Effects of a mixture of organisms, *Lactobacillus acidophilus* or *Streptococcus faecalis* on cholesterol metabolism in rats fed on a fat- and cholesterol-enriched diet

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The effect of a mixture of organisms (a probiotic mixture) comprising *Bacillus*, *Lactobacillus*, *Streptococcus*, *Clostridium*, *Saccharomyces* and *Candida* (10^{7-8} colony-forming units/g rice bran of each component) on lipid metabolism was compared with that of *L. acidophilus* and that of *S. faecalis*. There were four treatment groups: rice bran (control), the mixture of organisms, *L. acidophilus* or *S. faecalis* (30 g/kg) were given to rats in a fat- and cholesterol-enriched diet for 4 weeks. The serum total cholesterol concentration of the group fed on the mixture of organisms was reduced by 15–33% compared with the other groups at the end of the 4-week feeding period ($P < 0.05$). This group also had a lower hepatic cholesterol concentration (36–44%) than the two single-bacteria groups ($P < 0.05$). 3-Hydroxy-3-methylglutaryl-Co A reductase (NADPH; *EC* 1.1.1.34) activities of the mixed-organism and *L. acidophilus* groups were significantly lower (61–63%) than those of the other groups ($P < 0.05$); the activity of the *S. faecalis* group was also significantly lower (42%) than that of the control group ($P < 0.05$). The faecal cholesterol and bile acid concentrations of the mixed-organism group increased compared with those of the *L. acidophilus* and *S. faecalis* groups ($P < 0.05$). The capacity of the mixed-organism cells to bind bile salt *in vitro* was significantly higher (approximately 50%) than that of the single-bacteria cells ($P < 0.05$). On the other hand, cholesterol micelle formation for the mixed-organism cells was significantly (approximately 9%) lower than that of the single-bacteria cells ($P < 0.05$). These results indicate that the mixture of organisms decreased the synthesis of cholesterol in the liver and increased the loss of steroids from the intestine, in rats. Thus, the mixture of organisms had a hypocholesterolaemic role.

Probiotics: *Lactobacillus acidophilus*: *Streptococcus faecalis*: Cholesterol metabolism: HMG-CoA reductase

It has been reported that numerous fermented milks and yoghurts have hypocholesterolaemic functions in human subjects and in rats (Hepner *et al.* 1979; Grunewald, 1982; Jaspers *et al.* 1984). The cholesterol-lowering mechanism depends heavily on binding of cholesterol by *Lactobacillus acidophilus* (Gilliland *et al.* 1985). Our laboratory also reported previously that a probiotic mixture comprising *Bacillus*, *Lactobacillus*, *Streptococcus*, *Saccharomyces* and *Candida* improved the intestinal flora balance and lowered 3-hydroxy-3-methylglutaryl-Co A (HMG-CoA) reductase (NADPH; *EC* 1.1.1.34) activity in rats (Fukushima & Nakano, 1995). It was considered that the results obtained for the mixture of bacteria were due to the existence of a symbiotic relationship in the intestine.

The aim of the present study was to compare the effects of a mixture of organisms (a probiotic mixture) containing *Bacillus thermophilus* and *Clostridium butyricum* (Tsuda *et al.* 1982), which are constituents of fermenting mixtures isolated from brown forest soil rich

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in litter and humus, on cholesterol synthesis in the liver and the re-absorption of cholesterol and bile acids *in vitro*, with those of *Lactobacillus acidophilus* or *Streptococcus faecalis*, which have hypocholesterolaemic functions (Gilliland *et al.* 1985; Ishihara *et al.* 1989; Suzuki *et al.* 1991) when fed in a fat- and cholesterol-enriched diet.

MATERIALS AND METHODS

Probiotics

The composition of the mixture of organisms is shown in Table 1. The mixture containing *Bacillus thermophilus* and *Clostridium butyricum*, which are constituents of fermenting mixtures, was prepared as described elsewhere (Fukushima & Nakano, 1995). *L. acidophilus* and *S. faecalis* were also prepared in the same manner.

