Effects of Cultured Milk Products by *Lactobacillus* and *Bifidobacterium* Species on the Secretion of Bile Acids in Hepatocytes and in Rats

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Summary Whey preparations prepared from cultured milk by 19 *Lactobacillus* (2 species) and 20 *Bifidobacterium* (5 species) strains were examined for the effects of secretion and synthesis of bile acids in primary cultured rat hepatocytes. The stimulating effect of whey preparation on bile acid secretion depended on the species as well as the strains used for milk fermentation. Two strains belonging to *L. casei* SBT 2230 (LC2230) and *B. longum* SBT 2912 (BL2912) produced the whey which stimulates both the secretion of bile acid and the activity of cholesterol 7α-hydroxylase, a rate-limiting enzyme for bile acid synthesis. When the cultured products by these two strains were given to rats for 14 days, the products from *L. casei* (LC2230) were found to stimulate the biliary secretion of bile acids. These results suggest that primary cultured hepatocytes were a useful experimental system as an initial screening for an active principle modulating cholesterol metabolism.

Key Words cultured milk, *Lactobacillus casei*, *Bifidobacterium longum*, secretion of bile acids, primary cultured hepatocytes, cholesterol 7α-hydroxylase, secretion of biliary bile acids, whey preparation, lactic acid bacteria

The effects of fermented milk products on human cholesterol metabolism are conflicting among reports (1–4). In addition to differences in experimental designs, species and/or strains of lactic acid bacteria used for the production of fermented milk appear to be responsible for the inconsistency, because chemical and physical natures of fermentation products vary with different type of bacteria (5).

Since serum cholesterol is discharged ultimately as the biliary bile acids, transformation of cholesterol into bile acids by the liver is an important process by
which the serum cholesterol level is reduced. Primary cultured rat hepatocytes are extensively utilized for studies on the regulation of bile acid synthesis. Dietary manipulations such as cholestyramine treatment and a high-cholesterol diet have been reported to maintain regulatory influence on bile acid metabolism even in the primary cultured hepatocytes prepared from rats (6). In addition, in the previous experiment, we showed that rat whole milk and its whey preparation modify the secretion of bile acids by cultured hepatocytes (7). Thus, in the present study, we adopted the cultured rat hepatocytes to examine influences of the whey preparations prepared from cultured milk by 39 strains of lactic acid bacteria on the secretion of bile acids. *Lactobacillus* and *Bifidobacterium* species were chosen here as they are a constant inhabitant in human microflora. The present results show that production of bile acid varies depending on the products from different strains, and one of the strains of *L. casei* was found to be active for secretion of bile acid both *in vitro* and *in vivo.*

**MATERIALS AND METHODS**

Preparation of cultured milk products. Mediums composed of 10% skim milk powder (a reduced form) and 0.3–0.5% yeast extract (Asahi Brewery Co., Tokyo) in deionized water were sterilized at 121°C for 15 min and inoculated with two *Lactobacillus* species (11 strains of *L. acidophilus* and 8 strains of *L. casei*) and five *Bifidobacterium* species (3 strains of *B. bifidum*, 6 strains of *B. infantis*, 3 strains of *B. breve*, 5 strains of *B. adolescentis*, and 3 strains of *B. longum*) at 37°C for 16–48 h. After incubation the medium pH was in the range from 3.79 to 5.07. Whey fractions were prepared from the medium by centrifugating at 1,000 g for 10 min, and filtered through 0.45 μm filter. A definite volume of the filtrates was freeze-dried (1 ml is approximately equivalent to 53.8 mg of dried whey). Immediately before the addition of the whey preparations to the culture medium, they were dissolved in the original volume of water, equivalent to that before freeze-drying.

