Feeding Human Milk to Rats Increases *Bifidobacterium* in the Cecum and Colon Which Correlates with Enhanced Folate Status\(^1,2,3\)

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**ABSTRACT** The purpose of this investigation was to determine if feeding diets containing human milk resulted in increased numbers of microorganisms implicated in increased folate production and the effect on folate availability. Following a folate-depletion period (5 wk), 30 rats were fed folate-repletion diets (4 wk) with or without 20% milk solids (human, cow or goat) and containing either 906 or 4530 nmol folate acid/kg. At the end of the test period, the cecum and colon were removed in an anaerobic chamber, homogenized, diluted (10\(^{-2}\) to 10\(^{-8}\)), and the contents of each plated on selective and nonselective media. In addition to enumeration of the total anaerobic load, five genera of bacteria were counted (*Bacteroides, Bifidobacterium, Clostridium, Escherichia* and *Streptococcus*). Rats fed human milk solids had at least a seven- and one-fold mean increase in the *Bifidobacterium* concentration in the cecum (P < 0.006) and colon (P < 0.04), respectively, compared with rats fed other diets. The total anaerobic bacterial concentration in the cecum and the colon of rats fed human milk solids was also greater than that of rats fed the other diets (P < 0.05). The single exception was the total anaerobic count in the cecum of rats consuming goat milk solids, which did not differ from that of rats consuming human milk solids. Further, rats fed human milk solids had at least a 42 and 48% higher mean plasma folate concentration and total cecal material folate content, respectively, than rats in other dietary treatments containing 906 nmol/kg folate acid. Therefore, the improved folate status of rats fed human milk–containing diets appears to be due, at least in part, to increased folate synthesis by *Bifidobacteria* and other folate-synthesizing microbes in the cecum and colon. J. Nutr. 126: 1505-1511, 1996.

**INDEXING KEY WORDS:**
- folate
- milk
- microflora
- *Bifidobacterium*
- rats

Folate plays a critical role in DNA, RNA and amino acid biosynthesis; thus, an adequate supply of this nutrient is essential to facilitate the rapid rate of growth that occurs during early development. Milk consumption has been shown to be an important determinant of infant folate status (Smith et al. 1985), and data generated from rat studies suggest that various milk types will differentially affect the availability of folate from the remainder of the diet (Semchuk et al. 1994, Swiatlo et al. 1990). For example, Swiatlo et al. (1990) demonstrated that the availability of folic acid added to rat diets was 40, 133 and 75% greater from diets containing human milk solids compared with diets containing cow milk, goat milk or milk-free rat diets, respectively.

Previous research in our laboratory suggested that the marked improvement in the availability of folate to rats consuming human milk solids may have more to do with changes in the intestinal environment and enhanced microbial folate biosynthesis than specific constituents in milk that promote folate absorption per se (Semchuk et al. 1994). We found that addition of a sulfa drug to diets containing milk completely eliminated the difference in the folate status between rats fed human and goat milk (Semchuk et al. 1994). Two

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major short-comings of this latter investigation were that intestinal bacteria were not directly quantified and that sulfa drugs do not systematically eliminate all intestinal bacteria; rather, they prevent the utilization of p-aminobenzoic acid by intestinal bacteria for folate synthesis (Stokstad and Jukes 1987). Therefore, microorganisms such as Lactobacillus casei that require pre-formed folate are insensitive to sulfaamides.

It has been known since the 1940s that large quantities of folate are produced by microorganisms in the large intestine (Cooperstock and Zedd 1983, Denko et al. 1946), and recent work with rats (Rong et al. 1991) and organ-cultured endoscopic biopsy specimens from the human colon (Zimmerman 1990) suggest that this folate can, in fact, be absorbed across the large intestine and utilized by the host.