Animals and diets

Male F344 rats were purchased from Japan CLEA Co. Ltd (Tokyo, Japan). All animals were housed individually in cages and maintained on a 12 h light–dark cycle. Temperature and humidity were controlled at $23 \pm 1°$ and $60 \pm 5\%$, respectively. The composition of the diet was (g/kg): casein 200, palm oil 200, vitamin mixture (AIN-76; American Institute of Nutrition, 1977) 10, mineral mixture (AIN-76; American Institute of Nutrition, 1977) 35, choline bitartrate 2, DL-methionine 3, cholesterol 10, and sucrose to 1000. All animals were fed on this diet for 4 weeks as a preliminary treatment, to create hypercholesterolaemic rats. The rats (8 weeks old) were then divided into four groups of six animals by randomization. There were no significant differences in body weights and serum total cholesterol concentrations between groups at the start of the experimental period (average initial body weight (g) 142 (SD 6), 141 (SD 14), 144 (SD 15), 142 (SD 13); average initial serum cholesterol concentration (mmol/l) 6·9 (SD 1·3), 6·1 (SD 1·2), 7·2 (SD 1·6), 7·2 (SD 0·9) respectively for the four experimental groups). The experimental groups were fed for 4 weeks on one of the following diets which contained 30 g/kg of: the mixture of organisms fermented with rice bran, *L. acidophilus* fermented with rice bran, or *S. faecalis* fermented with rice bran. The control group was fed for 4 weeks on the diet containing 30 g rice bran/kg. 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 *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1985).

Analytical procedures

Blood samples were collected weekly between 08.00 and 09.00 hours from the jugular veins of fed rats. The samples were taken into tubes without anticoagulant and, after standing at room temperature for 2 h, serum was prepared by centrifugation at 1500 *g* for 20 min. At the end of the experimental period of 4 weeks, all faeces excreted during 1 d were collected. The rats were killed by diethyl ether inhalation between 10.00 and 12.00 hours, based on the diurnal variation of HMG-CoA reductase activity (Shefer *et al.* 1972), and the livers were removed quickly, washed with cold saline (9 g NaCl/l), blotted dry on filter paper and weighed before being frozen for storage at $-80°$.

Chemical analysis

Total cholesterol and HDL-cholesterol concentrations in the serum were determined enzymically using commercially-available reagent kits (assay kits for the TDX system; Abbott Lab. Co., Irving, TX, USA).

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Table 1. *Micro-organisms contributing to the mixture of organisms**

Bacillus subtilis
Bacillus natto
Bacillus megaterium
Bacillus thermophilus
Lactobacillus acidophilus
Lactobacillus plantarum
Lactobacillus brevis
Lactobacillus casei
Streptococcus faecalis
Streptococcus lactis
Streptococcus thermophilus
Clostridium butyricum
Saccharomyces cerevisiae
Candida utilis

* Each micro-organism was regulated at 10^{7-8} colony-forming units/g rice bran.

Total lipids were extracted from liver and faeces by a mixture of chloroform–methanol (2:1, v/v; Folch *et al.* 1957). Methyl ester derivatives of liver phosphatidylcholine (PC)-fatty acids were prepared using methanolic HCl (50 ml/l) for 2 h at 125° (Nakano & Fischer, 1977) and estimated using a Shimadzu 14A gas–liquid chromatograph (Shimadzu, Kyoto, Japan). Neutral steroids in liver and faeces were acetylated (Matsubara *et al.* 1990) and analysed by GLC. Acidic steroids in faeces were determined by the method of Grundy *et al.* (1965).

Rat-liver enzyme preparation

The liver was homogenized in 2 vol of a cold medium containing 50 mM-KCl, 2 mM-$MgCl_2$, 20 mM-Tris hydrochloride (pH 7·6) and 250 mM-sucrose at 4°. The mixture was centrifuged at 1000 *g* for 10 min, and the supernatant fraction was then centrifuged at 12000 *g* for 15 min. The supernatant fraction from this centrifugation was further fractionated by centrifugation at 105000 *g* for 60 min and the resulting pellet was designated the microsomal (Ms) fraction. The Ms fraction was washed by centrifugation at 12000 *g* for 15 min and at 105000 *g* for 60 min in the suspending medium followed by suspension in 150 mM-KCl (pH 7·6) containing 1 mM-EDTA.

