Effects of Lactic Acid-Fermented Soymilk on Lipid Metabolism in Rat Liver

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Contents

Abbrivation

ACAT : acetyl-CoA acetyltransferase

ACC : acetyl-CoA carboxylase

C : control

CPT-1 : carnitine palmitoyltransferase 1

CVD : cardiovascular disease

CYP7a1 : cytochrome p450 family 7 subfamily a polypeptide 1

F : fermented soymilk

F-10 : 10% soy protein containing-fermented soymilk

F4404 : fermented soymilk by lactic acid fermentation using *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4404L

F4408 : fermented soymilk by lactic acid fermentation using *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4408L

F-5 : 5% soy protein containing-fermented soymilk

FAS : fatty acid synthase

FSE : fermented soymilk extract

FXR : Farnesoid X receptor

GAPDH : glyceraldehyde-3-phosphate dehydrogenase

HC : high cholesterol

HDL-C : HDL-cholesterol

HMF : undigested insoluble fraction (high molecular fraction)

HMGCR : 3-hydroxy-3-methylglutaryl-CoA reductase

LDL-C : low density lipoprotein cholesterol

LDLR : low-density lipoprotein receptor

LL : lipid loading test

LXRα : liver X receptor alpha

PPARα : peroxisome proliferator activated receptor alpha

PXR : pregnane X receptor

S : soymilk

S+FSE : soymilk and fermented soymilk extract

S+SE : soymilk and soymilk extract

SE : soymilk extract

SHP : small heterodimer partner

SPI : soy protein isolate

SREBP-1 : sterol regulatory element binding protein 1

SREBP-2 : sterol regulatory element binding protein 2

TC : total cholesterol

TG : triglyceride

Introduction

Cardiovascular disease (CVD) is a severe problem in developed and developing countries. WHO warns that 17.3 million people died from CVD in 2008, and 23.6 million people a year will die from CVD by 2030. CVD is a disease mainly caused by atherosclerosis [1]. One of the risk factors of atherosclerosis is hypercholesterolemia [2)3)] and low density lipoprotein cholesterol (LDL-C) is the major cause of onset of the atherogenic process [1]. Some functional ingredients in daily food are known to be useful for prevention of hypercholesterolemia or atherosclerosis. Soy foods, such as soymilk, tofu, soy sause, miso, natto, and temphe, are traditional food products in Asia [4]. Soy is often referred to as "meat from the field" containing good quality of protein, and the intake of soy foods has been known to exhibit lipid metabolism-modulating effects through many animal and clinical studies.

The major bioactive ingredients of soy foods are soy proteins and isoflavones. Much evidence on soy protein has been reported from studies on experimental animals and human subjects. In 1940, plasma high cholesterol level reduced by soy protein isolated compared with casein was reported [5]. Then, Carroll *et al* reported that soy protein in the diet reduced the concentration of total cholesterol (TC) and prevented atherosclerosis in rabbits [6]. The availability of soy protein was demonstrated in human by Sirtori *et al* [7]. Additionally, Anderson *et al* reported that the composition of soy protein rather than animal protein significantly decreased serum concentration of total cholesterol, LDL cholesterol and triglycerides in meta-analysis of 38 controlled clinical trials [8]. By the results from many studies, FDA (The Food and Drug Administration) was recognized that the consumption of more than 25 g/day intake of soy protein

decreased plasma TC and LDL-C, and it may reduce the risk of heart disease. Additionally, it was also reported that soy protein decreased plasma TC level in Japanese [9]. Soy protein was recognized as the food for specified health use (FOSHU). The effective dosage of soy protein is proposed to be about 3~6 g/day in Japan. It was reported that the decrease of serum total cholesterol concentration associated with high intake of soy products in Japanese men and women [9]. The Japanese might have high response decreasing plasma TC level by ingestion of soy protein [9]. Soy peptides derived from soy protein also reduced the plasma cholesterol level by inhibiting absorption of cholesterol to intestines and promoting excretion through adsorption of bile acid [10)11)]. Additionally, Sugano *et al* found that the undigested insoluble fraction (HMF) obtained by treating soy protein with microbial protease and pepsin had a hypocholesterolemic effect in rats fed a cholesterol-enriched diet [12)]. HMF had high activity of bile acid binding and promoted excretion of feces [13)]

Soy protein also reduced the triglyceride (TG) concentrations in the plasma and liver in experimental animals and human. Iritani *et al* reported that soy protein isolate (SPI) increased fatty acid oxidation enzyme activity and decreased synthesis of fatty acid activity in rats [14)]. Anderson *et al* reported that soy protein reduced plasma TG level [8)]. Additionally, it was reported that component of soy protein improving TG metabolism is β-conglycinin. SPI is constructed with β-conglycinin and glycinin, but only β-conglycinin decreased plasma TG level and body fat [15)]. A β-conglycinin-rich diet, but not casein-rich or glycinin-rich diet, significantly decreased serum TG, glucose and insulin level accompanied with a decrease in body weights in ICR mice and KK-Ay mice [16)]. In a human randomized double-blind, placebo-controlled study, the consumption of β-conglycinin significantly decreased serum TG concentration [17)].

Isoflavones have a weak estrogenic activity and reduced the risk of cardiovascular disease [18]. Genistein and daizein modulated the hepatic glucose and lipid-regulating enzyme activities [19]. The uptake of isoflavone reduced the cholesterol concentration in the aorta of cholesterol-fed rabbits without soy protein [20]. Genistein decreased adipose deposition in a dose dependent manner in mice [21]. Furthermore, a recent analysis assessing 11 randomized controlled trials using human subjects indicated that isoflavones-enriched soy protein enhanced the reduction of serum total and LDL cholesterol compared with the same amount of isoflavone-depleted soy protein [22]. Additionally, the consumption of soy protein including isoflavone resulted in a significant reduction in the total cholesterol compared with a diet containing soy protein without isoflavone in female cynomolgus monkeys [23]. The cholesterol-lowering effect of isoflavone has not been adequately clarified. Thus, many researchers assumed that lipid metabolism-modulation effect of soy foods exerted in coexistence with soy protein and isoflavone. Moreover, it was reported that saponin from Platycodi Radix improved high fat diet induced obesity [24][25]. A methanol-washed soy protein increased plasma cholesterol level compared with intact soy protein [26][27]. Thus, saponin might have a hypocholesteromic effect. Therefore, recently increasing use of soy food in diets has aroused a surge of interest in the potential physiological benefits.