The first objective of the present investigation, then, was to assess directly the impact of incorporating human, cow and goat milk solids into rat diets on the total anaerobic load and quantity of Bacteroides, Bifidobacterium, Clostridium, Escherichia and Streptococcus species in the cecum and colon of rats. Second, we sought to determine whether the amount of folate produced in the cecum and colon of rats fed diets containing human milk was greater than the amount produced in rats fed diets containing cow or goat milk solids or milk-free diets.

**MATERIALS AND METHODS**

**Rat husbandry.** Thirty Sprague-Dawley rats (Charles River, LaSalle, St-Constant, PQ, Canada) weighing 50-79 g were housed individually in wire-bottom stainless steel cages with a controlled temperature (23-24°C) and a 12-h light:dark cycle. Rats were randomly assigned to cages and one of five experimental treatment groups. Rats were acclimated for 1 wk prior to study initiation and fed a standard nonpurified diet (Ralston Purina, Saint Louis, MO). Throughout the study, rats had free access to food and tap water. All aspects of the experimental protocol were reviewed and approved by the University of Guelph Animal Care Committee.

To deplete rats of their folate stores, they were fed a semi-purified diet containing 195 nmol folate/kg for 5 wk. To confirm depletion of folate stores, a 500-μL tail blood sample was collected from a random sample of 10 rats and analyzed for plasma folate concentration. After the 5-wk depletion period, rats were fed test diets containing folate for 4 wk. The test diets included human milk solids, cow milk solids, goat milk solids and a milk-free diet. These diets were formulated to contain 906 nmol of additional folic acid/kg diet. A fifth milk-free diet was prepared with 4530 nmol/kg additional folic acid to determine if an increase in dietary folate could alter the quantity or profile of microflora in the large intestine. The amount of folate from milk contributed between 2 and 4% of the total folate concentration in the diets containing milk. The protocol used in this rat bioassay and the rationale for the amount of milk solids and folic acid added are described in detail by Keagy and Oace (1982) and Semchuck et al. (1994).

**Formulation of diets.** Human milk was obtained locally from 43 healthy lactating women at any time after the first month postpartum and until the end of the first year of lactation. After each nursing, milk samples were cooled in the refrigerator (4°C) and then frozen at -20°C in opaque polypropylene containers to protect viable milk components. Milk was collected from the participants at 2-wk intervals, transported to the University of Guelph on ice and stored at -80°C until it was thawed, pooled and freeze-dried (Dura-Top Digital Control Bulk Tray Dryer, FTS Systems, Mississauga, ON, Canada). All procedures involving the use of human subjects were reviewed and approved by the Human Ethics Committee at the University of Guelph.

Fresh cow and goat milk (13 L each) was obtained from a local dairy (Hewitt Dairies, Hagersville, Ontario) immediately following pasteurization (79°C for 4 s). Milk was transported to the University of Guelph on ice and prepared for freeze-drying as described above for human milk.

All of the diets were formulated according to the American Institute of Nutrition recommendations (AIN 1980) and are described in Table 1. Milk samples were analyzed for their protein, fat, moisture and ash content via proximate analysis (AgriFood Laboratories, Guelph, ON, Canada). Analyses were conducted according to the AOAC methods of proximate analysis (AOAC 1990). Following proximate analysis, dietary constituents providing the protein (casein), fat (corn oil) and carbohydrate (cornstarch and sucrose) fractions of the milk-containing diets were adjusted to ensure that all of the diets were equal in energy and nitrogen content.

**Blood, cecum and colon collection.** After the 4-wk repletion period, each rat was placed in an anaerobic chamber airlock into which carbon dioxide gas was administered (2 min). Five milliliters of whole blood was collected via cardiac puncture using a syringe (5 mL) and needle (2.5 cm, 21-gauge) coated with heparin. The rat was returned to the airlock and killed using carbon dioxide gas. Blood was centrifuged (850 x g at 4°C) and plasma was separated and stored (−80°C) with 10 g/L ascorbate.

