A Mixture of Organisms Affects Cholesterol Metabolism Together with Rat Cecal Flora

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The effects of a mixture of organisms on cecal fermentation and cholesterol metabolism in sham-operated and ceccectomized rats were investigated. Male F344 rats, allocated into four groups: ceccectomized rats fed a mixture of organisms (CEMO), ceccectomized rats fed rice bran (CERB), sham-operated rats fed a mixture of organisms (SHMO), and sham-operated rats fed rice bran (SHRB) for 4 weeks. The diets had 0.5% cholesterol and 0.125% sodium cholate added. There were no significant differences in the body weight gain and food intake among the groups. The cecal pH in the SHMO group was significantly lower than that in the other groups. The total cholesterol and (VLDL + IDL + LDL)-cholesterol concentrations in serum were significantly lower in the SHMO group than that in the SHRB group, and the triacylglycerol concentration in the sham-operated rats tended to decrease compared to the ceccectomized rats. The fecal cholesterol excretion in the CERB group was higher than that in the other groups, and that in the SHMO group was significantly higher than in the SHRB group. The acetic acid, propionic acid, n-butyric acid, and total short-chain fatty acid concentrations in the cecum contents were significantly higher in the SHMO group than those in the other groups. Streptococcus, Bifidobacterium, and Lactobacillus in the SHMO group tended to be higher than the other groups and Bacteroidaceae in the CEMO and CERB groups were significantly higher than that in the SHMO group. The results demonstrate that the mixture of organisms was fermented with the cecal contents and that the metabolites such as short-chain fatty acid lowered the serum total cholesterol and liver cholesterol concentrations in the rats fed a cholesterol-containing diet.

Key words: mixture of organisms; short-chain fatty acid; cholesterol

Serum cholesterol has been identified as a major risk factor for coronary heart disease. Though many environmental and genetic variables influence serum cholesterol, a good correlation exists between total dietary fat intakes and mean serum cholesterol levels of different populations. Thus, numerous active substances with hypocholesterolemic functions have been investigated. It has also been reported that numerous probiotics have hypcholesterolemic functions in human subjects and in rats.\(^1\)\(^-\)\(^3\) Fukushima and Nakano\(^9\) reported that a mixture of organisms lowered serum cholesterol concentrations and hydroxy-3-methylglutararyl-Co A reductase (NADPH; \(EC\) 1.1.1.34), and increased fecal cholesterol and bile acid excretions in rats. It was considered that the results obtained for the mixture of bacteria were due to the existence of a symbiotic relationship in the cecum.

The aim of this study was to examine the effects of the mixture of organisms on intraintestinal fermentation and cholesterol metabolism in ceccectomized rats and in sham-operated rats, which have cecum of a major site of short-chain fatty acid (SCFA) production.\(^5\)

Materials and Methods

**Probiotics.** The composition of the mixture of organisms is shown in Table 1. The mixture was prepared as described elsewhere.\(^6\)

**Animals and diets.** Male F344/DuCrj rats (8 weeks old) were purchased from Charles River Japan Inc. (Yokohama, Japan). All animals were individually housed in cages and maintained on a 12-h light-dark cycle (light on from 0700 to 1900 h). Temperature and humidity were controlled at 23 ± 1°C and 60 ± 5%, respect-

<table>
<thead>
<tr>
<th>Table 1. Micro-organisms Contributing to the Mixture of Organisms</th>
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<tr>
<td><strong>Bacillus subtilis</strong></td>
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<td><strong>Bacillus natto</strong></td>
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<td><strong>Bacillus megaterium</strong></td>
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<td><strong>Bacillus thermophilus</strong></td>
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<td><strong>Lactobacillus acidophilus</strong></td>
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<td><strong>Lactococcus plantarum</strong></td>
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<td><strong>Lactobacillus brevis</strong></td>
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<td><strong>Lactobacillus casei</strong></td>
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<td><strong>Streptococcus faecalis</strong></td>
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<td><strong>Streptococcus lactis</strong></td>
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<td><strong>Streptococcus thermophilus</strong></td>
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<td><strong>Clostridium butyricum</strong></td>
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<td><strong>Saccharomyces cerevisiae</strong></td>
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<td><strong>Candida utilis</strong></td>
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\(^1\) Each micro-organism was regulated at 10\(^9\) colony-forming units/g rice bran.