As soymilk has a lot of soy protein and isoflavone, the intake of soymilk is known to reduce plasma and hepatic lipid levels [28][29]. The positive use of soymilk was focused in this thesis, because it is easy to ingest soymilk as a diet. However, the taste of soymilk is not always favorable for everybody. Therefore, I used soymilk fermented with lactic acid bacteria of vegetable origin in order to ingest it more easily. The

feature of fermented soymilk is to convert isoflavone glycosides to isoflavone aglycones [30]. It is necessary that isoflavone glycones are converted to aglycones before absorption into intestine. Thus, it is assumed that isoflavone aglycone enhanced the bioavailability of isoflavone [31]. The fermented soymilk prepared by using *bifidobavterium breve* YIT 4065 has been reported to improve the lipid metabolism. Lactic acid-fermented soymilk containing okara on the plasma and hepatic lipid profiles, and expression of the lipid metabolism-related genes in rats was reported [32]. Additionally, dose of soymilk for improving lipid metabolism was also reported [33]. However, the dose of fermented soymilk has not been solved. Thus, in this thesis, I hope to clarify the isoflavone aglycone ratio in soymilk on lipid metabolism, and the difference between the effects of soymilk and fermented soymilk on lipid metabolism were investigated. Moreover, as fermented soymilk is a suitable food for comparing the individual role of soy protein and isoflavone on lipid metabolism-modulation effect, the function of isoflavone aglycone on gene expression was investigated to clarify the significant role of lactic acid-fermented soymilk on lipid metabolism in liver.

Chapter 1. Dose of Lactic Acid-Fermented Soymilk for Improving Lipid Metabolism in Rat Liver

It is well known that soy protein has hypolipidemic action [5)-23) 34)-39)]. In almost studies, 20% soy protein in the diet has been used to investigate the improvement of lipid metabolism in rats. Although lactic-acid fermented soymilk was found to ameliorate hyperlipidemia, dose of fermented soymilk containing soy protein for hypolipidemic action has not been known. Thus, I investigated the dose of fermented soymilk for improving lipid metabolism in rats in Chapter 1.

Material and Methods

The fermented soymilk was prepared by lactic acid fermentation using the *Lactobacillus delbrueckii* subsp. *delbrueckii* strain of TUA4408L for 15 h and then immediately freeze-dried for the animal experiment. The compositions and energy of fermented soymilk was shown in Table 1-1. The other feed materials were purchased from Clea Japan (Tokyo, Japan) and Wako Pure Chemical Industries (Osaka, Japan).

Table 1-1. Composition of Freeze-dried Fermented Soymilk

Component	Fermented soymilk	
	%	Energy (kcal/100 g)
Water	8.1	-
Protein	40.0	160.0
Fat	32.4	291.6
Carbohydrate	7.4	29.6
Dietary fiber	6.7	13.4
Mineral	5.4	-
Total energy		494.6

Animals Eighteen male Sprague-Dawley rats (7 weeks old) were purchased from Clea Japan (Tokyo, Japan) and individually housed in cages at 23 ± 1°C and a humidity of 55 ± 7% with a 12-h light-dark cycle. All the rats were acclimatized on an AIN-93G diet for 1 week to stabilize the metabolic conditions before the feeding experiments. The rats were assigned to three groups (n=6) which did not exhibit any significant difference in the body weight and serum total cholesterol concentration from each other. The control (C) group was fed with the AIN-93G diet. The 5% soy protein containing-fermented soymilk (F-5) group was fed with a diet where 12.5% of the control diet had been replaced with dried fermented soymilk, and the 10% soy protein containing-fermented soymilk (F-10) group was fed with a diet where 25.0% of the control diet had been replaced with dried fermented soymilk (Table 1-2). The rats in each group were fed the appropriate diet for 5 weeks and provided with *ad libitum* access to the diet and water. The food intake and the body weight were measured on alternate days. All the rats were fasted for 8 h (8:00-16:00) on one day of each week during the feeding period, and blood was withdrawn from the tail vein under anesthesia. The plasma immediately obtained by centrifugation was stored at -80°C until needed. The non-fasted rats were sacrificed after the feeding period without any affliction, and the liver was quickly removed and washed with ice-cold physiological saline. After the weight of tissues had been measured, the liver tissues were kept at -80°C until needed. These animal experiments were performed according to the guidelines from the Animal Use Committee of Mukogawa Women's University.

Table 1-2. Composition of the Experimental Diets (weight %)

Ingredient	Diet group		
	C (AIN-93*)	F-5	F-10
Casein [1]	20.0	14.2	8.4
Cornstarch [1]	39.8	39.8	37.0
Dextrinized cornstarch [1]	13.2	13.1	11.3
Sucrose [1]	10.0	10.0	10.0
Soybean oil [1]	7.0	3.1	0.0
Cellulose [1]	5.0	4.1	3.2
Mineral mix (AIN-93G-MX) [1]	3.5	3.5	3.5
Vitamin mix (AIN-93-VX) [1]	1.0	1.0	1.0
L-Cystine [2]	0.3	0.3	0.3
Choline bitartrate [2]	0.25	0.25	0.25
tert -Butylhydroquinone [2]	0.0014	0.0014	0.0014
Soymilk [3]	0.0	0.0	0.0
Fermented soymilk [3]	0.0	12.5	25.0
Total	100	100	100

C, control; F-5, 12.5% of the control diet was replaced with dried fermented soymilk to 5% soy protein as final concentration; F-10, 25.0% of the control diet was replaced with fermented soymilk to 10% soy protein as final concentration;

[1] Clea Japan, Osaka, Japan

[2] Wako Pure Chemical Industries, Osaka, Japan

[3] Marusan-Ai, Okazaki, Japan

*Reeves PG, Nielsen FH, and Fahey JrGC, *J. Nutr* ., **123**, 1939–1951 (1993)

Measurement of the plasma and liver metabolic parameters Plasma total cholesterol (TC) and triglyceride (TG) were enzymatically measured by using commercial kits (Cholesterol E-test and Triglyceride E-test; Wako Pure Chemical Industries). The hepatic lipids were extracted by the method of Folch *et al*., [40], and the hepatic cholesterol and TG concentrations were enzymatically determined as already described.

