifier use, and episodes of colic (according to the mother’s interpretation of crying symptoms).

### Table 1

#### Nutritional composition of infant formulas

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Amount Per 100 mL as fed</th>
<th>Amount Per 100 g powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kJ)</td>
<td>2190</td>
<td>284</td>
</tr>
<tr>
<td>Energy (kcal)</td>
<td>524</td>
<td>68</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>11</td>
<td>1.5</td>
</tr>
<tr>
<td>Whey protein</td>
<td>6.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Casein protein</td>
<td>4.3</td>
<td>0.6</td>
</tr>
<tr>
<td>Carbohydrate as lactose (g)</td>
<td>53.6</td>
<td>7.0</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>29.4</td>
<td>3.8</td>
</tr>
<tr>
<td>Linoleic acid (mg)</td>
<td>2700</td>
<td>350</td>
</tr>
<tr>
<td>α-Linolenic acid (mg)</td>
<td>340</td>
<td>44</td>
</tr>
<tr>
<td>γ-Linolenic acid (mg)</td>
<td>250</td>
<td>33</td>
</tr>
<tr>
<td>Long-chain polyunsaturates (mg)</td>
<td>200</td>
<td>26</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>300</td>
<td>39</td>
</tr>
<tr>
<td>Chloride (mg)</td>
<td>310</td>
<td>40</td>
</tr>
<tr>
<td>Copper (µg)</td>
<td>320</td>
<td>42</td>
</tr>
<tr>
<td>Iodine (µg)</td>
<td>35</td>
<td>4.5</td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>5.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Magnesium (mg)</td>
<td>40</td>
<td>5.2</td>
</tr>
<tr>
<td>Manganese (µg)</td>
<td>26</td>
<td>3.4</td>
</tr>
<tr>
<td>Phosphorus (mg)</td>
<td>210</td>
<td>27</td>
</tr>
<tr>
<td>Potassium (mg)</td>
<td>440</td>
<td>57</td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>130</td>
<td>17</td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>2.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Vitamin A (µg)</td>
<td>800</td>
<td>100</td>
</tr>
<tr>
<td>Thiamine (µg)</td>
<td>320</td>
<td>42</td>
</tr>
<tr>
<td>Riboflavin (µg)</td>
<td>420</td>
<td>55</td>
</tr>
<tr>
<td>Vitamin B-6 (µg)</td>
<td>270</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin B-12 (µg)</td>
<td>1.1</td>
<td>0.14</td>
</tr>
<tr>
<td>Biotin (µg)</td>
<td>7.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Folic acid (µg)</td>
<td>26</td>
<td>3.4</td>
</tr>
<tr>
<td>Nicin (mg)</td>
<td>5.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Pantothenic acid (mg)</td>
<td>1.8</td>
<td>0.2</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>53</td>
<td>6.9</td>
</tr>
<tr>
<td>Vitamin D (µg)</td>
<td>8.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Vitamin E (mg)</td>
<td>3.7</td>
<td>0.5</td>
</tr>
<tr>
<td>Vitamin K (µg)</td>
<td>21</td>
<td>2.7</td>
</tr>
<tr>
<td>Choline (mg)</td>
<td>37</td>
<td>4.8</td>
</tr>
<tr>
<td>Taurine (mg)</td>
<td>39</td>
<td>5.0</td>
</tr>
</tbody>
</table>

* Nucleotide composition of the nucleotide-supplemented formula was as follows: cytidine monophosphate (15 mg/L), uridine monophosphate (5 mg/L), adenosine monophosphate (6 mg/L), guanosine monophosphate (2 mg/L), and inosine monophosphate (3 mg/L). Measurable nucleotide in the control formula was cytidine monophosphate (3 mg/L).
Fecal bacteriology

All mothers still participating in the study at the 20-wk home visit were given collecting pots and asked to collect a fresh sample of their infant’s stool by frequently checking the nappy during the day (excluding the first sample in the morning). The sample was collected by the research nurse (or taken by a courier) and frozen at \(-20 \, ^\circ C\) within 1 h. The samples were subsequently frozen at \(-80 \, ^\circ C\) for later analysis of fecal flora.