In the anaerobic chamber (80% N2, 10% CO2, 10% H2), the colon and cecum were removed, cleaned and weighed. Cecal tissue and contents were weighed separately. The colon, cecum (plus cecal contents) were placed separately into vials containing 10 mL of 0.05% yeast extract (Difco, Detroit, MI) and homogenized. Serial dilutions were then prepared (10−2−10−8). Ten microliters of diluent were plated in triplicate on five selective and one nonselective media. The media utilized
TABLE 1
Composition of depletion and experimental diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Depletion diet</th>
<th>Diets containing milk</th>
<th>Diets lacking milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk solids†</td>
<td>—</td>
<td>200</td>
<td>—</td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
<td>142–181</td>
<td>200</td>
</tr>
<tr>
<td>DL-Methionine*</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Cornstarch†</td>
<td>300</td>
<td>229–252</td>
<td>300</td>
</tr>
<tr>
<td>Sucrose†</td>
<td>300</td>
<td>229–252</td>
<td>300</td>
</tr>
<tr>
<td>Fiber*</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Corn oil†</td>
<td>100</td>
<td>55–62</td>
<td>100</td>
</tr>
<tr>
<td>Vitamin mix†</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Salt mix†</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Added folic acid†</td>
<td>906 nmol/kg</td>
<td>906 nmol/kg</td>
<td>4530 nmol/kg</td>
</tr>
</tbody>
</table>

† Incorporation of milk solids at 200 g/kg diet provided the following: human milk: 3.1 g total N, 125.3 g carbohydrate, 38.2 g fat; cow milk: 8.37 g total N, 86.7 g carbohydrate, 43.0 g fat; goat milk: 9.3 g total N, 148.1 g carbohydrate, 45.1 g fat.

* Vitamin-free casein, Teklad, Madison, WI.
† Sigma Chemical, Saint Louis, MO.
§ Best Foods Canada, Etobicoke, ON, Canada.
5 Redpath Industries Limited, Toronto, ON, Canada.
6 Alphacell, ICM Biochemicals, Cleveland, OH.
8 No. S10001 (Research Diets) (Swiatlo et al. 1990).

were as follows: Bacteroides Bile Esculin Agar for Bacteroides sp.; BL Agar for Bifidobacterium sp. (Difco); Egg Yolk Agar for Clostridium sp. (Difco); MacConkey’s Agar for Escherichia sp. (BBL, Baltimore, MD); SF Agar for Streptococcus sp. (Difco); and Brucella agar enriched with sheep blood hemin and phyloquinone (BBL) for an overall anaerobic bacteria count. The plates were incubated at 37°C for 48 h and then counted. The number of bacteria per gram of colon and cecum was calculated. The dry weight of the colon and cecum contents was also obtained by drying 5 mL of sample at 75°C for ~1 wk.

Folate analyses. Folate was extracted from cecal and colon contents, and diets and drinking water using the double extraction method as described by Wilson and Horne (1984) and Gregory et al. (1990). Extracts were treated with partially purified folate conjugase prepared from fresh hog kidney to convert folates into an assayable form (Engelhardt and Gregory 1990). The amount of folate in the supernatant was determined using the test microorganism, Lactobacillus casei (ATCC 7469) (Wilson and Horne 1984, Tamura 1990). The CV for repeated determinations of folate in pooled samples of plasma, cecal and colon samples were 3.9, 6.8 and 4.5%, respectively.

Bacteria verification. To verify the presence of a particular genus of bacteria on its respective selective plating medium, the following tests were performed as suggested by Summanen et al. (1993). Each selective plate was examined for colony characteristics including size, shape, edge, color, opacity and other noteworthy attributes such as hemolysis. The colonies were then picked off and viewed under a phase contrast microscope for motility. Bacterial morphology (rods or cocci) as single cells or chains and the presence of spores were noted. Gram stains were also performed. Aerotolerance tests were undertaken in ambient air. For Clostridium, a Shaeffer Fulton spore test was executed [Summanen et al. 1993]. The Analytical Profile Index (API) [bioMerieux Canada, Quebec City, PQ, Canada] was used to confirm the findings of the above conventional tests. The API An-Ident was used for the identification of Bacteroides and Clostridium, the API Rapid 20E for the identification of Escherichia and the API 20 Strep for the identification of Streptococcus. Because API Strips were not available for confirmation of the presence of Bifidobacterium, suspected colonies were analyzed for acetic acid and lactic acid using gas liquid chromatography [Microbiology Specialists, Houston, TX]. See Summanen et al. (1993) for a description of this procedure.