Statistical analysis The results were presented as the mean ± standard error,

and were analyzed by Tukey's multiple-comparison test at $p<0.05$. Statistical analyses were performed with SPSS 12.0 J for Windows.

Results and Discussion

Body weight, food intake, food efficiency and tissue weight No significant differences in the final body weight, food intake, food efficiency and liver weight were found among the three groups (Table 1-3).

Table 1-3. Initial and Final Body Weights, Food Intake, Food Efficiency, and Liver Weights of Rats Fed on the Experimental Diets for 5 Weeks.

	C	F-5	F-10
Initial body weight (g)	254.3 ± 3.6 [a]	253.5 ± 3.9 [a]	254.4 ± 4.3 [a]
Final body weight (g)	487.8 ± 15.4 [a]	475.0 ± 9.6 [a]	475.3 ± 19.2 [a]
Food intake (g/d)	23.8 ± 0.8 [a]	24.0 ± 0.7 [a]	24.5 ± 1.1 [a]
Food efficiency (g b.w. gain/g diet)	0.29 ± 0.01 [a]	0.28 ± 0.02 [a]	0.27 ± 0.01 [a]
Tissue weight (% b.w.)			
Liver	4.1 ± 0.1 [a]	4.0 ± 0.1 [a]	3.9 ± 0.1 [a]

Each value is the mean ± SE for 6 rats. [a,b] Means not sharing a common superscript differ significantly by Tukey's multiple-comparison test ($p<0.05$).

Plasma and hepatic lipid level The plasma TC and TG levels of the F-5 and F-10 groups were decreased *vs.* C group (Table 1-4). Especially, the F-10 group was tend to decrease the plasma TC level ($p<0.1$). The hepatic TC and TG levels were decreased in the F-5 and F-10 groups. Those in the F-10 group were significantly decreased *vs.* C group. It was suggested that fermented soymilk improved the lipid metabolism in dose dependent manner. Additionally, increasing administration amount of fermented soymilk in diet decreased the administration amount of casein in diet. Thus, decreasing administration amount of casein might affect the reduction of plasma

TC, TG, hepatic TC and TG levels.

Moreover, it was found that the administration of amount of fermented soymilk corresponding to more than 10% soy protein was necessary to keep a significant physiological effect during 1 month.

Table 1-4. Plasma and Liver Parameters of Rats Fed on the Experimental Diets for 5 Weeks

	C	F-5	F-10
Plasma			
Total cholesterol (mmol/L)	2.28 ± 0.14 [a]	1.98 ± 0.20 [a]	1.66 ± 0.16 [a]
Triglyceride (mmol/L)	2.03 ± 0.55 [a]	1.78 ± 0.56 [a]	1.56 ± 0.41 [a]
Liver			
Total cholesterol (mg/g)	3.0 ± 0.1[a]	2.5 ± 0.2[ab]	2.2 ± 0.1[b]
Triglyceride (mg/g)	34.6 ± 4.8[a]	22.5 ± 1.8[ab]	15.7 ± 0.9[b]

Each value is the mean ± SE for 6 rats. [a,b] Means not sharing a common superscript differ significantly by Tukey's multiple-comparison test (p <0.05).

Chapter 2. Effects of Lactic Acid-Fermented Soymilk on Lipid Metabolism-Related Gene Expression in Rat Liver

In the chapter 1, the fermented soymilk exhibited lipid metabolism-modulating effect. More than 10% soy protein-containing fermented soymilk was necessary to keep significant lipid metabolism-modulating effect during 1 month. The mechanism involved in the lactic fermentation of soymilk for the lipid metabolism-related gene expression in rat liver has not yet been elucidated. Furthermore, the effect of the administration period of fermented soymilk on lipid metabolism-related gene expression is not clearly understood.

Therefore, the different effects between soymilk and fermented soymilk on lipid metabolism and lipid metabolism-related gene expression were investigated to clarify the regulation of hepatic gene expression induced by lactic acid-fermented soymilk. Short-term feeding for 1 week and long-term feeding for 5 weeks were also examined to confirm the effect of administration period.

Materials and Methods

Diets The fermented soymilk was prepared as described in previous chapter. Unfermented soymilk was also freeze-dried for the animal experiments. The compositions and energy of soymilk and fermented soymilk are shown in Table 2-1. The other feed materials were purchased from Clea Japan (Tokyo, Japan) and Wako Pure Chemical Industries (Osaka, Japan).

Table 2-1. Composition of Freeze-dried Soymilk and Fermented Soymilk

Component	Soymilk		Fermented soymilk	
	%	Energy (kcal/100 g)	%	Energy (kcal/100 g)
Water	4.6	-	8.1	-
Protein	43.1	172.4	41.6	166.4
Fat	32.7	294.3	32.4	291.6
Carbohydrate	5.3	21.2	5.8	23.2
Dietary fiber	8.7	17.4	6.7	13.4
Mineral	5.6	-	5.4	-
Total energy	-	505.3	-	494.6

Animals Forty eight male Sprague-Dawley rats (7 weeks old) were purchased from Nihon SLC (Hamamatsu, Japan) and feeding procedure was carried out as described in previous chapter.

Experiment 1: feeding for 5 weeks Twenty four rats were assigned to three groups (n=8) which did not exhibit any significant difference in the body weight and serum total cholesterol concentration from each other. The control (C) group was fed with the AIN-93G diet. The soymilk (S) group was fed with a diet where 23.2% of the control diet had been replaced with dried soymilk so that 10% soy protein was contained as the final concentration, and the fermented soymilk (F) group was fed with a diet where 24.1% of the control diet had been replaced with dried fermented soymilk so that 10% soy protein was contained as the final concentration (Table 2-2). The rats in each group were fed the appropriate diet for 5 weeks. The rats in each group were fed the appropriate diet for 5 weeks and provided with *ad libitum* access to the diet and water. The food intake and the body weight were measured as described in previous chapter. Analysis of blood were carried out as described in previous chapter The liver was quickly removed and washed with ice-cold physiological saline. After the weight

of the tissues had been measured, the liver tissues were immediately immersed in RNA Later (Qiagen, Tokyo, Japan) and kept at -80°C until needed.

