Prebiotic oligosaccharides and the enterohepatic circulation of bile salts in rats

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Prebiotic oligosaccharides and the enterohepatic circulation of bile salts in rats. Am J Physiol Gastrointest Liver Physiol 294: G540–G547, 2008. First published December 13, 2007; doi:10.1152/ajpgi.00396.2007.—Human milk contains prebiotic oligosaccharides, which stimulate the growth of intestinal bifidobacteria and lactobacilli. It is unclear whether the prebiotic capacity of human milk contributes to the larger bile salt pool size and the more efficient fat absorption in infants fed human milk compared with formula. We determined the effect of prebiotic oligosaccharides on bile salt metabolism in rats. Rats were fed a control diet or an isocaloric diet containing a mixture of galactooligosaccharides (GOS), long-chain fructooligosaccharides (lcFOS), and acidified oligosaccharides (AOS) for 3 wk. We determined synthesis rate, pool size, and fractional turnover rate (FTR) of the primary bile salt cholate by using stable isotope dilution methodology. We quantified bile flow and biliary bile salt secretion rates through bile cannulation. Prebiotic intervention resulted in significant changes in fecal and colonic flora: the proportion of lactobacilli increased 434% (P < 0.01) in colon content and 139% (P < 0.01) in feces compared with the control group. The number of bifidobacteria also increased 366% (P < 0.01) in colon content and 282% in feces after the prebiotic treatment. Furthermore, pH in both colon and feces decreased significantly with 1.0 and 0.5 pH point, respectively. However, despite this alteration of intestinal bacterial flora, no significant effect on relevant parameters of bile salt metabolism and cholate kinetics was found. The present data in rats do not support the hypothesis that prebiotics naturally present in human milk contribute to a larger bile salt pool size or altered bile salt pool kinetics.}

THE ENTEROHEPATIC CIRCULATION of bile salts, the major constituents of bile, serves two important functions in the human body. Bile salts enhance the absorption of long-chain saturated fatty acids and fat-soluble vitamins from the intestine. Furthermore, the enterohepatic circulation of bile salts promotes the excretion of lipophilic molecules via the bile into the feces (e.g., cholesterol and bilirubin) and is critically important for cholesterol homeostasis in the body. The dietary fat intake in infants is relatively high compared with the dietary fat intake in adults, whereas the bile salt pool size of the former is lower (33). Efficient absorption of dietary fat is essential for optimal growth and development during infancy.

Human milk is the gold standard in infant feeding. One of the beneficial qualities of human milk involves the more efficient absorption of dietary fat compared with that from formula (34). Interestingly, the bile salt pool is larger in premature infants fed human milk compared with formula, but it has not been clarified whether this contributes to the mechanism underlying the more efficient fat absorption (34). Previously, we reported that the maturation of fat absorption in human neonates is functionally related to an increased capacity to absorb long-chain fatty acids from the intestine, possibly due to developmental changes in bile salt composition and bile salt pool (28).

Prebiotics are nondigestible food ingredients that stimulate the growth and activity of specific bacteria in the colon (i.e., bifidobacteria and lactobacilli) (14). Oligosaccharides are a major constituent of human milk and have been demonstrated to increase the proportion of bifidobacteria and lactobacilli in the infant’s colon (4, 9). The prebiotic galactooligosaccharides (GOS) and fructooligosaccharides (FOS) are known to increase the number of bifidobacteria and lactobacilli in the gut in both human and animal studies (3, 26, 32). Previous studies in rats, using the prebiotic substrate FOS, have shown an increase in the amount of intestinal bifidobacteria and lactobacillus (25, 31).

It is not known whether oligosaccharides, the prebiotic constituent of human milk, influence the bile salt metabolism and thereby play a role in the more efficient fat absorption of breast milk. Interactions between intestinal flora and bile salts are well known. The bacterial metabolism of bile salts in the intestinal lumen can involve deconjugation and dehydroxylation, converting primary bile salts such as cholate and chenodeoxycholate into secondary bile salts (i.e., deoxycholate and lithocholate). Bacterial metabolism of bile salts is partly responsible for the fractional turnover rate (FTR) of primary bile salts, i.e., the portion of the pool that is newly synthesized per day. Bacterial metabolism may also influence the physiological activity of bile salts since secondary bile salts are more hydrophobic than primary bile salts and therefore have a greater capacity to interact with dietary fat.