Statistical analyses. For statistical analysis, all non-parametric data were transformed by using the natural log. The data presented in the results are the untransformed means ± SEM. Differences among treatments were evaluated using one-way ANOVA followed by least significant difference tests utilizing the Statistical Analysis System (SAS 1989). In the analyses examining the relationship between milk composition of diets on gut microflora content vs. the folate status of rats, the 2 × folate dietary treatment was removed from the database. Differences between treatments were determined by least significant difference tests. Correlation coefficients between variables were determined using Pearson correlation coefficients [Snedecor and Cochran 1989]. A probability level of 5% was chosen as the level of statistical significance.

RESULTS

Status of rats after depletion and repletion periods. Mean food consumption (22.6 ± 0.1 g/d) and weight gain (60.4 ± 0.5 g/wk) of rats during the depletion period did not differ among dietary treatments. The mean weight of rats at the end of the depletion period was 409.7 ± 8.6 g. The mean plasma folate concentration of rats at the end of the depletion period was 23.1 ± 0.7 nmol/L which is comparable to that of rats previously depleted of folate in this laboratory (33.0 ± 14.0 nmol/L) [Semchuk et al. 1994]. Similarly, the mean food intake (28.0 ± 0.2 g/d) and weight gain (35.6 ± 0.3 g/wk) of rats did not differ among experimental groups during the repletion period. The mean body and liver weights at necropsy were...
552.4 ± 3.3 g and 24.5 ± 0.3 g, respectively. Again no differences were observed among experimental treatments.

In contrast, mean cecum weight (cecal sac plus contents) did differ among dietary treatment groups (Table 2). Rats fed human milk solids had a significantly heavier mean cecum weight than did rats fed goat milk ($P < 0.05$), milk-free or 2x folate milk-free diets ($P < 0.01$). Mean cecum weight did not differ between rats fed diets containing human milk solids and those fed diets containing cow milk solids. When cecum weights were compared without their contents, the mean cecum weight of rats fed diets containing human milk was greater than that of rats fed milk-free ($P < 0.01$) and 2x folate milk-free diets ($P < 0.05$) but not from rats fed cow and goat milk-containing diets. The mean colon weight of rats did not differ among dietary treatments. The mean water content of the colon and cecum of rats was similar among all treatment groups.

**Bacterial counts.** The mean total anaerobic load and concentration of *Bacteroides, Bifidobacterium, Clostridium, Escherichia* and *Streptococcus* in the cecum and colon for each dietary treatment are presented in Figures 1 and 2 and Tables 3 and 4. In cecal samples, the mean concentration of *Bifidobacteria* sp. was at least sevenfold greater among rats consuming human milk solids vs. all other dietary treatments ($P < 0.0007$). Similarly the mean total anaerobic count in the cecum of rats fed human milk solids was greater than that of all other treatments ($P < 0.01$) with the exception of rats fed cow milk solids ($P < 0.06$).

**Folate concentration of plasma, cecal and colon samples.** The mean plasma folate concentration of rats fed human milk was significantly greater than that of rats fed cow or goat milk or a milk-free diet at the end of the folate-repletion period ($P < 0.05$) (Table 5). The mean plasma folate concentration of rats fed cow, goat and milk-free diets did not differ. As expected, the mean plasma folate concentration of rats fed the 2x folate milk-free diet (4530 nmol folate/kg diet) was high (151.8 ± 15.2 nmol/L) compared with rats in the other dietary treatments that were fed 906 nmol folate/kg diet. Plasma folate concentrations were significantly correlated with the number of *Bifidobacterium* sp. in the ceca ($r = 0.69$, $P < 0.0007$) and colons ($r = 0.57$, $P < 0.02$). Trends were observed between the overall anaerobic counts in the cecal ($r = 0.42$, $P < 0.07$) and colon ($r < 0.03$) with the exception of rats fed cow milk solids ($P < 0.06$).