Experiment 2: feeding for 1 week Twenty four rats were assigned to three groups (n=8) which did not exhibit any significant difference in the body weight and serum total cholesterol concentration from each other. Feeding the diets was the same as described for experiment 1. The liver tissues were treated by same method as that described in experiment 1.

These animal experiments were performed according to the guidelines from the Animal Use Committee of Mukogawa Women's University.

Table 2-2. Composition of the Experimental Diets (weight %)

Ingredient	Diet group		
	C (AIN-93*)	S	F
Casein [1]	20.0	8.4	8.4
Cornstarch [1]	39.8	38.2	37.6
Dextrinized cornstarch [1]	13.2	12.3	11.7
Sucrose [1]	10.0	10.0	10.0
Soybean oil [1]	7.0	0.0	0.0
Cellulose [1]	5.0	2.9	3.3
Mineral mix (AIN-93G-MX) [1]	3.5	3.5	3.5
Vitamin mix (AIN-93-VX) [1]	1.0	1.0	1.0
L-Cystine [2]	0.3	0.3	0.3
Choline bitartrate [2]	0.25	0.25	0.25
tert-Butylhydroquinone [2]	0.0014	0.0014	0.0014
Soymilk [3]	0.0	23.2	0.0
Fermented soymilk [3]	0.0	0.0	24.1
Total	100	100	100

C, control; S, soymilk; F, fermented soymilk

[1] Clea Japan, Osaka, Japan

[2] Wako Pure Chemical Industries, Osaka, Japan

[3] Marusan-Ai, Okazaki, Japan

*Reeves PG, Nielsen FH, and Fahey JrGC, *J. Nutr*., **123**, 1939–1951 (1993)

Measurement of the plasma and liver metabolic parameters Plasma total cholesterol (TC), HDL-cholesterol (HDL-C), and triglyceride (TG) were enzymatically measured by using commercial kits (Cholesterol E-test, HDL-cholesterol E-test, and Triglyceride E-test; Wako Pure Chemical Industries). The non-HDL-C concentration was calculated as [non-HDL-C] = [TC] - [HDL-C]. The measurement of hepatic metabolic parameters was carried out as described in previous chapter.

RNA extraction Total RNA was extracted from the liver tissues with the RNeasy Mini kit (Qiagen, Tokyo, Japan). The quality and quantity of isolated total RNA were checked by agarose gel electrophoresis and spectrophotometry. Equal amounts of isolated total RNA from eight rats in each group were mixed and used in the DNA microarray analysis.

DNA microarray analysis Six RNA samples (10 μg each) were analyzed by the MAMR-01 GeneSQUARE® multiple assay DNA microarray metabolic syndrome gene expression kit for rats (Kurabo Industries, Osaka, Japan). Alexa Fluor®555- labeled cDNA was prepared from hepatic total RNA by cDNA synthesis, and *in vitro* transcription was performed by the MADL-1 GeneSQUARE® cDNA direct labeling system (Kurabo Industries) according to the manufacturer's protocol. Labeled cDNA was purified with a MinElute PCR purification kit (Qiagen, Hilden, Germany) and added to a hybridization buffer (5x SSC at pH 7.0, 4x Denhardt's solution (Sigma- Aldrich, St. Louis, MO, USA), 1 μg of salmon sperm DNA (Invitrogen, Carlsbad, CA, USA) and 0.5% (w/v) SDS. Hybridization was performed in a final volume of 8 μL per well by the GeneSQUARE® multiple assay DNA

microarray metabolic syndrome gene expression kit for rats (Kurabo Industries Ltd.) in a an MAHC hybridization chamber (Kurabo Industries Ltd.) at 65°C for 16 h. The hybridized slides were washed by the following steps after hybridization: (i) immersion in a 1x SSC and 0.1% SDS solution for 5 min, (ii) immersion in a 0.2x SSC and 0.1% SDS solution for 5 min, (iii) immersion in a 0.2x SSC and 0.1% SDS solution at 55°C for 5 min, (iv) rocking in 0.2x SSC, and (v) immersion in 0.05% SSC for 2 min. After being dried by centrifuge, the slides were scanned by GenePix 4000B with GenePix Pro 6.0.1.27 (Molecular Devices, Sunnyvale, CA, USA). The fluorescence intensity of each scanned image was quantified, and then normalized to the intensity of GAPDH.

Real-time reverse transcription-polymerase chain reaction (RT-PCR) The expression of mRNA was quantitatively measured by real-time RT-PCR, using the model 7500 (Applied Biosystems, Foster City, CA, USA) and related reagent kits according to the manufacturer's protocol. Total RNA was extracted from liver tissues with the RNeasy mini kit (Qiagen). The genomic DNA in total RNA was digested by Turbo DNase (Applied Biosystems). Complementary DNA (cDNA) was synthesized from the DNase-treated RNA by using a high capacity cDNA reverse transcription kit with an RNase inhibitor (Applied Biosystems). The following TaqMan® gene expression assays were conducted, using Rn01495769_ml for *Srebf1* (mRNA of SREBP-1), Rn00569117_ml for *Fasn* (mRNA of FAS), Rn00573474_ml for *Acaca* (mRNA of ACC), Rn00581185_ml for *Nr1h3* (mRNA of LXRα), Rn01502638_ml for *Srebf2* (mRNA of SREBP-2), Rn00565598_ml for *Hmgcr* (mRNA of HMGCR) and Rn00564065_ml for *Cyp7a1* (Applied Biosystems) as the PCR primer sets for

real-time PCR. Rn99999916_s1 for GAPDH was used as an endogenous control. Real-time PCR was performed with the TaqMan® Universal PCR master mix (Applied Biosystems). Data were normalized to GAPDH RNA expression, and the number of fold is presented as a ratio to that of the C group.