Recently we developed a method to quantify cholate fluxes in the enterohepatic circulation of experimental animals (19).

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This method is based on a stable isotope dilution methodology used in humans that was successfully downscaled to allow measurement of cholate fluxes in experimental animals (19). The enterohepatic circulation of bile salts can be quantitatively characterized by specific kinetic parameters: the pool size (the amount of bile salts in the body), the FTR (the fraction of the pool that is newly synthesized each day), the synthesis rate, and, finally, the cycling time (the time it takes the cholate pool to undergo one full cycle in the enterohepatic circulation). In the present study, we determined the effect of dietary prebiotic oligosaccharides on the enterohepatic circulation of cholate, the major primary bile salt in rats.

MATERIALS AND METHODS

Animals and materials. Six-week-old male rats (Harlan Laboratories, Zeist, The Netherlands) were housed in a light- and temperature-controlled facility with free access to tap water and to either the prebiotic diet or the control diet. Experimental protocols were approved by the Ethical Committee for Animal Experiments, Faculty of Medical Sciences (University Medical Center Groningen, The Netherlands). 2,2,4,4-Tetradeuterated cholic acid ([2H4]-CA; isotopic purity, 98%) was obtained from Isotec (Miamisburg, OH). All other chemicals and solvents used were of the highest purity commercially available. Animals received semi-purifiedAIN-93-based diets pressed into pellets (Research Diet Services, Wijk bij Duurstede, The Netherlands). In the prebiotic diet, the supplemented oligosaccharides were exchanged for the same amount of carbohydrates. The experimental erlands). In the prebiotic diet, the supplemented oligosaccharides were into pellets (Research Diet Services, Wijk bij Duurstede, The Neth- erlands). 2,2,4,4-Tetradeuterated cholic acid ([2H4]-CA; isotopic pu- rity, 98%) was obtained from Isotec (Miamisburg, OH). All other chemicals and solvents used were of the highest purity commercially available. Animals received semi-purified AIN-93-based diets pressed into pellets (Research Diet Services, Wijk bij Duurstede, The Netherlands). In the prebiotic diet, the supplemented oligosaccharides were exchanged for the same amount of carbohydrates. The experimental diet was supplemented with GOS, lCFOS, and AOS in a dose of 7.65, 0.85, and 0.90 wt%, respectively (Table 1).

Experimental procedures. Male Wistar rats (253 ± 1 g body wt) were fed the control diet for 3 wk as a run in, after which they were randomly assigned to the prebiotic or the control diet for another 3 wk (n = 8 per group). Body weight was measured weekly; food intake and fecal production were measured during 72 h in the third week after randomization. Three weeks after randomization, relevant parameters of synthesis and enterohepatic circulation of cholate were determined by using the previously mentioned stable isotope dilution technique (19). In short, 3.0 mg of [2H4]-CA in a solution of 0.5% NaHCO3 in phosphate-buffered saline was slowly injected via the exchanged for the same amount of carbohydrates. The experimental

The cycling time of the enterohepatic circulation is the time it takes for a bile salt to undergo one full cycle. The cycling time for cholate can be estimated by dividing the cholate pool size by the biliary secretion rate of cholate (which was calculated by using bile salt composition in bile and bile flow rates after bile cannulation). The fraction of cholate lost per cycle was calculated by

Table 1. Composition of the prebiotic diet and the control diet

<table>
<thead>
<tr>
<th>Ingredients, g/kg</th>
<th>Control Diet (AIN-93G)</th>
<th>Prebiotic Diet (GOS-lCFOS-AOS supplemented)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornstarch</td>
<td>397.5</td>
<td>397.5</td>
</tr>
<tr>
<td>Dextrinized cornstarch</td>
<td>132.0</td>
<td>65.9</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100.0</td>
<td>72.3</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Pure carbohydrate mix Numico*</td>
<td>70.0</td>
<td>93.8</td>
</tr>
<tr>
<td>Soybean oil</td>
<td></td>
<td>70.0</td>
</tr>
</tbody>
</table>

*85 g galactooligosaccharides (GOS), long-chain fructooligosaccharides (lCFOS) (9:1), and 8.8 g acidified oligosaccharides (AOS).
dividing fractional cholate synthesis rate by cholate cycling frequency.