Determination of HMG-CoA reductase (NADPH) (EC 1.1.1.34) activity

The present procedure followed the method of Lippe *et al.* (1985) with some modifications (Yu-Ito *et al.* 1982). A 1·5 mg sample of protein was suspended in 200 μl of a solution containing 250 mM-NaCl, 50 mM-potassium phosphate (pH 7·2), 10 mM-EDTA and 10 mM-dithiothreitol. The sample was pre-incubated for 20 min at 37° and the reaction started by adding 25 μl of a solution containing 300 mM-glucose-6-phosphate, 25 μl 30 mM-NADP, 1 IU glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49) and 50 μl 0·14 mM-[3-^{14}C]HMG-CoA (0·25 MBq/ml, specific activity 59568 disintegrations/min per nmol). After 30 min incubation at 37° the reaction was stopped with 0·1 ml 2 M-HCl and the sample left for 30 min at 37° to allow lactonization of mevalonic acid. It was then cooled in ice and centrifuged for 10 min at 3000 *g*. To the supernatant fraction was added 10 μl 0·5 M-mevalonolactone (carrier) and 100 mg $Na_2S_2O_3$. The final pH of the solution was 6·5. After double extraction with 2 ml benzene the extract was applied to a silica-gel TLC plate and developed in benzene–acetone (1:1, v/v). The silica gel of the mevalonolactone region, detected with I vapour, was scraped off, transferred to a scintillation vial containing Pico-

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aqua cocktail (Packard Instrument Co. Inc., Meriden, CT, USA) and the radioactivity measured with a scintillation spectrometer (Packard Instrument Co. Inc., Downers Grove, IL, USA).

Determination of bacterial cells binding bile salt in vitro

A 30 mg portion of the freeze-dried cells of the mixture of organisms, *L. acidophilus* or *S. faecalis* was suspended in 2 ml of a solution containing 200 mM-potassium phosphate (pH 6·0) and 0·5 mM-sodium taurocholate. The control contained no bacteria. After 18 h incubation at 37° the sample was centrifuged at 12000 *g* for 30 min (Suzuki *et al.* 1991). The cholic acid concentration of the supernatant fraction was determined by GLC.

Determination of cholesterol-solubilizing capacity in vitro

The present procedure followed the method of Saunders & Wells (1969). Freeze-dried cells (30 mg) for the mixture of organisms and the two single bacteria were suspended in 2 ml 3 mM-cholesterol, 30 mM-bile salt and 20 mM-lecithin. The control contained no bacteria. The mixture was cooled in a water bath containing ice, then placed under a stream of N_2, and sonicated at 20 kcycles/s for 5 min. The sonicated mixtures were incubated overnight at 37°. The mixtures were centrifuged at 15000 *g* for 30 min. To estimate the transmittance for these supernatant solutions, their absorbances were measured against a blank of distilled water using a Shimadzu UV 260 spectrophotometer at 450 nm.

Statistical analysis

Data are presented as means and standard deviations. The mean and standard deviation for serum total cholesterol for each time-point was calculated. The serum total cholesterol responses were also expressed as the total area under the curve (AUC) between 0 and 28 d. The significance of differences between treatment groups was determined using the general linear model with Duncan's multiple-range test (Statistical Analysis Systems, 1990); differences were considered significant at $P < 0{\cdot}05$.

RESULTS

Feed intake, rat growth and liver weight

Feed intake, feed efficiency, body-weight gain and liver weight are shown in Table 2. There were no differences in body-weight gain among the groups. The mixture of organisms reduced liver weight significantly compared with the other treatment groups ($P < 0{\cdot}05$). Feed intakes of the control and mixed-organism groups were significantly lower than those of the single-bacteria groups and the feed efficiency of the control and mixed-organism groups tended to be higher than those of the single-bacteria groups.

Tissue lipid concentration

The data for serum total cholesterol concentrations are presented in Table 3. The total cholesterol concentrations at week 4 were significantly lower than those for week 0 for all treatment groups (data not shown). The total cholesterol concentrations in rats fed on the diet containing the mixture of organisms were lower than those of other treatment groups throughout the experimental period. The total cholesterol AUC for the mixed-organism group were also lower than those for the control group. There were no significant differences in the cholesterol AUC concentrations between the three probiotic treatment groups.