Isoflavones in the diet and plasma Isoflavone was measured after extracting 0.25 g of the diet with 5 mL of 70% ethanol at room temperature for 24 h and then centrifuged at 10,000×*g* for 30 min at room temperature. The supernatant was collected and filtered through a cellulose acetate membrane (Tosoh, Japan). Isoflavones in the S and F diets were analyzed by HPLC (Tosoh, Japan) equipped with an ODS-80Ts column, using gradient elution by acetonitrile from 15% to 35% in 0.1% acetic acid. Isoflavones in the plasma were analyzed by HPLC, using gradient elution by a solvent of sodium acetate (pH4.8)-methanol (80:20) and sodium acetate (pH4.8)-methanol-acetonitrile(40:20:20) after enzymatic hydrolyzation.

Statistical analysis The results were presented as the mean ± standard error, and were analyzed by Tukey's multiple-comparison test at $p<0.05$. Statistical analyses were performed with SPSS 12.0 J for Windows.

Results

Body weight, food intake, food efficiency and tissue weights No significant differences in the final body weight, food intake and food efficiency were found among the three groups in either experiment 1 (long term administration) or 2 (short term administration) (Table 2-3). The liver weights of the S and F groups after 5 weeks

were significantly lower than that of the C group, but there was no significant difference among the three groups after 1 week (Table 2-3). These results indicated that the long-term administration of soymilk and fermented soymilk obviously affected the lipid metabolism and decreased the liver weight.

Table 2-3. Initial and Final Body Weights, Food Intake, Food Efficiency, and Tissue Weights of Rats Fed on the Experimental Diets for 1 Week and 5 Weeks

	C	S	F
Experiment 1 (feeding for 5 weeks)			
Initial body weight (g)	261.4 ± 3.5 [a]	257.1 ± 3.9 [a]	258.3 ± 1.9 [a]
Final body weight (g)	420.8 ± 11.7 [a]	419.3 ± 12.1 [a]	410.9 ± 5.3 [a]
Food intake (g/d)	20.3 ± 0.3 [a]	20.1 ± 0.3 [a]	20.1 ± 0.2 [a]
Food efficiency (g b.w. gain/g diet)	0.24 ± 0.01 [a]	0.25 ± 0.01 [a]	0.24 ± 0.01 [a]
Tissue weight (% b.w.)			
Liver	3.7 ± 0.1 [a]	3.4 ± 0.0 [b]	3.3 ± 0.0 [b]
Experiment 2 (feeding for 1 week)			
Initial body weight (g)	259.2 ± 3.0 [a]	259.4 ± 3.0 [a]	261.0 ± 2.3 [a]
Final body weight (g)	329.2 ± 5.3 [a]	333.3 ± 4.5 [a]	336.4 ± 5.9 [a]
Food intake (g/d)	20.3 ± 0.5 [a]	19.9 ± 0.3 [a]	20.5 ± 0.3 [a]
Food efficiency (g b.w. gain/g diet)	0.40 ± 0.01 [a]	0.42 ± 0.02 [a]	0.42 ± 0.02 [a]
Tissue weight (% b.w.)			
Liver	4.2 ± 0.1 [a]	4.1 ± 0.1 [a]	4.3 ± 0.1 [a]

Each value is the mean ± SE for 8 rats. [a,b] Means not sharing a common superscript differ significantly by Tukey's multiple-comparison test (p <0.05).

Plasma lipid level The plasma TC levels of the S and F groups were significantly lower than that of the C group from 1 week to 5 weeks (Table 2-4). Although the ingestion of S and F diets did not affect plasma non-HDL-C for the first 3 weeks, non-HDL-C of the S and F groups was temporarily lower than that of the C group in the 4 th week. The ratio of HDL-C/TC in the S and F groups also temporarily increased in the 4th week. On the other hand, the plasma TG level of the S and F

groups tended to decrease.

Table 2-4. Plasma Parameters of Rats Fed on the Experimental Diets for 5 Weeks

	C	S	F
Total cholesterol (mmol/L)			
0W	2.05 ± 0.12 [a]	2.07 ± 0.11 [a]	2.07 ± 0.07 [a]
1W	1.89 ± 0.17 [a]	1.43 ± 0.09 [b]	1.38 ± 0.05 [b]
2W	1.78 ± 0.16 [a]	1.29 ± 0.09 [b]	1.20 ± 0.06 [b]
3W	1.93 ± 0.17 [a]	1.35 ± 0.09 [b]	1.24 ± 0.07 [b]
4W	2.04 ± 0.17 [a]	1.46 ± 0.09 [b]	1.36 ± 0.05 [b]
5W	2.08 ± 0.15 [a]	1.55 ± 0.09 [b]	1.42 ± 0.08 [b]
HDL-cholesterol/Total cholesterol (%)			
0W	60.3 ± 2.5 [a]	60.2 ± 1.4 [a]	64.3 ± 0.9 [a]
1W	57.3 ± 1.7 [a]	49.2 ± 2.3 [a]	52.3 ± 1.1 [a]
2W	61.7 ± 3.9 [a]	55.7 ± 5.2 [a]	55.0 ± 5.5 [a]
3W	56.1 ± 4.5 [a]	49.0 ± 5.1 [a]	51.9 ± 2.9 [a]
4W	54.5 ± 3.1 [a]	66.8 ± 2.7 [b]	64.5 ± 2.1 [b]
5W	61.1 ± 7.4 [a]	66.5 ± 2.9 [a]	66.9 ± 1.9 [a]
non-HDL-cholesterol (mmol/L)			
0W	0.71 ± 0.11 [a]	0.82 ± 0.06 [a]	0.74 ± 0.04 [a]
1W	0.77 ± 0.14 [a]	0.74 ± 0.07 [a]	0.66 ± 0.03 [a]
2W	0.61 ± 0.12 [a]	0.56 ± 0.06 [a]	0.53 ± 0.06 [a]
3W	0.76 ± 0.17 [a]	0.71 ± 0.11 [a]	0.61 ± 0.06 [a]
4W	0.81 ± 0.14 [a]	0.49 ± 0.05 [b]	0.49 ± 0.04 [b]
5W	0.70 ± 0.16 [a]	0.54 ± 0.07 [a]	0.47 ± 0.04 [a]
Triglyceride (mmol/L)			
0W	0.76 ± 0.13 [a]	0.99 ± 0.08 [a]	0.97 ± 0.09 [a]
1W	1.23 ± 0.21 [a]	1.11 ± 0.09 [a]	0.99 ± 1.12 [a]
2W	1.12 ± 0.18 [a]	0.98 ± 0.08 [a]	0.95 ± 0.11 [a]
3W	1.46 ± 0.24 [a]	1.25 ± 0.11 [a]	1.10 ± 0.13 [b]
4W	1.42 ± 0.26 [a]	1.27 ± 0.12 [a]	1.13 ± 0.12 [a]
5W	1.20 ± 0.19 [a]	1.34 ± 0.18 [a]	1.14 ± 0.13 [a]