Stool samples were assessed for 4 microbial species previously suggested to be influenced by breastfeeding compared with formula feeding (19) and by nucleotide supplementation of infant formula (20). After mechanical disruption of the bacterial cells with the use of a mini bead-beater, total RNA was extracted from stool samples with the use of the phenol-chloroform method described by Dore et al (24). The RNA was then blotted, hybridized, and washed with the use of procedures described by Hopkins et al (25), with minor modifications. The initial nucleic acid concentrations were measured spectrophotometrically at 260 nm (an absorption at 260 nm of 1.0 corresponded to an RNA concentration of 40 \(g/mL\)). The RNA was then denatured in a 0.5 (by vol) glutaraldehyde solution, diluted to 1.5 ng/L, and blotted onto Hybond XL nylon hybridization membranes (Amersham Pharmacia Biotech Inc, Bucks, United Kingdom). Oligonucleotide probes were used to quantify the abundance of RNA from bifidobacteria [Bio1412: 5’-CCGTTTMTAGGGATCC-3′ (26)], enterobacteria [Entero 1418: 5’-CTTTTGCARCCACACT-3′ (27)], the BPP group [Bacto1080: 5’-GCATTTAGCCGACACCT-3′ (24)], and lactic acid bacteria [Lacto 722: 5’-YCAACGCTACACTGAGTCTCCACT-3′ (28)], and the values obtained were expressed as a percentage of the total bacterial rRNA, which was quantified with the use of the probe [Eub338: 5’-GCTGCGCTCCGCCGAGGT-3′ (29)]. The sequences were 5’ end-labeled with \(^{32}P\) with the use of Microspin G-25 columns (Amersham Pharmacia Biotech Inc). The membranes were hybridized with the labeled probes overnight, before the wash procedure at the designated temperature for each probe. RNA abundance was quantified by measuring the \(^{32}P\) signal with the use of an Instant Imager (Canberra Packard, Pangbourne, Berks, United Kingdom) and IMAGEQUANT software (Molecular Dynamics, Sunnyvale, CA). Reference concentrations of RNA representative of the bacterial group were also run on each gel, and standard curves were calculated by linear regression to measure group-specific RNA.

Diarrhea incidence and stool characteristics

Diarrhea or vomiting episodes were recorded as separate illnesses. Diarrhea was defined as a discrete illness lasting \(>48 \, h\) (2, 30) with \(>3\) loose stools in a 24-h period (5) and distinguished from chronic diarrheal disease, such as intolerance to cow milk or malabsorption (30). Mothers also recorded the stool consistency for 3 d before each home visit according to Weaver et al (31).

Statistical analysis

Our a priori outcomes were the effect of nucleotide supplementation on the ratio of BPP to Bifidobacterium spp. rRNA and the incidence of diarrhea. Diarrheal illness was assessed both as the total number of diarrheal episodes and as the proportion of infants with \(\geq 1\) episode of diarrhea from birth to 20 wk of age. Sample size was initially calculated to detect a 0.5 SD difference in the number of diarrheal episodes between randomized formula-fed groups with 80% power at 5% significance. However, successful recruitment meant that the trial was continued...
beyond that originally planned to give a power of 0.4 SD difference in outcomes between the randomized groups at 80% power and \( P < 0.05 \).

Randomized formula-fed groups were compared with the Student’s \( t \) test for normally distributed variables, the Mann-Whitney \( U \) test for not-normally distributed variables, and the chi-square test for dichotomous variables. The ratio of BPP to \textit{Bifidobacterium} spp. was loge transformed and then multiplied by 100 before statistical analyses (32). Therefore, for 100 loge-transformed data the SD represents the CV, and the difference in means between randomized groups represented the percentage difference between groups (32). Multiple linear regression was used to adjust for confounding factors that could potentially affect stool microbial pattern (sex, age at stool analysis, age at which complementary foods were introduced, socioeconomic status, and mother’s education). Values for 16S rRNA from the 4 different groups of bacteria quantified in stool (expressed as a percentage of total bacterial rRNA) could not be transformed to normality; hence, nonparametric statistics were used.