**Fecal flora composition.** Frozen fecal or colon samples were thawed by adding 1 ml of MilliQ water and heating at 90°C. The suspensions were subsequently homogenized and frozen overnight at −20°C. The homogenized samples were thawed at room temperature, followed by DNA isolation by using the NucliSense Isolation Extraction Kit (BioMerieux, Boxtel, The Netherlands). For the relative quantification of the genera *Bifidobacterium* and *Lactobacillus* in relation to the total bacterial load, a duplex 5′ nuclease quantitative real-time PCR assay was used (15).

Briefly, different primers and probes for the genus *Bifidobacterium* or *Lactobacillus* in combination with primers and probes for total bacteria were used in a temperature profile consisting of 2 min at 50°C, 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 60°C for 1 min, run on ABI Prism 7700 PCR equipment (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The relative amounts of the genus *Bifidobacterium* or *Lactobacillus* in the samples were calculated with respect to the total bacterial load according to Liu et al. (23) and expressed in percentages. All samples were analyzed in triplicate.

**Statistics.** Values represent means ± SD for the indicated number of animals per group. Differences between the two groups were determined by Student’s *t*-test for normally distributed values, and Mann-Whitney exact two-tailed *U*-test was used for nonnormally distributed data. *P* < 0.05 was considered significant. Analysis was performed using SPSS 12.0 for Windows software (SPSS, Chicago, IL).

**RESULTS**

**Animal characteristics.** Animals fed the prebiotic diet or the control diet were comparable in body weight, growth, fecal production, and food intake (Table 2). The prebiotic diet significantly elevated serum lathosterol, an intermediate in the cholesterol synthesis pathway, whereas plasma concentrations of cholesterol, triglycerides, alanine transaminase, aspartate transaminase, and alkaline phosphatase were not altered upon treatment (Table 2).

Table 2. Animal characteristics, plasma and hepatic parameters of lipid metabolism, and liver function after feeding rats a prebiotic or a control diet

<table>
<thead>
<tr>
<th></th>
<th>Control Diet</th>
<th>Prebiotic Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Animal characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight</td>
<td>252 ± 12</td>
<td>254 ± 11</td>
</tr>
<tr>
<td>Body weight at termination</td>
<td>456 ± 35</td>
<td>476 ± 28</td>
</tr>
<tr>
<td>Feces (wet), g/day</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Food intake, g/24 h</td>
<td>27.9 ± 1.7</td>
<td>26.7 ± 1.6</td>
</tr>
<tr>
<td><strong>Hepatic parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver weight (absolute, g)</td>
<td>13.8 ± 1.4</td>
<td>14.8 ± 1.7</td>
</tr>
<tr>
<td>Liver weight (relative, % of body wt)</td>
<td>3.0 ± 0.2</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>Triglycerides, nmol/mg liver</td>
<td>14.3 ± 5.4</td>
<td>14.7 ± 5.6</td>
</tr>
<tr>
<td>Cholesterol, nmol/mg liver</td>
<td>5.4 ± 0.7</td>
<td>5.7 ± 1.7</td>
</tr>
<tr>
<td><strong>Plasma parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine transaminase, units/l</td>
<td>46 ± 10</td>
<td>45 ± 7</td>
</tr>
<tr>
<td>Aspartate transaminase, units/l</td>
<td>153 ± 75</td>
<td>155 ± 49</td>
</tr>
<tr>
<td>Alkaline phosphatase, units/l</td>
<td>6 ± 7</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Cholesterol, mmol/l</td>
<td>1.8 ± 0.3</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>1.4 ± 0.5</td>
<td>1.9 ± 1.0</td>
</tr>
<tr>
<td>Lathosterol, µmol/l</td>
<td>0.7 ± 0.1</td>
<td>1.3 ± 0.5*</td>
</tr>
</tbody>
</table>

*P* < 0.05.