Determination of uptake of [14C]cholesterol and secretion of labeled bile acid, and synthesis of cholesterol from [14C]acetate by primary cultured hepatocytes. Male Sprague-Dawley rats, weighing approximately 200 g and maintained on the commercial nonpurified diet (NMF, Oriental Yeast Co., Tokyo) were used. Liver parenchymal cells were prepared by the collagenase perfusion method as described previously (7,8). The cells suspended in L-15 medium (pH 7.2) containing antibiotics (penicillin G, streptomycin sulfate, and amphotericin B), insulin (10⁻⁷ M), dexamethazone (10⁻⁷ M), nicotinic acid (10 mM) and 20% fetal calf serum were seeded on the culture dish (35 × 10 mm; FALCON® 3046, Becton Dickinson & Co., N.J.), precoated with rat tail collagen (Type I, Biomedical Product Division, MA), at 37°C for 2 h. Subsequently the cells (1 × 10⁶) were incubated with 2 ml of the same fresh medium as above containing 1% whey fractions for 24 h. When necessary, the medium was adjusted to pH 7.2 with a diluted NaOH solution. The cells thus obtained were incubated with the freshly prepared medium.
containing 6–7 μl of 1 μCi[4-14C]cholesterol (56.1 mCi/mmol, Amersham Japan, Tokyo)–rat serum complex for a further 24 h. Lipids of the medium were extracted according to the Bligh-Dyer method and the methanol phase was subjected to determination of the radioactive bile acids (6). Namely, the cells were washed repeatedly with phosphate-buffered saline, solubilized with 0.2 N NaOH and its portion was used for determination of the [14C]cholesterol uptake by the cells and proteins as described previously (7).

To determine the synthesis of cholesterol, the hepatocytes which were preincubated for 24 h were incubated with the fresh medium containing 1 μCi[1-14C] acetate (40 mCi/mmol, Amersham Japan) for 24 h, and incorporation of the label compound into cholesterol was determined by thin-layer chromatography using a precoated plate (Silica gel 60, Merck, Darmstadt) and a developing solvent of petroleum ether : diethyl ether : acetic acid = 82 : 18 : 1 by vol, as described previously (7).

**Determination of cholesterol 7α-hydroxylase activity in primary cultured hepatocytes.** Cholesterol 7α-hydroxylase activity in primary monolayer-culture of hepatocytes was determined according to the method of Princen et al. (9). The liver parenchymal cells were prepared as described above and seeded on culture dishes (100×10 mm; FALCON®3003). The cells (5×10⁶) were incubated for 48 h with 10 ml of medium as described above. After removal of the medium, the cells washed with cold phosphate-buffered saline were scraped off with 2 ml of 100 mM phosphate buffer (pH 7.4) containing 20 mM cysteamine HCl (Wako Pure Chemicals Co., Osaka) and 4 mM MgCl₂. The cells were homogenized with Ultra disperser (Type TP18/10S4, Jank & Kunhol GmbH & CoKG, IKA-Werk, Staufen). The homogenate containing 4 mg protein (0.6 ml) was incubated with 0.2 ml of 100 mM phosphate buffer (pH 7.4) containing 20 mM glucose-6-phosphate (Oriental Yeast Co., Tokyo), 2 mM NADP (Oriental Yeast Co.) and 1 U glucose-6-phosphate dehydrogenase (Oriental Yeast Co.) and with 0.2 ml of substrate solution containing 1 μCi[14C]cholesterol in the phosphate buffer containing Tween 80 (1.5 mg/ml) for 30 min at 37°C. The reaction was stopped by the addition of a chloroform–methanol (2 : 1, vol/vol) mixture containing butylated hydroxytoluene (50 mg/liter). The lipid fraction separated in the chloroform layer was subjected to thin-layer chromatography (Silica gel 60, Merck) using ethyl acetate–benzene (7 : 3, vol/vol) as a developing solvent. The plate sprayed with EN³HANCE spray (New England Nuclear, Boston, MA) was subjected to autoradiography for 7–10 days, and the radioactivity of a band corresponding to 7α-hydroxycholesterol was counted by liquid scintillation counter (LSC-900, Aloka, Tokyo). The blank value was obtained by removing the NADPH generating system.