In a secondary analysis, the ratio of BPP to \textit{Bifidobacterium} spp. in breastfed infants was compared with the 2 formula-fed groups with the use of one-factor analysis of variance and Bonferroni corrections. Statistical analyses were conducted with the use of SPSS for WINDOWS (version 12.0; SPSS Inc, Chicago, IL).

**RESULTS**

Of the 301 infants recruited into this study, 88 of 100 nucleotide-supplemented, 85 of 100 control formula-fed, and 96 of 101 breastfed infants were followed at age 5 mo (Figure 1). Infants dropped out of the study usually for reasons that were not given or were nonclinical, social reasons. Only 5 infants (4 from the control group and 1 from the nucleotide formula group) dropped out because of perceived problems with the milk such as vomiting (\( n = 3 \)) or apparent hunger (\( n = 2 \)). These infants were changed by their mothers to formulas completely different from those used in the study. Mothers of infants dropping out of the study did not continue to collect clinical data, allow follow-up visits, or provide stool samples, so they were not included in the analyses at age 20 wk. The 2 trial formulas were well tolerated, and the time spent crying, incidence of colic, nighttime wake periods, and use of pacifiers did not significantly differ between randomized groups (data not shown). No infant from any dietary group had a diagnosis of chronic diarrhea or other gastrointestinal illness.

Nucleotide-supplemented and control formula-fed groups were closely matched for age at assessment, sex, socioeconomic status, percentage of children born by cesarean delivery, and anthropometric variables (Table 2). The age at which complementary feeding was started and the use of antibiotics during the follow-up period were also not significantly different between randomized formula-fed groups (Table 2). As expected, mothers of breastfed infants were better educated and of a higher socioeconomic status than were the mothers of formula-fed infants (data not shown). More breastfed infants completed the study than did formula-fed infants, but no significant difference was observed in the completion rate between nucleotide-supplemented or control formula-fed infants (Table 3).

### Table 2

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control ((n = 100))</th>
<th>Nucleotide supplemented ((n = 100))</th>
<th>Breastfed reference ((n = 101))</th>
<th>(P) for comparison of 3 dietary groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex [(n (% ))]</td>
<td>56 (56)</td>
<td>61 (61)</td>
<td>51 (51)</td>
<td>0.4(^2)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>3.5 ± 0.5(^d)</td>
<td>3.5 ± 0.6</td>
<td>3.5 ± 0.5</td>
<td>0.9(^d)</td>
</tr>
<tr>
<td>Weight z score</td>
<td>0.3 ± 1.0</td>
<td>0.1 ± 1.2</td>
<td>0.1 ± 1.0</td>
<td>0.6(^d)</td>
</tr>
<tr>
<td>Gestation (wk)</td>
<td>39.2 ± 1.3</td>
<td>39.5 ± 1.4</td>
<td>39.6 ± 1.1</td>
<td>0.1(^d)</td>
</tr>
<tr>
<td>Social class: nonmanual [(n (% ))]</td>
<td>39 (39)</td>
<td>41 (41)</td>
<td>81 (81)</td>
<td>&lt;0.001(^d)</td>
</tr>
<tr>
<td>Mothers with degree [(n (% ))]</td>
<td>8 (8)</td>
<td>8 (8)</td>
<td>52 (52)</td>
<td>&lt;0.001(^d)</td>
</tr>
<tr>
<td>Cesarean delivery [(n (% ))]</td>
<td>40 (40)</td>
<td>32 (32)</td>
<td>31 (31)</td>
<td>0.4(^d)</td>
</tr>
</tbody>
</table>

\(^d\) Treated by the chi-square test. There was a slight loss of \( n \) for some variables (<2%).

\(^d\) Treated by ANOVA.