Table 4 shows the HDL-cholesterol and VLDL-+IDL-+LDL-cholesterol concentrations in the serum and the cholesterol concentrations in livers of rats at the end of the

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Table 2. *Body-weight gain, liver weight, and feed intake of rats fed on a fat- and cholesterol-enriched diet alone (control) or containing 30 g/kg of a mixture of organisms,* Lactobacillus acidophilus *or* Streptococcus faecalis *for 4 weeks**

(Mean values and standard deviations for six rats per treatment group)

	Body-wt gain (g/4 weeks)		Liver dry wt (g/kg body wt)		Feed intake (g/4 weeks)		Feed efficiency‡	
Treatment group	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	84·6[a]	2·2	23·3[bc]	1·7	367·7[b]	16·1	0·23[a]	0·01
L. acidophilus	79·1[a]	4·4	25·6[ab]	0·7	435·6[a]	62·8	0·19[b]	0·02
S. faecalis	79·9[a]	6·6	27·2[a]	2·6	435·8[a]	41·1	0·18[b]	0·02
Mixture of organisms†	85·0[a]	7·1	20·6[c]	3·6	368·3[b]	50·0	0·24[a]	0·04
Pooled SD (23 df)	7·5		3·4		55·0		0·03	

[a,b,c] Mean values in a vertical column with different superscript letters were significantly different (Duncan's multiple-range test; $P < 0·05$).

* For details of diets and procedures, see p. 858.

† For details, see Table 1.

‡ Body-wt gain/feed intake.

4-week feeding period. The VLDL-+IDL-+LDL-cholesterol concentration of the mixed-organism group was lower than that of the other treatment groups and that of the *L. acidophilus* group was also lower than those of control and *S. faecalis* groups. There were no differences in the HDL-cholesterol concentration in serum between the four treatment groups. The cholesterol concentrations in livers from the mixed-organism, *L. acidophilus* and *S. faecalis* groups were reduced to 23–57% of that found in the control group ($P < 0·05$). The cholesterol concentration of the mixed-organism group was lower than that of the single-bacteria groups.

Fatty acid composition of the liver lipid

The fatty acid composition of liver PC is shown in Table 5. The proportion of arachidonate for the mixed-organism group was significantly higher than that of the control and *S. faecalis* groups. When the degree of Δ6-desaturation was estimated as 20:4/18:2 (Table 6), it was found that the proportions were significantly higher in the liver PC of rats fed on the mixture of organisms compared with the control group ($P < 0·05$), but the differences between rats fed on the mixture of organisms, *L. acidophilus* or *S. faecalis* were not significant.

HMG-CoA reductase (NADPH) activity in the liver

The effects of the various bacteria on HMG-CoA reductase activity are shown in Table 6. The mixture of organisms, *L. acidophilus*, or *S. faecalis* significantly lowered the activity compared with the control group ($P < 0·05$). In particular, the activity of the mixed-organism group tended to be lower than those of the single-bacteria groups.

Faecal steroid concentration

Table 7 shows the effects of the probiotics on faecal neutral steroid and bile acid concentrations in rats at the end of the experimental period. The faecal cholesterol concentration of rats fed on the mixture of organisms increased compared with the other groups ($P < 0·05$). The faecal coprostanol concentration in the mixed-organism group

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Table 3. *Serum total cholesterol concentrations (mmol/l) of rats fed on a fat- and cholesterol-enriched diet alone (control) or containing 30 g/kg of a mixture of organisms,* Lactobacillus acidophilus *or* Streptococcus faecalis *for 4 weeks**

(Mean values and standard deviations for six rats per treatment group)

	Serum total cholesterol										Serum total cholesterol AUC (mmol/l)	
Week of experiment...	0		1		2		3		4			
Treatment group	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	6·88[a]	1·30	5·74[a]	0·87	4·95[a]	1·05	5·30[a]	1·22	4·55[a]	0·51	151·9[a]	29·0
L. acidophilus	6·08[a]	1·21	4·76[bc]	0·66	4·53[ab]	0·25	4·21[b]	0·04	3·58[b]	0·29	128·2[ab]	8·1
S. faecalis	7·23[a]	1·63	5·46[ab]	0·61	4·75[ab]	0·42	4·09[b]	0·16	4·29[a]	0·43	140·3[ab]	13·7
Mixture of organisms†	7·16[a]	0·89	4·53[c]	0·20	4·14[b]	0·40	2·81[c]	0·96	3·04[c]	0·36	117·1[b]	10·7
Pooled SD (23 df)	1·29		0·77		0·65		1·16		0·71		20·8	

[a,b,c] Mean values in a vertical column with different superscript letters were significantly different (Duncan's multiple-range test; $P < 0·05$).
AUC, area under curve.
* For details of diets and procedures, see p. 858.
† For details, see Table 1.