**Effect of prebiotic treatment on the composition of intestinal flora.** Figure 1 shows that the prebiotic diet had a prominent bifidogenic effect. After 3 wk the relative numbers of bifidobacteria and of lactobacilli were significantly higher in

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Fig. 1. Effect of prebiotic treatment (solid bars) on percentage of total bacterial load of bifidobacteria (*A*) and lactobacilli (*B*) in feces and colon content compared with control rats (open bars). Effect of prebiotic treatment on pH in feces and colon content (*C*). Prebiotic-treated rats are significantly different from controls. Data are expressed in means ± SD of n = 8 per group; *P* < 0.05; **P** < 0.01.
colon content and in feces in rats fed the prebiotic diet compared with controls. The proportion of lactobacilli increased 344% ($P < 0.01$) in colon content and 139% ($P < 0.05$) in feces compared with rats on control diet (Fig. 1).

Prebiotic treatment also increased the contribution of bifidobacteria 366% ($P < 0.01$) in colon content and 282% ($P < 0.01$) in feces compared with the control group (Fig. 1). The prebiotic diet significantly lowered pH in both colon content and feces with 1.0 and 0.5 pH point, respectively (Fig. 1).

**Effect of prebiotic intervention on parameters of the enterohepatic circulation of bile salts.** Bile flow and biliary secretion rates of bile salts, phospholipids, and cholesterol were similar between the groups (Fig. 2), as were biliary bile salt composition (Fig. 3), fecal neutral sterol composition, and fecal bile salt excretion (Fig. 4). Prebiotic treatment did not affect the enterohepatic circulation of bile salts. Kinetic parameters of cholate, the main bile salt in rodents, were not significantly altered by the prebiotic diet. Cholate synthesis rate, pool size, and FTR, as well as fecal excretion, were similar in both groups (Table 3 and Fig. 5).

**Effect of prebiotic treatment on hepatic and plasma lipids.** The prebiotic treatment did not affect hepatic and plasma concentrations of cholesterol and triglycerides (Table 2). Upon FPLC separation of plasma lipoproteins, the distribution of triglycerides in the different fractions was comparable in prebiotic-treated rats and controls (Fig. 6).

**DISCUSSION**

We investigated whether prebiotic treatment and subsequent alteration of the intestinal bacterial flora affects the enterohepatic circulation and composition of bile salts in rats. The results show that prebiotic treatment significantly increased the relative contributions of both lactobacilli and bifidobacteria in the colon and the feces of rats. Furthermore, pH in both colon and feces significantly decreased after feeding rats a prebiotic diet. This prebiotic effect, however, did not significantly affect bile flow or bile salt composition or the synthesis and enterohepatic circulation of cholate.

The liver parenchymal cells synthesize the primary bile salts cholate and chenodeoxycholate. Primary bile salts are conjugated before their secretion into bile (in rats predominantly to taurine). Upon entering the proximal small intestine, conjugated bile salts stimulate the emulsification and absorption of dietary fat. Conjugated bile salts are efficiently reabsorbed in the terminal ileum, mediated by the apical sodium-dependent bile salt transporter (Asbt) and the basolateral transporter Ost α/β. Primary bile salts that escape absorption in the terminal
ileum may be deconjugated by the bacterial flora in the colon and then undergo (7-alpha-) dehydroxylation resulting in the formation of secondary bile salts such as deoxycholate and lithocholate. Various intestinal bacterial species, including lactobacilli and bifidobacteria, have the capacity to metabolize bile salts (10). The secondary bile salts are partly reabsorbed by passive diffusion or are lost via the feces. Intestinal reabsorption of secondary bile salts, dependent on passive diffusion, is less efficient than the Asbt-mediated transport of primary bile salts.