**Stool microbiology**

A stool sample was collected from 116 (43%) of 269 infants remaining in the study at 20 wk of age. Formula-fed infants who provided a stool sample (\( n = 72 \)) compared with infants who did not (\( n = 128 \)) had a higher proportion of girls, but they did not differ significantly in age, gestation, randomized formula-fed group, anthropometric characteristics at birth, socioeconomic status, and mother’s education (data not shown). Nucleotide-fed infants (\( n = 37 \)) had a lower ratio of fecal BPP to bifidobacterial rRNA than did controls (\( n = 35 \)) (mean difference: −118%; 95% CI: −203%,
TABLE 3
Stool bacteriology and episodes of diarrhea

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n = 100)</th>
<th>Nucleotide supplemented (n = 100)</th>
<th>P for comparison between formula-fed groups</th>
<th>Breastfed reference (n = 101)</th>
<th>P for comparison of 3 dietary groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Completed study [n (%)]</td>
<td>85 (85)</td>
<td>88 (88)</td>
<td>0.4(^{a})</td>
<td>96 (96)</td>
<td>0.07(^{a})</td>
</tr>
<tr>
<td>Age at final visit and stool collection (wk)</td>
<td>20.5 ± 1.3(^{ab})</td>
<td>20.4 ± 1.4</td>
<td>0.6</td>
<td>20.3 ± 1.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Age at which complementary foods introduced (mo)</td>
<td>14.0 ± 2.3</td>
<td>13.6 ± 2.3</td>
<td>0.3</td>
<td>15.4 ± 1.8</td>
<td>&lt;0.001(^{ab})</td>
</tr>
<tr>
<td>Courses of antibiotic [n (%)]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>14 (14)</td>
<td>21 (21)</td>
<td>0.2(^{a})</td>
<td>12 (12)</td>
<td>0.4(^{a})</td>
</tr>
<tr>
<td>2</td>
<td>5 (5)</td>
<td>12 (12)</td>
<td></td>
<td>2 (2)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1 (1)</td>
<td>1 (1)</td>
<td></td>
<td>1 (1)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0 (0)</td>
<td>1 (1)</td>
<td></td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

Fecal bacteriology\(^{7}\)

- Ratio of BPP to bifidobacteria\(^{6}\)
  - Control: 20.2 (158)
  - Nucleotide supplemented: 6.2 (187)
  - P = 0.007
  - Breastfed: 3.6 (146)
  - P = <0.001\(^{ac}\)

- BPP (%)
  - Control: 34.6 (20.2)
  - Nucleotide supplemented: 29.1 (31.9)
  - P = 0.02\(^{10}\)
  - Breastfed: 17.5 (24.3)
  - P = 0.001\(^{11}\)

- Bifidobacteria (%)
  - Control: 1.8 (2.9)
  - Nucleotide supplemented: 2.8 (6.5)
  - P = 0.09\(^{10}\)

- Enterobacteria (%)
  - Control: 0.7 (0.3)
  - Nucleotide supplemented: 0.8 (0.3)
  - P = 0.2\(^{10}\)

- Lactic acid bacteria (%)
  - Control: 0.5 (1.0)
  - Nucleotide supplemented: 0.5 (1.0)
  - P = 0.3\(^{10}\)

Episodes of diarrhea

- No. by age 20 wk
  - Control: 0 (0–3)\(^{1/2}\)
  - Nucleotide supplemented: 0 (0–10)
  - P = 0.2\(^{10}\)
  - Breastfed: 0 (0–2)
  - P = 0.01\(^{11}\)

- >1 episode of diarrhea by age 20 wk [n (%)]
  - Control: 26 (31)
  - Nucleotide supplemented: 33 (38)
  - P = 0.2\(^{11}\)

Stool consistency by age\(^{12}\)

- 8 wk
  - Control: 3.2 ± 0.5
  - Nucleotide supplemented: 3.0 ± 0.5
  - P = 0.03

- 16 wk
  - Control: 3.3 ± 0.5
  - Nucleotide supplemented: 3.1 ± 0.5
  - P = 0.09

- 20 wk
  - Control: 3.5 ± 0.7
  - Nucleotide supplemented: 3.4 ± 0.6
  - P = 0.5

Stool frequency by age (movements/d)

- 8 wk
  - Control: 1.5 ± 1.0
  - Nucleotide supplemented: 1.6 ± 1.1
  - P = 0.7

- 16 wk
  - Control: 1.4 ± 0.7
  - Nucleotide supplemented: 1.5 ± 0.9
  - P = 0.3

- 20 wk
  - Control: 1.4 ± 0.8
  - Nucleotide supplemented: 1.5 ± 0.7
  - P = 0.6