Table 4. *Serum HDL- and VLDL-+IDL-+LDL-cholesterol concentrations and liver cholesterol concentrations of rats fed on a fat- and cholesterol-enriched diet alone (control) or containing 30 g/kg of a mixture of organisms,* Lactobacillus acidophilus *or* Streptococcus faecalis *for 4 weeks**

(Mean values and standard deviations for six rats per treatment group)

Treatment group...	Control		*L. acidophilus*		*S. faecalis*		Mixture of organisms†		Pooled SD (23 df)
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Serum HDL-cholesterol (mmol/l)	1·53[a]	0·30	1·61[a]	0·17	1·44[a]	0·19	1·48[a]	0·17	0·21
Serum VLDL-+IDL-+LDL-cholesterol (mmol/l)	3·02[a]	0·42	1·97[b]	0·28	2·84[a]	0·49	1·35[c]	0·42	0·79
Liver cholesterol (μmol/g dry wt)	163·7[a]	16·2	126·2[b]	28·7	108·9[b]	31·0	70·1[c]	18·9	41·3

[a,b,c] Mean values in a horizontal row with different superscript letters were significantly different (Duncan's multiple-range test; $P < 0·05$).
* For details of diets and procedures, see pp. 858–859.
† For details, see Table 1.

Table 5. *Fatty acid composition (mol/100 mol) of liver phosphatidylcholine from rats fed on a fat- and cholesterol-enriched diet alone (control) or containing 30 g/kg of a mixture of organisms,* Lactobacillus acidophilus *or* Streptococcus faecalis *for 4 weeks**

(Mean values and standard deviations for six rats per treatment group)

Treatment group...	Control		*L. acidophilus*		*S. faecalis*		Mixture of organisms†		Pooled SD
Fatty acid	Mean	SD	Mean	SD	Mean	SD	Mean	SD	(23 df)
16:0	26·8[a]	6·7	25·3[a]	5·2	26·6[a]	5·0	20·7[a]	3·5	5·4
18:0	19·7[a]	4·9	21·6[a]	5·6	14·7[a]	3·9	23·8[a]	0·5	5·2
18:1	20·3[ab]	4·4	18·2[b]	2·9	24·2[a]	4·9	16·6[b]	1·2	4·4
18:2 (*n*-6)	13·3[a]	4·0	11·2[a]	1·1	12·2[a]	2·6	11·6[a]	0·8	2·4
20:4 (*n*-6)	19·2[b]	5·1	22·9[ab]	4·5	19·6[b]	6·3	27·2[a]	4·0	5·6
20:4/18:2	1·47[b]	0·34	2·05[ab]	0·37	1·71[ab]	0·82	2·35[a]	0·32	0·58

[a,b] Mean values in a horizontal row with different superscript letters were significantly different (Duncan's multiple-range test; $P < 0·05$).

* For details of diets and procedures, see pp. 858–859.

† For details, see Table 1.

Table 6. *3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (NADPH;* EC *1.1.1.34) activity (pmol/h per mg protein) in liver microsomes of rats fed on a fat- and cholesterol-enriched diet alone (control) or containing 30 g/kg of a mixture of organisms,* Lactobacillus acidophilus *or* Streptococcus faecalis *for 4 weeks**

(Mean values and standard deviations for six rats per treatment group)

Treatment group...	Control		*L. acidophilus*		*S. faecalis*		Mixture of organisms†		Pooled SD
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	(23 df)
HMG-CoA reductase	306·3[a]	52·4	119·8[c]	35·9	176·7[b]	19·9	114·1[c]	22·8	85·5

[a,b,c] Mean values in a horizontal row with different superscript letters were significantly different (Duncan's multiple-range test; $P < 0·05$).