**Secretion of biliary bile acid and cholesterol in vivo.** Male Sprague-Dawley rats, 4 weeks old, were raised for 2 weeks with a purified diet composed of (in weight %): 20 casein, 10 lard, 15 α-corn starch, 3.5 mineral mixture (AIN76), 1.0 vitamin mixture (AIN76), 0.2 choline bitartrate, 0.3 DL-methionine, 5 cellulose, 0.43 freeze-dried cultured milk preparation, and sucrose to 100 (10). Bile duct was
cannulated with polyethylene tubing (PE 10, Becton-Dickinson Co., Parsippany, NJ) under Nembutal anesthesia (5 mg/100 g body weight). The bile was collected for 1.5 h. Bile acids were determined by hydroxy-steroid dehydrogenase assay (11). Cholesterol and triacylglycerol were determined enzymatically by using commercially available kits (Cholesterol C-Test and Triglyceride G-Test, Wako Pure Chemicals Co.).

Statistics. Data were analyzed by Duncan’s multiple range test preceded by analysis of variance (ANOVA).

RESULTS

In vitro experiment

Table 1 shows the protein contents, synthesis of cholesterol from [14C]acetate, uptake of [14C]cholesterol and secretion of [14C]bile acids in hepatocytes. Protein contents of hepatocytes incubated with the whey-containing medium relative to those incubated with the whey-free medium (control) were as follows (% to the control value); 103±2.1 for L. acidophilus, 95.4±1.5 for L. casei, 98.3±2.0 for B. infantis, 97.0±3.0 for B. breve, 108±5.0 for B. adolescentis, 103±5.0 for B. longum and 95.3±2.4 for B. bifidum. As a whole, the addition of the whey to the medium did not largely modify the protein content of the cells. Some cultured products from different strains, however, elevated the hepatocytes protein content, while others lowered it despite strain of the same species.

The synthesis of cholesterol from [14C]acetate was suppressed by supplementation of the medium with the whey preparations except for the ones prepared from 2 L. acidophilus strains (LA0280 and LA0290). The relative cholesterol synthesis in the strain of Bifidobacterium was less than 70% while that in the strain of Lactobacillus was more than 70% except 5 strains (LA0269, LA0270, LA2062, LC2227 and LC2230). The relative uptake of [14C]cholesterol by the hepatocytes was suppressed when they were incubated with the milk cultured products except for those from 3 strains (LC2227, BL2912 and BB2752). Since the uptake also varied within a species, the secretion of bile acids was corrected for the amount of the [14C]cholesterol taken up by the cells. Among the whey preparations which stimulated the secretion of bile acids (LA0269, LC2226, LC2230, LC2235, BI2852, BI2853, BB2813, BB2814, BA2701, BA2705, BL2907, BL2912 and BB2915), those from L. casei SBT 2230 (LC2230) and B. longum SBT 2912 (BL2912) were further examined for their effect on the activity of cholesterol 7α-hydroxylase. As shown in Fig. 1, the whey preparations from L. casei (LC2230) and B. longum (BL2912) had an increased effect on the activity of cholesterol 7α-hydroxylase.

In vivo experiment

Purified diets containing the cultured milk products from L. casei (LC2230) and B. longum (BL2912) were given to rats for 2 weeks, and the bile duct was cannulated. Although the food intake was lower in rats fed the whey from the B. longum, the liver weight of the rats fed the whey from the B. longum was significantly lower than that of the control group.
Table 1. Effects of cultured milk on the protein content, synthesis and uptake of cholesterol, and secretion of bile acids in hepatocytes (relative %).