**FIGURE 1** Enumeration of *Bifidobacterium* in the cecum (cecal sac plus contents) of rats consuming diets containing milk solids (human, cow and goat) and diets devoid of milk solids. Results are expressed as untransformed means ± SEM ($n = 5–6$). Statistical analyses were performed on the log scale to normalize these data. Unlike letters denote significant differences ($P < 0.05$).
MILK TYPE AND MICROFLORA IN THE CECUM AND COLON

FIGURE 2 Enumeration of total anaerobic load in the cecum (cecal sac plus contents) of rats consuming diets containing milk solids (human, cow and goat) and diets devoid of milk solids. Results are expressed as untransformed means ± SEM (n = 5–6). Statistical analyses were performed on the log scale to normalize these data. Unlike letters denote significant differences (P < 0.05).

= 0.41, P < 0.08) samples vs. plasma folate concentrations but these were not significant. Plasma folate concentrations were not correlated with the number of Bacteroides sp., Clostridium sp., Escherichia sp. or Streptococcus sp. in the rat cecum or colon.

The total amount of folate found in the cecum (cecal sac plus contents) of rats fed human milk solids tended to be greater than that of all other dietary treatments. Similarly, the total folate content in the cecal contents (excluding the sac) of rats fed human milk was at least 56% greater than that of all other dietary treatments. In contrast, the folate content of the colon did not significantly differ among dietary treatments.

DISCUSSION

Findings from this study suggest that the superior folate status of rats consuming diets containing human milk solids is due to alterations in the microbial milieu of the cecum and colon. Rats fed human milk solids in the present study had at least a seven- and onefold increase in cecal (P < 0.0007) and colonic (P < 0.04) Bifidobacterium density, respectively, compared with rats fed diets containing cow or goat milk solids or diets prepared without milk solids. This dramatic increase in Bifidobacterium content was reflected in an increase in the total anaerobic bacterial population density in both the cecum and colon. Further, rats fed human milk solids had ceca that were at least 30% larger than those of rats fed diets containing goat milk or milk-free diets.

Logically, if an increase in the total anaerobic bacterial numbers generally and the Bifidobacterium population density, specifically, is responsible for the improved folate status of human milk–fed rats, one would expect an association between the number of bacteria in the cecum and colon and blood folate values. Indeed, in the present investigation, the plasma folate concentrations of rats were correlated with the Bifidobacterium concentrations in both the cecum (r = 0.69, P < 0.0007) and colon (r = 0.57, P < 0.02). Trends were also observed between the total anaerobic bacterial density in the cecal (r = 0.42, P < 0.07) and colon (r = 0.41, P < 0.08) material and plasma folate concentrations, but this relationship did not reach significance. Further, the concentration of folate in the cecal material of rats fed human milk solids was greater (at least 48%) than that of all other dietary treatments containing 906 nmol/kg folic acid. Considered together, these data suggest that the consumption of human milk solids promotes the colonization of Bifidobacterium in the colon and cecum of rats. The increase in Bifidobacterium, a known synthesizer of folate (Deguchi et al. 1985), appears to enhance the net microbial production of folate, thereby improving the folate status of the host organism.