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Abbreviations: HDL, high-density lipoprotein; VLDL, very-low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; GLC, gas-liquid chromatography; SCFA, short-chain fatty acid
tively. The composition of the diet was (wt%): casein 20, palm oil 10, maize starch 15, cellulose powder 5, vitamin mixture (AIN-76)\textsuperscript{7} 1, mineral mixture (AIN-76)\textsuperscript{7} 3.5, choline bitartrate 0.2, dl-methionine 0.3, cholesterol 0.5, sodium cholate 0.125, rice bran or mixture of organisms fermented with rice bran 10, and sucrose to 100. The rats were divided into four experimental groups at random. There were no significant differences in serum total cholesterol concentrations between groups at the start of the experimental period (6.24±0.73–6.71±0.99 mmol/l). Nine of the cecotomized rats (CEMO) and ten of the sham-operated rats (SHMO) were fed for 4 weeks on diets which contained the mixture of organisms fermented with rice bran. Eight of the cecotomized rats (CERB) and ten of the sham-operated rats (SHRB) were fed for 4 weeks on diets containing the rice bran. The rats were allowed free access to experimental diets and water. Body weight and feed consumption were recorded weekly and every day, respectively. All animal procedures described conformed to the principles in the Guide for the Care and Use of Laboratory Animals.\textsuperscript{9}

**Analytical procedures.** At the end of the experimental period, blood samples were collected between 0800 and 0900 h from the jugular veins of the rats. The blood was taken into tubes without anticoagulant and then left at room temperature for 2 h. Serum was prepared by centrifugation at 1500 x g for 20 min. During the 3 days before the end of the experimental period of 4 weeks, feces were collected. The rats were killed by ether inhalation, and the liver, cecum, and colon quickly removed. The liver was washed with cold 0.9% saline, blotted dry on filter paper, and weighed before freezing for storage.

**Chemical analysis.** Total cholesterol and triacylglycerol (TG) concentrations in the serum were measured enzymatically using commercially available reagent kits (assay kits for the TDX system; Abbott Laboratory Co., Irving, TX, USA). Serum HDL-cholesterol was measured in a similar manner after dextran sulfate-Mg\textsuperscript{2+} precipitation of VLDL and LDL cholesterol.\textsuperscript{9} The very low density lipoprotein (VLDL)+intermediate density lipoprotein (IDL)+LDL cholesterol concentration was calculated as follows: VLDL+IDL+LDL cholesterol=total cholesterol−HDL-cholesterol.

Total lipids were extracted from liver and feces by the method of Folch et al.\textsuperscript{10} The neutral sterols in the total lipids obtained by saponification were acetylated\textsuperscript{11} and then analyzed by gas-liquid chromatography (GLC) using a Shimadzu 14A chromatograph (Kyoto, Japan) with a DB17 capillary column (0.25 mm×30 m; J&W Scientific, Folsom, CA) with nitrogen as the carrier gas. The peaks corresponding to the reference cholesterol standard were determined. Cecal and colonic contents were taken out into desalted water in a vial without exposure to air, and suspended. The suspension was deproteinized by perchloric acid (final concentration 50 g/l) cooled in ice, and centrifuged at 10000 rpm for 10 min at 4°C. The supernatant was added to a 4 N NaOH solution to precipitate perchloric acid and to form potassium salts of SCFA. Individual SCFA was measured by GLC with a glass column (2000 mm×3 mm) packed with 80–100 mesh chromosorb W-AW DMCS with He\textsubscript{2}PO\textsubscript{4} (100 ml/l) as a liquid phase after adding H\textsubscript{2}PO\textsubscript{4} by the procedure of Hara et al.\textsuperscript{12}

**Growth of bacteria in the cecum and colon.** After thorough mixing of cecal and colonic contents, a series of 10-fold dilutions (10\textsuperscript{-1} to 10\textsuperscript{-8}) was done using anaerobic diluents. From the appropriate dilution, 0.05 ml samples were spread onto agar plates. E. coli and Streptococcus in the cecal and colonic contents were incubated and then grown for 2 d on deoxycholate agar and streptococcal agar (KF; Becton Dickinson Co. Ltd, Cockeysville, USA) plates at 37°C, respectively. Bifidobacterium, Lactobacillus, Bacteroidaceae, and Clostridium in the cecal and colonic contents were incubated for 5 d on Bifidobacterium-selective agar medium, Lactobacillus-selective agar medium (Becton Dickinson Co. Ltd), Neomycin-Brilliant Green Tetrachololate-blood agar medium, and Neomycin Nagler agar medium at 37°C by the gaspak method.

**Statistical analysis.** Data are presented as means and standard deviations. The significance of differences among treatment groups was evaluated using the general linear model with Duncan’s multiple-range test (Statistical Analysis Systems, 1990) and Student’s t-test. Differences were considered significant at \(p<0.05\).

**Results**

**Feed intake, rat growth and liver weight**

Feed intake, body-weight gain, liver weight, and cecal and colonic pH are shown in Table 2. There were no significant differences in body-weight gain or feed intake among the groups. The liver weight in the CEMO group was significantly higher than that in the SHMO and CERB groups. The pH of cecal contents was significantly lower in the SHMO group than that in the SHRB group and the pH of colonic contents in the cecotomized rats.