Under steady-state conditions, fecal bile salt loss is compensated by synthesis of primary bile salts in the liver. In theory, modification of intestinal bacterial flora and subsequent altered distribution of bile salts between primary and secondary type could influence intestinal absorption and fecal excretion of bile salts and therefore affect kinetic parameters of bile salt homeostasis. In preterm infants fed human milk, the bile salt pool size is larger compared with formula-fed infants, indicating that dietary factors affect the pool size (34). Also, fecal bile salt concentrations are higher in infants fed human milk and contain a smaller fraction of secondary bile salts compared with those fed formula (16). Breast-fed infants have an intestinal bacterial flora that is characterized by relatively high amounts of bifidobacteria and lactobacilli (4, 17, 20, 29). Because of the fermenting ability of these bacteria, a lower stool pH is found in infants fed human milk compared with formula-fed infants. The prebiotic capacity of human milk induces a different intestinal environment, which could, in theory, alter the enterohepatic circulation of bile salts. Our present data show that a prebiotic treatment altered the intestinal bacterial flora in adult rats, indicated by a significant induction of colonic and fecal bifidobacteria and lactobacilli and a significant decrease in fecal pH. Interestingly, however, the prebiotic diet did not affect either the bile salt composition or the kinetic parameters of the enterohepatic bile salt circulation (Fig. 5).

Theoretically, these observations could be due to a different response to a prebiotic diet in rats compared with other species. The present prebiotic treatment resulted in lactobacilli, accounting for 2.0 and 1.8% of total bacterial load in colon and feces, respectively. The same diet (AIN-93G) supplemented with 10 wt% GOS-IcFOS (ratio 9:1) resulted in 7.5% lactobacilli in feces of mice (32). Haarman et al. (15) found fecal flora to contain 4.4% lactobacilli in infants receiving a formula supplemented with 0.8 g/100 ml GOS-IcFOS (ratio 9:1) for 6 wk, similar to values observed in breast-fed infants. Despite the still limited absolute contribution of lactobacilli, the deceased colonic and fecal pH indicates that a physiological response is achieved.

However, the bacterial flora of infants fed human milk contains >60% bifidobacteria within 1 wk after birth (17). The addition of a GOS-FOS mixture to infant formula resulted in an increase of fecal bifidobacteria of almost 60% in healthy infants (20). Vos et al. (32) used an identical prebiotic diet (10 wt% GOS-IcFOS; ratio 9:1) in a murine model, resulting in 40% bifidobacteria of total bacterial load in fecal samples.
Surprisingly, the present study showed a contribution of bifidobacteria of 1.3 and 1.5% in feces and colon content, respectively, after prebiotic treatment. Thus, although the presently used diet resulted in an increase of lactobacillus in the same range as mice and humans and a significant decrease in fecal and colonic pH, the less pronounced bifidogenic response could theoretically contribute to the observed lack of effect on the enterohepatic circulation of bile salts.

It should be realized that the situation studied in the present rat model differs in several aspects from the physiology in human infants. Besides the less pronounced prebiotic response, it cannot be excluded that the absence of a gallbladder in rats influences the dynamics of the enterohepatic circulation of bile salts. Furthermore, immaturity of intestinal absorption of nutrients (28) and bile salts (6) intervene with the enterohepatic circulation of bile salts, which may render the dynamics of bile salt metabolism in human infants not completely comparable to the presently studied rat model. Finally, only in rats, the primary bile salt chenodeoxycholate is further metabolized to α-muricholic acid and β-muricholic acid, rendering the bile salt pool more hydrophilic compared with the human situation. In the present experiment, however, the amount of muricholic bile salts measured in bile did not exceed 25%. Nevertheless, we are aware of the possibility that one or more of the aforementioned differences may limit the extrapolation of the present results to the human situation.

Besides the addressed differences in bile salt physiology between human infants and the presently used rat model, human milk contains high concentrations of cholesterol (2.6–3.9 mmol/l) compared with formulas (0.3–0.9 mmol/l) (5). The colonic flora of human infants develops its dehydroxylating capacity over the first months of life (2), whereas in the adult rat the dehydroxylating bacteria are well established. It is tempting to speculate that the larger bile salt pool size found in breast-fed infants could be related to the substantially higher dietary intake of cholesterol and not, based on our present observations, on differences in dietary oligosaccharides.