1. BPP, Bacteroides-Porphyromonas-Prevotella group. There was a slight loss of n for some variables (<2%).
2. Infants were randomly assigned to formula groups.
3. Analyzed with the Student’s t test.
4. Determined by ANOVA. Values with different superscript letters are post hoc comparisons with Bonferroni’s test; breastfed compared with control formula-fed infants: \(^{a}\) P < 0.001, \(^{b}\) P = 0.02, \(^{c}\) P = 0.03; breastfed compared with nucleotide-supplemented formula-fed infants \(^{d}\) P < 0.001; nucleotide-supplemented compared with control formula-fed infants: \(^{e}\) P = 0.01.
5. Determined by the chi-square test.
6. \(^{1}\) \(\bar{x} \pm SD\) (all such values).
7. Stool microbiota were assessed in a subset of infants: 35 control formula-fed, 37 nucleotide-supplemented formula-fed, and 44 breastfed.
8. Values are geometric mean; CV in parentheses.
9. Values are median; interquartile range in parentheses.
10. Determined by the Mann-Whitney U test.
11. Determined by the Kruskal-Wallis test.
12. Median; range in parentheses (all such values).
13. Analyzed as hard (code 5), mushy soft (code 4), formed soft (code 3), runny (code 2), or watery (code 1).

-34%; P = 0.007). This difference remained significant after adjustment of potential confounding factors (age, sex, age at which complementary feeds were introduced, socioeconomic status, and mother’s education) (mean difference: -131%; 95% CI: -217%, -45%; P = 0.003). The abundance of BPP was significantly lower in nucleotide-supplemented formula-fed infants than in controls (Table 3). However, the abundance of bifidobacteria, lactic acid bacteria, and enterobacteria did not differ significantly between randomized formula-fed groups (Table 3).

In a secondary analysis that used analysis of variance, the ratio of BPP to Bifidobacterium spp. RNA statistically differed in the 3 dietary groups (P < 0.001) and between the 2 randomized formula-fed groups (P = 0.01). The ratio of BPP to bifidobacteria in breastfed infants was lower than in infants fed control formula (P < 0.001), but it was not significantly different from infants assigned to the nucleotide-supplemented formula (P = 0.5). In the whole study population, the percentage of rRNA from bifidobacteria correlated with both BPP (r = -0.3, P = 0.004) and lactic acid bacteria (r = 0.2, P = 0.04).

Diarrhea incidence, stool frequency, and stool characteristics

The total number of diarrheal episodes or presence of ≥1 episode up to 20 wk of age did not differ significantly between...
randomized formula-fed groups (Table 3). Nucleotide-supplemented infants produced softer stools at 8 wk of age but not at 16 or 20 wk of age, but stool frequency did not differ significantly between randomly assigned formula-fed infants at any age. As expected, breastfed infants had significantly fewer diarrheal episodes during the first 20 wk of age and also between 8 and 20 wk of age (during which comparable, prospective collected data were available in both formula-fed and breastfed infants) (data not presented). Breastfed infants also had a higher stool frequency and softer stools than did formula-fed infants (Table 3). The ratio of BPP to Bifidobacterium spp. did not significantly correlate with the number of episodes of diarrhea in the whole study population and when the analysis was confined to formula-fed infants.

DISCUSSION

The addition of nucleotides to infant formula was suggested to have beneficial effects on health (3–12), but relatively few experimental studies have tested this hypothesis in humans. In a prospective randomized trial, we found that supplementation of infant formula with 31 mg nucleotides/L had advantages for the whole study population and when the analysis was confined to formula-fed infants.

In contrast to previous reports (3–6), we did not find an effect of nucleotide supplementation on the incidence of diarrhea. One possible explanation for this is that, unlike findings from a developing country (3), a low incidence of diarrhea in a more-developed setting could have reduced our study’s power to detect small but clinically important differences between nucleotide-supplemented and control formula-fed infants. Another potential explanation is a low concentration of nucleotides (31 mg/L) in our supplemented formula, which, although in line with European regulations (≤33.5 mg/L) (23), was lower than that used in most randomized studies that showed a benefit of nucleotides on diarrheal illness (72 mg/L) (4–6). The latter concentration is an estimate of both free and enzymatically liberated nucleosides in human milk (ie, total potentially available nucleosides) and is higher than the free nucleotide and nucleoside content alone (11, 33). Our study therefore supports the hypothesis that nucleotide supplementation of formula to a higher concentration, more similar to the total available to breastfed infants, is required for a protective effect against diarrhea. Theoretically, differences in the pattern of nucleotide supplementation could also explain differences in study findings, although, to our knowledge, no evidence supports this hypothesis.