Each value is the mean ± SE for 8 rats. [a,b] Means not sharing a common superscript differ significantly by Tukey's multiple-comparison test (p <0.05).

Hepatic lipid level Although the hepatic TG and cholesterol contents were

not affected in comparison with the C group after 1 week, the levels were significantly lower in the S and F groups after 5 weeks as shown in Table 2-5. These data suggest that the long-term administration of S and F affected the fatty acid and cholesterol metabolism. No difference in the hepatic lipid profile was found between the S and F groups.

Table 2-5. Liver Parameters of Rats Fed on the Experimental Diets for 1 Week and 5 Weeks

	C	S	F
Experiment 1 (feeding for 5 weeks)			
Cholesterol (mg/g)	2.59 ± 0.08 [a]	2.14 ± 0.05 [b]	2.20 ± 0.05 [b]
Triglyceride (mg/g)	18.3 ± 1.1 [a]	10.0 ± 0.5 [b]	10.3 ± 0.8 [b]
Experiment 2 (feeding for 1 week)			
Cholesterol (mg/g)	2.17 ± 0.10 [a]	1.98 ± 0.03 [a]	1.89 ± 0.09 [a]
Triglyceride (mg/g)	12.6 ± 0.9 [a]	11.8 ± 0.6 [a]	12.1 ± 1.0 [a]

Each value is the mean ± SE for 8 rats. [a,b] Means not sharing a common superscript differ significantly by Tukey's multiple-comparison test ($p < 0.05$).

DNA microarray analysis The ingestion the S or F diets decreased the lipid levels in the plasma and liver, so the change of lipid metabolism-related gene expression in the rat liver was cyclopaedically investigated by using a DNA microarray analysis. The effects on gene expression of the administration period for 1 week and 5 weeks were also compared. The lipid metabolism-related genes whose expression levels in the S and F groups *vs*. the C group differed by more than 1.5-fold or less than 0.7-fold are respectively shown as being up-regulated and down-regulated.

Administering the diet for 1 week (experiment 2) respectively suppressed the gene expression ratio of SREBP-1 in the S and F groups to 0.7 and 0.8 (Table 2-6). The expression ratio of FAS was also obviously down-regulated to 0.5 in both the S

and F groups. The expression ratio of ACC was also suppressed in both the S and F groups to 0.8. These data suggest that the ingestion of the S and F diets down-regulated the fatty acid synthesis-related genes after 1 week, although the fatty acid catabolism-related gene, PPARα, was not affected. However, the CPT-1 genes in the S and F groups were respectively decreased to ratios of 0.7 and 0.8. The expression ratio of CYP7a1, cholesterol catabolism-related gene, in the S group was obviously decreased to 0.7 after administering the diet for 1 week, whereas the ratio of this gene in the F group was increased to 1.6 (Table 2-7). The effect on gene expression was different in experiment 1 with diet administration for 5 weeks (Table 2-6). The suppression ratio of FAS in both the S and F groups was maintained at 0.7, while the SREBP-1 gene expression ratio was scarcely suppressed in the S and F groups (at 0.8 and 0.9, respectively). The expression ratio of ACC was virtually unaffected in the S and F groups (at 1.2 and 1.1, respectively). The, fatty acid catabolism-related genes were also scarcely affected, and the CPT-1 gene expression ratios in S and F groups were respectively suppressed at 0.8 and 0.7. The expression of the cholesterol metabolism-related genes was affected by the ingestion of the S and F diets, the expression ratio for CYP7a1 in the S group was up-regulated to 1.6, and that of F group was up-regulated to 3.1 (Table 2-7), while the ratio for the ACAT2 gene in the S and F groups was also respectively up-regulated to 1.6 and 1.7.

Table 2-6. DNA Microarray Data for Fatty Acid Synthesis and Catabolism Genes in the Liver of Rats Fed on the Experimental Diets for 1 Week and 5 Weeks

Gene name	Abbreviation	Weeks	S	F
Sterol regulatory element binding transcription factor 1	SREBP-1	1W	0.7	0.8
		5W	0.8	0.9
Fatty acid synthase	FAS	1W	0.5	0.5
		5W	0.7	0.7
Acetyl-coenzyme A carboxylase alpha	ACC	1W	0.8	0.8
		5W	1.2	1.1
Peroxiome proliferator activated receptor alpha	PPARα	1W	1.0	1.0
		5W	1.3	0.9
Carnitine palmitoyltransferase 1a, liver	CPT-1	1W	0.7	0.8
		5W	0.8	0.7

Data were normalized to GAPDH and are presented as a ratio to the C group; means of more than 1.5 or less than 0.7 are respectively shown as up-regulated and down-regulated.

Table 2-7. DNA Microarray Data for Cholesterol Metabolism and Catabolism Genes in the Liver of Rats Fed on the Experimental Diets for 1 Week and 5 Weeks

Gene name	Abbreviation	Weeks	S	F
Low density lipoprotein receptor	LDLR	1W	1.1	0.9
		5W	1.0	1.1
Acetyl-coenzyme A acetyltransferase 2	ACAT2	1W	1.0	1.1
		5W	1.6	1.7
Cytochrome P450, family 7, subfamily a, polypeptide 1	CYP7a1	1W	0.7	1.6
		5W	1.6	3.1

Data were normalized to GAPDH and are presented as a ratio to the C group; means more than 1.5 or less than 0.7 are respectively shown as up-regulated and down-regulated.