| Strains | Protein content | Cholesterol synthesis | Cholesterol uptake | Bile acid synthesis |
|---------|----------------|-----------------------|--------------------|--------------------|
| L. acidophilus |                 |                       |                    |                    |
| 0237   | 103            | 86                    | 88                 | 92                 |
| 0269   | 117            | 58                    | 97                 | 118                |
| 0270   | 117            | 63                    | 78                 | 89                 |
| 0278   | 96             | 70                    | 88                 | 75                 |
| 0280   | 107            | 106                   | 50                 | 58                 |
| 0290   | 109            | 104                   | 48                 | 58                 |
| 0301   | 97             | 77                    | 71                 | 53                 |
| 2048   | 96             | 73                    | 68                 | 58                 |
| 2056   | 92             | 94                    | 67                 | 56                 |
| 2057   | 97             | 87                    | 58                 | 43                 |
| 2062   | 103            | 68                    | 90                 | 92                 |
| L. casei |                |                       |                    |                    |
| 2226   | 96             | 77                    | 81                 | 117                |
| 2227   | 90             | 44                    | 103                | 85                 |
| 2228   | 92             | 78                    | 73                 | 96                 |
| 2230   | 93             | 53                    | 85                 | 121                |
| 2231   | 98             | 82                    | 72                 | 56                 |
| 2232   | 100            | 74                    | 87                 | 66                 |
| 2233   | 92             | 88                    | 85                 | 60                 |
| 2235   | 102            | 76                    | 87                 | 119                |
| B. infantis |             |                       |                    |                    |
| 2851   | 91             | 63                    | 75                 | 89                 |
| 2852   | 100            | 44                    | 88                 | 115                |
| 2853   | 100            | 48                    | 83                 | 114                |
| 2854   | 100            | 43                    | 89                 | 99                 |
| 2855   | 94             | 63                    | 92                 | 94                 |
| 2856   | 105            | 69                    | 74                 | 69                 |
| B. breve |               |                       |                    |                    |
| 2813   | 100            | 67                    | 89                 | 108                |
| 2814   | 109            | 68                    | 89                 | 111                |
| 2815   | 82             | 57                    | 82                 | 72                 |
| B. adolescentis |              |                       |                    |                    |
| 2701   | 110            | 65                    | 87                 | 115                |
| 2702   | 109            | 54                    | 81                 | 93                 |
| 2703   | 110            | 68                    | 53                 | 72                 |
| 2704   | 115            | 54                    | 47                 | 97                 |
| 2705   | 97             | 67                    | 73                 | 112                |
| B. longum |              |                       |                    |                    |
| 2907   | 98             | 57                    | 72                 | 109                |
| 2911   | 113            | 52                    | 83                 | 82                 |
| 2912   | 98             | 60                    | 110                | 121                |
| B. bifidum |              |                       |                    |                    |
| 2751   | 100            | 65                    | 72                 | 100                |
| 2752   | 94             | 50                    | 140                | 101                |
| 2915   | 92             | 63                    | 97                 | 112                |

Hepatocytes were incubated with whey-free (5 dishes) or whey-containing (2 dishes) medium. The values are expressed as a relative mean-value to those in hepatocytes incubated with the whey-free medium.
Fig. 1. Effects of cultured milk prepared from *L. casei* (LC2230) and *B. longum* (BL2912) on the activity of cholesterol 7α-hydroxylase in hepatocytes. Hepatocytes were incubated with the whey-free medium (control), and the whey-containing medium prepared from the cultured milk by *L. casei* (LC2230) and *B. longum* (BL2912). Results show mean±SE for 5 separate experiments. *ab* Different superscript letters show significant difference between the groups at *p* < 0.05.

Table 2. Effects of cultured products on growth parameters, and bile and serum lipids in rats.

| Parameters                | Control       | *L. casei* (LC2230) | *B. longum* (BL2912) |
|---------------------------|---------------|---------------------|----------------------|
| Body weight (g)           |               |                     |                      |
| Initial                   | 115±3.4       | 115±3.4             | 115±2.6              |
| Final                     | 218±5.4       | 228±5.8             | 211±5.1              |
| Food intake (g/day)       | 28.9±5.0*     | 29.5±5.8*           |                      |
| Bile                      |               |                     |                      |
| Flow (ml/h)               | 0.63±0.05*    | 0.72±0.06*          | 0.48±0.06*           |
| Bile acids (μmol/h)       | 9.72±0.82*    | 12.5±0.60*          | 8.40±0.80*           |
| Bile acids (μmol/ml)      | 15.4±0.71     | 17.8±0.95           | 17.9±1.10            |
| Cholesterol (μmol/l)      | 3.55±0.41     | 3.34±0.49           | 2.63±0.42            |
| Cholesterol (μmol/ml)     | 5.63±0.32     | 4.64±0.51           | 5.48±0.55            |
| Serum                     |               |                     |                      |
| Cholesterol (mmol/liter)  | 1.49±0.09*    | 1.85±0.10*          | 1.46±0.08*           |
| Triacylglycerol (mmol/liter) | 1.82±0.43 | 1.62±0.41           | 1.15±0.14            |

Values are mean±SE for 6 rats per group. *ab* Different superscript letters show significant difference at *p* < 0.05.