We considered a number of study limitations. First, we did not measure live bacterial counts and, like most studies that used 16S rRNA, we could only estimate the proportions of bacterial groups and not specific species or strains. Our study therefore makes the assumption that the group-specific percentage of bacterial RNA in feces reflects the viable bacterial counts in the colon. In addition, changes in abundance of fecal bacterial population expressed as a proportion of total rRNA could represent shifts in total community composition and ribosomal abundance and not necessarily changes in absolute amounts (25). Nevertheless, 16S rRNA measurements were shown to correlate well with viable bacterial counts (25) and to provide a good measure of the gut microbial composition, which is associated with both neonatal intestinal health (18, 22) and infant feeding (19). Furthermore, the previous observation that nucleotides may affect the relative proportions of bifidobacteria rather than absolute amounts (20) supports our assessment in terms of microbial patterns, rather than absolute bacterial counts.

Second, the effect of nucleotides on bifidobacteria did not reach statistical significance, possibly because the percentage of Bifidobacterium spp. in our infants aged 143 d was lower than that seen previously in infants aged < 20 d (19), but more similar to amounts seen in older infants (85–225 d) (24) or adults (<1%) (34). However, this difference was not unexpected in view of the fall in fecal bifidobacterial numbers that occurs with the introduction of solid feeding and with the transition from infant to adult stool microbiota in the first year of life (16–18). Furthermore, the percentage of BPP rRNA in the present study was similar to a previous report (≈30%) (24), which supports the validity of our 16S rRNA assay. We assessed fecal microbiology at 5 mo of age to allow the investigation of associations between nucleotide supplementation, diarrheal disease, and fecal microbiology. Nonetheless, the advantage of nucleotide supplementation for fecal microbiology was independent of the age at which complementary foods were introduced, which itself did not differ between nucleotide-supplemented or control formula-fed infants. The fact that differences between randomized formula-fed infants...
groups were seen in older infants, after the introduction of complementary foods and therefore large amounts of dietary nucleotides, raises the possibility that the effect of nucleotides on fecal microflora could be even greater in neonates, a hypothesis that requires further testing.

Finally, our study could not address mechanisms. Although >90% of ingested nucleotides are absorbed as nucleosides in the upper intestine (7, 8), some probably pass into the colon, where they could act as cofactors for the growth of bifidobacteria. For instance, the separate addition of adenosine monophosphate, CMP, guanosine monophosphate, uridine monophosphate, and inosine monophosphate was shown to stimulate the in vitro growth of bifidobacteria, whereas the simultaneous addition of these nucleosides had an even greater effect (15). Thus, nucleotide supplementation could have a direct nutritional or prebiotic effect as suggested previously (10) and as supported by findings from in vitro studies (13–15). Dietary nucleotides were also suggested to promote intestinal mucosal and epithelial growth (9), which could provide another nutrient source for colonic bacteria (22). Finally, nucleotides could directly inhibit the growth of specific bacterial groups (eg, BPP), thereby altering the overall microbiota pattern, although little evidence supports this hypothesis. A more likely explanation for the lower percentage of BPP in nucleotide-supplemented infants is the inhibition of BPP growth by the bifidobacteria-mediated increase in intestinal acidity (7–9), a hypothesis supported by the negative correlation between the percentage of rRNA from BPP and Bifidobacterium spp. in the present study.

Evidence that the neonatal gut microbiota have long-term effects on immune function (22) and atopic disease (35) raises the possibility that the benefits of nucleotide supplementation of infant formula for immune function could be mediated in part through an effect on the gut microbiota (4, 10). Further research is required to test this hypothesis and the potential long-term benefits for nucleotide supplementation of infant formula.

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