Real-time PCR analysis The DNA microarray results show that the ingestion of the S and F diets affected the fatty acid synthesis-related genes and cholesterol metabolism-related genes. A real-time RT-PCR analysis was therefore carried out on the important of these genes and other important genes to confirm the

hepatic gene expression by using a DNA microarray analysis. The S and F diets scarcely down- regulated SREBP-1 gene expression after feeding for 1 week in experiment 2 (Fig.2-1). The gene expression of FAS was significantly lower in the S group (p<0.05) and F group (p<0.01) *vs.* the C group after 1 week, whereas the ACC gene expression was scarcely affected. These data indicate that the ingestion of the S and F diets down- regulated the fatty acid synthesis-related gene after 1 week, but that the S and F diets scarcely down-regulated FAS and did not affect the down-regulation of ACC after feeding for 5 weeks in experiment 1 (Fig.2-1).

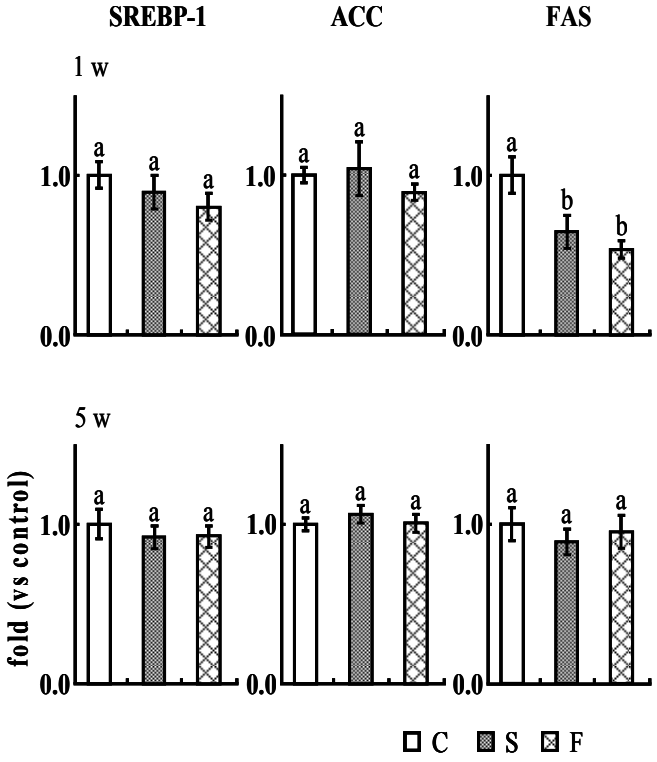

Fig. 2-1. Real-time PCR data for fatty acid synthesis and catabolism-related genes in the liver of rats fed on the experimental diets for 1 week and 5 weeks.The data were normalized to GAPDH RNA expression and are presented as a ratio to the C value. Each value is the mean±SE for 8 rats. [a,b] Means not sharing a common superscript differed significantly by Tukey's multiple-comparison test (p<0.05).

The S and F groups did not significantly change the expression of the cholesterol metabolism-related genes, except for LXRα after 1 week of feeding in experiment 2 (Fig 2-2). However, after 5 weeks of feeding in experiment 1, the gene expression of LXRα was significantly higher in the S group (p<0.05) and F group (p<0.05) than in the C group. In particular, the gene expression of Cyp7a1 in the F group was significantly up-regulated (p<0.001) compared with that in the C group and (p<0.05) compared with that in the S group. The gene expression of SREBP-2 was also significantly higher in the S group (p<0.01) and F group (p<0.001) compared with that in the C group. However, higher HMGCR gene expression was not apparent.

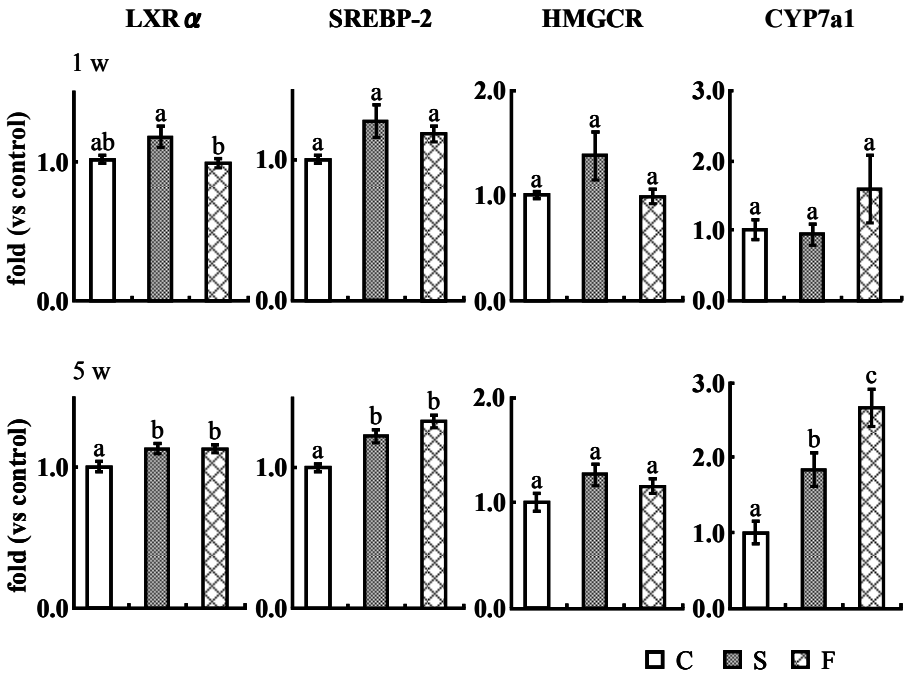

Fig. 2-2. Real-time PCR data for cholesterol metabolism-related genes in the liver of rats fed on the experimental diets for 1 week and 5 weeks. The data were normalized to GAPDH RNA expression and are presented as a ratio to the C value. Each value is the mean±SE for 8 rats. [a,b,c] Means not sharing a common superscript differed significantly by Tukey's multiple-comparison test (p<0.05).