* For details of diets and procedures, see pp. 858–860.

† For details, see Table 1.

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Table 7. *Faecal steroid concentrations (μmol/kg body wt per d) in rats fed on a fat- and cholesterol-enriched diet alone (control) or containing 30 g/kg of a mixture of organisms,* Lactobacillus acidophilus *or* Streptococcus faecalis *for 4 weeks**

(Mean values and standard deviations for six rats per treatment group)

Treatment group...	Control		*L. acidophilus*		*S. faecalis*		Mixture of organisms†		Pooled SD (23 df)
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Coprostanol	18·4[a]	7·1	21·8[a]	13·5	25·0[a]	19·4	32·0[a]	10·9	14
Cholesterol	371[b]	128	494[b]	114	364[b]	145	669[a]	121	173
CA	0·5[ab]	0·3	0·6[ab]	0·2	0·4[b]	0·2	0·8[a]	0·3	0·3
CDCA	0·3[b]	0·2	0·4[ab]	0·2	0·5[ab]	0·2	0·6[a]	0·3	0·2
DCA	4·7[ab]	5·1	2·6[ab]	1·8	1·2[b]	0·9	5·8[a]	2·6	3·3
LCA	2·1[b]	1·4	2·9[b]	0·9	2·0[b]	1·3	5·9[a]	4·0	2·7

[a,b] Mean values in a horizontal row with different superscript letters were significantly different (Duncan's multiple-range test; $P < 0·05$).
CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid.
* For details of diets and procedures, see pp. 858–859.
† For details, see Table 1.

Table 8. *Effects of the mixture of organisms,* Lactobacillus acidophilus *and* Streptococcus faecalis *on bile salt binding and cholesterol micelle formation* in vitro

(Mean values and standard deviations for six samples)

Bacterial cell type...	Control (no bacteria)		*L. acidophilus*		*S. faecalis*		Mixture of organisms*		Pooled SD (23 df)
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Cholic acid (μmol)†	0·78[a]	0·12	0·54[b]	0·08	0·51[b]	0·06	0·25[c]	0·06	0·21
Cholesterol micelle transmittance‡ (%)	83·2[a]	5·7	73·7[b]	4·8	74·1[b]	4·6	67·8[c]	1·7	7·0

[a,b,c] Mean values in a horizontal row with different superscript letters were significantly different (Duncan's multiple-range test; $P < 0·05$).
* For details, see Table 1.
† Freeze-dried cells (30 mg) were used and cholic acid concentration was measured by GLC; for details, see p. 860.
‡ Freeze-dried cells (30 mg) were used and absorbance was measured at 450 nm to give transmittance (%).

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tended to increase compared with the other groups. However, there were no significant differences in the concentration among the treatment groups. Of the faecal bile acid concentrations, those of deoxycholic acid and lithocholic acid of rats fed on the mixture of organisms were increased significantly more than those of rats fed on *S. faecalis* and more than those of rats fed on *L. acidophilus* and *S. faecalis* respectively ($P < 0.05$).

Capacity for binding to bile salt and cholesterol solubilizing

Table 8 shows the effects of the bacteria on the binding to sodium taurocholate and cholesterol solubilizing *in vitro*. The mixture of organisms bound to the bile salt significantly more than the single bacteria ($P < 0.05$) and the inhibition of micelle formation with the mixture of organisms was significantly higher than that for the other treatment groups ($P < 0.05$).

DISCUSSION

In the present study the effect of a mixture of organisms on the cholesterol mechanism in rats was compared with those of single bacteria species, *L. acidophilus* and *S. faecalis*. There were no significant differences in the serum HDL-cholesterol concentrations among the three bacteria-containing treatment groups. However, the serum total cholesterol concentration in the mixed-organism group decreased significantly compared with the *L. acidophilus* and *S. faecalis* groups. The concentration of VLDL-+IDL-+LDL-cholesterol was also reduced significantly in rats fed on the mixture of organisms and *L. acidophilus* as compared with *S. faecalis*. There are only two possible ways to reduce VLDL-+IDL-+LDL-cholesterol: (1) the lowering of apolipoprotein B-100 synthesis (Cardin *et al.* 1984), (2) the increase in lecithin:cholesterol acyltransferase (*EC* 2.3.1.43) activity (Glomset, 1970). Experiments to investigate these possibilities are in progress.