Enhanced conjugation of bile salts by bile hydrolase-producing bacteria such as lactobacilli could, in theory, be expected to increase the amount of deconjugated bile salts and the subsequent fecal excretion hereof. In the present study, both amount and composition of fecal bile salts were unaffected upon prebiotic treatment. We hypothesize that this can be due to the relative low contribution of lactobacillus and bifidobacteria to the total intestinal deconjugating capacity. Bile salt hydrolase is apart from lactobacillus and bifidobacteria, also detected in other bacterial species (12). Furthermore, efficient passive absorption of deconjugated bile salts may have masked a deconjugating, enhancing effect of prebiotic treatment.

The presently applied methodology allowed determination of bile salt kinetic parameters in a physiologically uncompromised animal model. The parameters of cholate kinetics determined in this experiment were in line with previous experiments performed in rats by using the same method (19). Under steady-state conditions, the bile salt pool size is regulated by hepatic de novo synthesis of bile salts and by the efficiency by
which bile salts are reabsorbed in the intestine. Under steady-state conditions, the amount lost via the feces is equal to the amount of bile salts newly synthesized by the liver. A difference neither in cholate synthesis rate nor in fecal bile salt excretion was observed, indicating that prebiotic treatment does not affect the two important rate-limiting parameters of the enterohepatic circulation. We also observed that the fecal excretion of chenodeoxycholate-derived metabolites (muricholate and hydoxycholate) was not altered either, suggesting no effect of prebiotic treatment on the synthesis of the other primary bile salt, chenodeoxycholate.

Apart from an important role in absorption of dietary lipids, bile salts are critically involved in cholesterol homeostasis in the body. Prebiotic substances are suggested to have a plasma lipid-lowering effect in both animal and human studies. Serum cholesterol concentrations in bottle-fed infants decreased as the number of lactobacilli in their stools increased (18). Possible mechanisms could be modulation of the intestinal bacterial flora with production of short-chain fatty acids inhibiting hepatic fatty acid or cholesterol synthesis or alteration of the bacterial bile acid deconjugating capacity and a subsequent altered fecal bile salt excretion (8). Studies in rats have consistently shown a decrease in plasma triglycerides after a diet supplemented with inulin and oligofructose (reviewed in Ref. 7). Data on plasma cholesterol, however, are less straightforward. A 10 wt% oligofructose-supplemented diet decreased serum cholesterol concentrations in rats in one study (11), but the addition of oligofructose to the diet of lean rats did not induce a decrease in plasma cholesterol levels in another study (21). Human studies investigating the effect of oligofructans on lipid metabolism have shown variable results (reviewed in Ref. 8). Our prebiotic diet did not significantly affect plasma concentrations of cholesterol in rats, whereas it increased the serum lathosterol concentration. Lathosterol is an intermediate in the cholesterol synthesis pathway, which could suggest that cholesterol synthesis was enhanced during prebiotic treatment. However, cholesterol synthesis was not determined in this experiment, and no other indications of increased cholesterol synthesis were obtained. For example, the hepatic mRNA expression of HMG-CoA reductase, encoding for the rate-limiting enzyme of cholesterol synthesis, was not upregulated by prebiotic treatment (data not shown). The presently used diet did not affect serum triglycerides or hepatic triglycerides. Also, the amount of triglycerides in the VLDL fraction of lipoproteins was comparable in prebiotic-treated and control rats, indicating that the present treatment did not affect hepatic VLDL composition or triglyceride distribution over the various plasma lipoproteins (Fig. 6). Rat studies on lipid metabolism or bile salt metabolism using diets supplemented with the presently used GOS-lcFOS, have, to the best of our knowledge, not been performed. In accordance with the present results, a study recently performed in human infants showed unaffected plasma levels of cholesterol and of triglycerides in infants fed a formula supplemented with identical prebiotic substances in the same ratio used in the present study (1).

In summary, we conclude that feeding rats a prebiotic diet induces modification of the intestinal flora and decreases the intestinal pH in the colon and the feces. However, the prebiotic diet does not influence the metabolism of bile salts in rats. The present data in rats do not support the hypothesis that prebiotics naturally present in human milk contribute to a larger bile salt pool size or altered pool kinetics.

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