It has been known since the 1940s that substantial quantities of folate are produced by microorganisms in the large intestine (Cooperstock and Zedd 1983, Denko et al. 1946). Rodriguez (1978), in her comprehensive

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Human milk</th>
<th>Cow milk</th>
<th>Goat milk</th>
<th>Milk free</th>
<th>2x Folate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14 ± 3b</td>
<td>64 ± 22a</td>
<td>49 ± 12a</td>
<td>42 ± 17ab</td>
<td>36 ± 10ab</td>
</tr>
<tr>
<td>Clostridium</td>
<td>7980 ± 532</td>
<td>2681 ± 440</td>
<td>5272 ± 1979</td>
<td>1322 ± 138</td>
<td>2239 ± 621</td>
</tr>
<tr>
<td>Escherichia</td>
<td>1 ± 0.4</td>
<td>1 ± 0.7</td>
<td>9 ± 4</td>
<td>5 ± 4</td>
<td>8 ± 5</td>
</tr>
<tr>
<td>Streptococcus</td>
<td>313 ± 298</td>
<td>221 ± 115</td>
<td>98 ± 42</td>
<td>18 ± 14</td>
<td>26 ± 18</td>
</tr>
</tbody>
</table>

1 Results are expressed as untransformed means ± SEM (n = 4–5). Statistical analyses were performed on transformed data. Unlike superscripts within each row denote significant differences (P < 0.05).
review of the research literature, concluded that the feces of humans may contain 5–15 times more folate than is ingested daily. Until very recently, however, researchers have not considered the metabolic fate of this large and potentially very useful store of newly synthesized folate. New evidence from work with rats (Rong et al. 1991) and organ-cultured endoscopic biopsy specimens from human colon (Zimmerman 1990) suggest that folate synthesized in the large intestine can, in fact, be absorbed across the large intestine and utilized by the host organism. We propose that the availability of microbially synthesized folate in the large intestine may have a significant impact on the folate status of the host organism and that dietary manipulations that alter the profile of microorganisms in the large intestine may, in turn, affect the folate status of the host.

Our work with suckling and weanling rats was motivated by an interest in understanding the constituents in food that might be exploited to promote the folate status of humans. Given this intention, then, it is important to consider the limitations of extrapolating these data from rats to the human situation. The two most obvious considerations are that rats are coprophagic and have a larger cecum relative to their total body weight than do humans. Because rats are highly efficient at consuming their own feces, folate synthesized by microorganisms in the large intestine and excreted by way of feces can be absorbed in the small intestine. Therefore, while we expect that folate is absorbed across the large intestine of both humans and rats, the potential benefit of enhanced microbial biosynthesis of folate may be greater for rats than humans.

Second, the cecum as a proportion of total body weight in the rat is much greater than that of the human. While we recognize this difference, the population density of various anaerobic species in the large intestine of humans does, in fact, approximate that in the cecum of rats (Goldin et al. 1994, Raibaud et al. 1966). Given the length (approximately 1.5 m) and total bacterial count (10^{14}–10^{15} colony forming units/L) in the large intestine, there is ample opportunity for microorganisms to produce large quantities of folate.

Our data using rats as well as early human work consistently demonstrate that rats and infants fed human milk maintain higher blood folate concentrations than their counterparts fed cow milk, goat milk or home-made cow milk preparations (Matoh et al. 1979,
Semchuk et al. 1994, Swiatlo et al. 1990). In contrast, the serum and erythrocyte folate concentrations of infants <6 mo old fed proprietary formula are reported to be similar to or greater than those of breast-fed infants (Davis et al. 1986, Picciano 1995, Smith et al. 1985). These observations make sense because proprietary formulas are highly fortified with synthetic folic acid and thus contain ~ twice the folate concentration of human milk (Picciano 1995). Thus, while human milk consumption may alter gut microflora to enhance folate synthesis of infants, the high folic acid concentration of proprietary formula protects the bottle-fed infant against suboptimal folate status.

In conclusion, the improved folate status of rats fed diets containing human milk appears to be due, at least in part, to changes in the microbial ecosystem in the colon and cecum and enhanced production of microbiologically synthesized folate. In the present study, these changes were noted in young rats that had been previously weaned, suggesting that components in human milk may be used to develop novel strategies to improve the folate status of humans and other mammals during stages of life beyond infancy.

**LITERATURE CITED**