Although no significant difference was found in the body-weight gain among the four groups, the liver weight of the mixed-organism group was reduced more than those of the single-bacteria groups. The reason for this is unknown. However, as shown in Table 1, this is a microbial mixture; it may be that the mixture functions via a symbiotic relationship in the intestine in contrast to that of single bacteria such as *L. acidophilus* and *S. faecalis*, which have individual biological activities (Ozawa & Yokota, 1981; Gilliland *et al.* 1985; Furushiro *et al.* 1990).

The liver cholesterol concentration of rats fed on the fat- and cholesterol-enriched diet was also reduced significantly in the mixed-organism group (Table 4). It may be possible that cholesterol synthesis in the liver was lower. In fact, the mixture of organisms and *L. acidophilus* lowered the HMG-CoA reductase activity significantly more than *S. faecalis*. It has been reported that hydroxymethylglutaric acid, orotic acid and uric acid in milk (Richardson, 1978) and casein hydrolyte in fermented milk (Papa *et al.* 1982) inhibit cholesterol synthesis. But, Gilliland *et al.* (1985) reported that *L. acidophilus* bound to cholesterol directly and Suzuki *et al.* (1991) reported that *L. acidophilus* did not inhibit cholesterol synthesis. However, our data from *L. acidophilus* suggested a lowering effect on cholesterol synthesis. The actual situation remains the subject of future discussion. The findings from the mixed-organism group confirm our previous data (Fukushima & Nakano, 1995). Although it is considered that the mixture of organisms may regulate the feedback control of cholesterol synthesis in the liver, the exact mechanism (for example, secretions from the mixture of organisms, such as compactin (Kaneko *et al.* 1978), or fermentation products in the caecum of the rats fed on the mixture of organisms, such as short-chain fatty acids (Anderson *et al.* 1990)) is still to be elucidated.

Consistent with the change in Δ6-desaturase activity, the linoleate desaturation index

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(20:3+20:4)/18:2, in rat liver Ms PC was decreased when cholesterol was fed at a level higher than 5 g/kg (Lee *et al.* 1991). There were no significant differences in linoleate desaturation among the bacteria-fed groups in the present experiment. However, the ratio for the mixed-organism group compared with that for the control group agreed with our previous findings (Fukushima & Nakano, 1995). The changes in the fatty acid composition may in turn cause a change in membrane fluidity. It is suggested that the mixture of organisms promotes membrane fluidity in the liver more than the control treatment.

Loss of cholesterol and lithocholic acid was promoted by mixture of organisms. These findings were supported by 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* (Table 8). It has been reported that intestinal micro-organism cells bind steroids (Hartman & Holmlund, 1962; Midtvedt & Norman, 1972) and that intestinal lactic acid bacteria absorb cholesterol (Bottazzi *et al.* 1986). We also reported that a mixture of organisms increased the number of intestinal lactic acid bacteria (Fukushima & Nakano, 1995). From these findings it may be suggested that cells of this mixture of organisms and/or increased intestinal lactic acid bacteria bind cholesterol and bile acid and have an inhibitory effect on cholesterol micelle formation in the intestine. In the present experiment *Clostridium butyricum*, which can influence the metabolism of amino acids and proteins (Tsuda *et al.* 1982), and *Bacillus thermophilus* were present in the mixture of organisms as constituents of fermenting mixtures. Our aim was to activate the symbiotic flora (cf. our previous probiotic; Fukushima & Nakano, 1995). The effect was maintained for 4 weeks in the present feeding experiment which used 30 g mixture of organisms/kg diet compared with our previous experiment (Fukushima & Nakano, 1995) when 150 g mixture of organisms/kg diet was used.

In conclusion, *L. acidophilus* and *S. faecalis* when taken as probiotics in rats fed on high-cholesterol diets tended to be hypocholesterolaemic and a mixture of organisms was most effective. Mechanisms included decrease in HMG-CoA reductase activity, binding of steroids to the organisms *in vitro*, and so possibly *in vivo*, and decreased cholesterol micelle formation *in vitro*, and so possibly *in vivo*.

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