*longum*, final body weight and feed efficiency (data not shown) did not differ among the groups (Table 2). Bile flow tended to be lower in rats fed the whey from the *B. longum*. Secretion rate of bile acid increased significantly in rats fed the whey from the *L. casei*, although the concentration did not differ among the groups. Biliary cholesterol secretion did not differ among the groups. Ingestion of the whey from the *L. casei* increased the concentration of serum cholesterol, while no significant
difference was observed for the triacylglycerol concentration.

DISCUSSION

The present study showed that the effects of the whey prepared from cultured skim milk on the secretion of bile acids by primary cultured hepatocytes varied widely depending on the bacterial strains, rather than on the species used for the culture. Among the strains examined, *L. casei* (LC2230) and *B. longum* (BL2912) produced the whey preparations which increased the activity of cholesterol 7α-hydroxylase, a rate-limiting enzyme for bile acid synthesis. Thus, the whey from these strains appears to contain a factor which influences directly the synthetic pathway of bile acids by the liver. The stimulative factor of bile acid synthesis may be cow's milk-derived substance but modified by lactic acid bacteria, or a newly secretory product by the bacteria. Preliminary experiment showed that this factor may not be fat-soluble materials as the lipid fraction prepared from the cultured skim milk from *L. casei* (LC2230) and *B. longum* (BL2912) did not stimulate the secretion of bile acids by primary cultured hepatocytes (unpublished observation). The possibility also remains that this factor could be a substance present originally in the skim milk and unmodified by milk fermentation, since milk prepared from rats and humans have been reported to stimulate bile acid secretion by cultured rat hepatocytes (7) and organ culture of rabbit liver (12), respectively.

In order to examine if the cultured milk products effective for bile acid synthesis in the hepatocyte system are also the case for in vivo system, the whey preparations from *L. casei* (LC2230) and *B. longum* (BL2912) were given to rats. The whey preparation only from the *L. casei*, but not from the *B. longum*, stimulated the secretion of biliary bile acids. Thus, an active component of the whey prepared from the *L. casei* can be absorbed and reach the liver to exert a stimulative influence on bile acid synthesis, whereas that from the *B. longum* may not be absorbed intactly.

Early reports showed that milks fermented by *Streptococcus thermophilus* and *L. acidophilus* exhibit a reduction of the serum cholesterol concentration when given to rats (13,14), although an active component and bile secretion were not investigated. As shown in the present study, however, an elevation of biliary bile acid secretion in rats fed the whey from *L. casei* (LC2230) did not accompany the reduction of the serum cholesterol concentration. The discrepancy between the present and the previous one is not clear, but the species and strain of lactic acid bacteria used for the preparation of cultured milk and also the feeding conditions may differently affect the metabolism of serum cholesterol.

The present study showed that synthesis of cholesterol from [14C]acetate by cultured hepatocytes was suppressed by the whey preparation from *Bifidobacterium* species as compared with *Lactobacillus* species. This result may raise a possibility that the former species, as compared with the latter, produce an inhibitory factor for cholesterol synthesis.
In summary, the present study showed that strains as well as species of lactic acid bacteria influence differently the metabolism of bile acids and possibly cholesterol. This result partly explains why yogurt intake in humans gives conflicting results on the serum lipid and lipoprotein levels, as no literature has ever clarified the strains of lactic acid bacteria used for the preparation of yogurt that was given to humans. The present result also indicates that primary cultured hepatocytes are a useful experimental system as an initial screening for an active principle modulating cholesterol metabolism.

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