**Tissue lipid concentration**

The data for serum total cholesterol, (VLDL+IDL+LDL)-cholesterol, HDL-cholesterol, and TG concentrations are presented in Table 3. The total cholesterol and (VLDL+IDL+LDL)-cholesterol concentrations in the SHMO group were significantly lower than in the SHRB group. The serum HDL-cholesterol concentration in the CEMO group was significantly higher than in the other groups. The serum TG concentration in the cecotomized rats tended to increase compared with the sham-operated rats.

Table 3 illustrates the liver cholesterol concentrations. The liver cholesterol concentration in the CERB group was significantly higher than that in the other groups. Though there were no differences in the liver cholesterol concentrations between the SHMO and SHRB groups, the concentration of the sham-operated rats fed on the mixture of organisms was reduced by 22% compared
with the sham-operated rats fed on rice bran.

**Fecal cholesterol excretion**

Figure 1 shows fecal cholesterol excretions in rats during the 3 days before the end of the experimental period. The excretion of cholesterol was increased significantly in the CERB group. The fecal excretion of cholesterol in the sham-operated rats fed on the mixture of organisms was higher than that in the sham-operated rats fed on rice bran.

**Cecal and colonic short-chain fatty acid concentrations**

The SCFA in the cecal and colonic contents are shown in Table 4. Acetate acid, propionate acid, and n-butyric acid concentrations in the SHMO group were significantly higher than those in the other groups. The total SCFA concentration in the SHMO group was also significantly higher than that in the other groups.

**Cecal and colonic microflora composition**

Table 5 shows the compositions of the cecal and colon-
ich microflora. *Streptococcus*, *Bifidobacterium*, and *Lactobacillus* in the SHMO group tended to be higher than the other groups and Bacteroidaceae in the CEMO and CERB groups were significantly higher than the SHMO group.

**Discussion**

In this study the effects of a mixture of organisms on large bowel fermentation and cholesterol metabolism in cecostomized rats was compared with those in sham-operated rats, which have a cecum that is a major site of SCFA production. The total serum cholesterol and (VLDL + IDL + LDL)-cholesterol concentrations in the sham-operated rats fed on a mixture of organisms were significantly lower than those in the sham-operated rats fed rice bran. The results agreed with those of previous reports.\(^6,6\) Fukushima and Nakano\(^6\) have reported that a mixture of organisms lowered hydroxy-3-methylglutaryl-Co A reductase (NADPH; EC 1.1.1.34) activity, and increased fecal cholesterol and bile acid excretions in rats as a result of the observation that the cell bodies of the mixture of organisms had the capacity to bind bile salt and decreased cholesterol micelle formation in vitro. It was considered that the results obtained for the mixture of bacteria were due to the existence of a symbiotic relationship in the cecum.\(^9\) In this study, there were no significant differences in the serum cholesterol concentration between the cecostomized rats and the sham-operated rats. The fecal cholesterol concentration of the SHMO was significantly higher than that of the SHR group, which was similar to those in previous reports.\(^4,6\)

The serum (VLDL + IDL + LDL)-cholesterol concentration in the SHMO group was lower than that in the SHR group. Danielson et al.\(^10\) reported that *Lactobacillus acidophilus* yogurt reduced serum total and LDL cholesterol concentrations, but it had no effect on serum TG. Our data agree well with this report.

There were no differences in the liver cholesterol concentration among the two sham-operated rat groups. However, the liver cholesterol concentration in the SHMO group was significantly lower than that in the SHR group by Student's t-test. The liver cholesterol concentration of the CEMO was also significantly lower than that of the CERB group. The results for sham-operated rats agreed with the report of Fukushima and Nakano.\(^6\) However, it was reported that numerous probiotics had no effect on liver cholesterol.\(^3,3,17\) which did not agree with our data. Gilliland et al.\(^10\) reported that the serum cholesterol-lowering mechanism depends heavily on binding of cholesterol by *L. acidophilus*. The SCFA production in the SHMO was significantly higher than in other groups. Probiotics consist of one or two bacterial species generally.\(^19-22\) However, we designed a system to ferment and adsorb many microbes on rice bran.\(^6\) Thus, it is possible that the SCFA were increased by symbiotic relationships within the cecal flora. In fact, though there were no significant differences in the *Streptococcus*, *Bifidobacterium*, and *Lactobacillus* levels between the SHMO and SHR groups, the microflora in the SHMO group tended to be higher than the other groups. However the vicissitudes of the mixture of organisms and the movement of extremely-oxygen-sensitive anaerobes that produce the SCFA in rats will be investigated further.

In conclusion, the effect of the mixture of organisms was most clearly seen when the sham-operated rats were compared with the SHR group. Its effects depended on the increase of the SCFA production by symbiotic relationships with the cecal flora, and the decrease in the serum and liver cholesterol concentrations, though there were no significant differences in the *Streptococcus*, *Bifidobacterium*, and *Lactobacillus* levels between the SHMO and SHR groups.

**References**


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