Isoflavones in the diet and plasma The glucoside and aglycone of the major isoflavones in S and F are shown in Table 2-8. The fermented soymilk contained a higher amount of the isoflavone aglycone, daidzein and genistein than soymilk. Plasma isoflavones contained equally in the S and F groups were greater than in the C group. Although the amount of plasma isoflavones in the F group was higher than that in the S group, the difference was not significant (data are not shown).

Table 2-8. Isoflavone Level of Fermented Soymilk (μ mol/100 g of dried sample)

Isoflavone	Soymilk	Fermented soymilk
Daidzin	288.6	11.1
Genistin	311.1	15.7
Daidzein	9.3	217.2
Genistein	9.2	272.1
Total isoflavone	618.2	516.1
Aglycone ratio (%)	3.0	94.8

Discussion

The effects of the S and F diets on lipid metabolism in the plasma and liver of rats were investigated in the present study. After feeding for 5 weeks, the S and F groups had significantly decreased plasma TC and hepatic lipid levels, although the plasma TG level was not affected (Tables 2-4 and 2-5). No significant difference in plasma lipid profile between the S and F groups was apparent.

The liver weights in the S and F groups also decreased after 5 weeks (Table 2-3). Although the S and F groups showed a significantly decreased plasma TC level by feeding for 1 week, the plasma TG and hepatic lipid levels were not affected (Tables 2-4 and 2-5). These results indicate that feeding for 1 week was too short to induce the

effects of soymilk and fermented soymilk on the lipid profile in the plasma and liver.

The gene expression of FAS was obviously decreased in the S and F groups after 1 week, although the expression of FAS was scarcely changed after 5 weeks. It has been reported that the gene expression of SREBP-1 and FAS was controlled by LXR [41]. Although the gene expression of LXRα was up-regulated in the S and F groups, the target genes, SREBP-1 and FAS, were not up-regulated. It seems that a gene other than LXRα controlled the SREBP-1 and FAS gene expression. The hepatic TG level was significantly decreased in the S and F groups after 5 week. However, no rapid decrease of hepatic TG was apparent after 1 week. These data suggest that the change in hepatic TG level by ingesting the S and F diets lagged behind the down-regulation of gene expression, because the effect of FAS down-regulation was attenuated by the longer administration of the S and F diets.

CYP7a1, the rate-limiting enzyme for cholesterol metabolism in the formation of bile acid from cholesterol [42], is controlled by LXRα [43]. The gene expression of LXRα was obviously up-regulated in the S and F groups after 5 weeks. It also seems that soy protein and isoflavone increased the CYP7al activity [44][45]. Although the expression of CYP7a1 in the S and F groups was not significantly affected after 1 week (Fig. 2-2), the respective CYP7a1 ratio in the S and F groups was significantly up-regulated after 5 weeks to 1.8 ($p<0.05$) and 2.5 ($p<0.001$). CYP7a1 gene expression was therefore significantly more up-regulated in the F group ($p<0.05$) than in the S group. It seems that lowering the plasma TC level depended on the acceleration of cholesterol catabolism by up-regulating the hepatic CYP7a1 gene [46] although the change in TC level at the early stage of the S and F diets-feeding appears not to have been related to the up-regulation of CYP7a1. It seems that the up-regulation of CYP7a1 was mediated

by the signal transfer of LXRα in the S and F groups after 5 weeks. In addition, the gene for SREBP-2 controlled by LXRα [47] was up-regulated in the S and F groups after 5 weeks (Fig. 2-2). It has been reported that CYP7a1 gene expression was not mediated by SREBP2 [48], and that soy protein stimulated CYP7a1 expression [49] for the catabolism to bile acid from cholesterol [50]. The up- regulation of SREBP-2 accelerating cholesterol synthesis in the present study seems to have compensated for the loss of hepatic cholesterol induced by up-regulation of the CYP7a1 gene. On the other hand, the gene expression of HMGCR, the rate-limiting enzyme in cholesterol synthesis [51][52], was scarcely increased in the S and F groups after 5 weeks.

It has been reported that isoflavone derived from Kudzu roots had hypo-cholesterolemic activity mediated by increased CYP7a1 gene expression [53]. Furthermore, a soy protein isolate (SPI), after removing isoflavone by ethanol washing, exhibited lower CYP7a1 gene expression than untreated SPI [54]. isoflavone-poor soy protein similarly reduced CYP7a1 gene expression [55]. Isoflavone therefore seems to have been important in the gene expression of CYP7a1. To enhance the bioavailability of isoflavone, it is necessary for the isoflavone glucoside to be converted to the aglycone, the form most easily absorbed in intestines, by intestinal microbial β-glucosidase. It has been reported that the isoflavone glucoside in soymilk was converted to the aglycone by fermentation with lactic acid bacteria [30]. I examined in the present study the isoflavone components in each diet. The fermented soymilk contained a higher level of the isoflavone aglycone, daidzein and genistein, than the soymilk (Table 2-8). The rapid up-regulation of CYP7a1 might therefore have been due to conversion of the isoflavone glucoside to the aglycone whose absorption in intestine was stimulated [31]. On the other hand, it has been reported that the

oligopeptide derived from bovine milk β-lactoglobulin, lactostatin, decreased the plasma TC level mediated by an increased CYP7a1 gene expression [56]. It is likely that soy peptides would exhibit a hypocholesteromic effect if lactic acid fermentation could convert soy protein to bioactive peptides [57][58]. However, the proteins in fermented soymilk were found to be scarcely digested by comparing the HPLC patterns of unfermented and fermented soymilk proteins.

Although the administration of S and F in the present study for 1 week was too short to induce an effect on the lipid profile in the plasma and liver, it appears that the gene expression for fatty acid synthesis was more markedly down- regulated after 1 week than after 5 weeks in the S and F groups. On the contrary, the cholesterol metabolism-related gene expression was more markedly up-regulated after 5 weeks than after 1 week in the S and F groups. It seems that the administration time required for gene expression was different between fatty acid metabolism and cholesterol metabolism. It is assumed that the bioactive components produced by lactic acid fermentation, isoflavones and others, induced the up-regulation of hepatic CYP7al to reduce the plasma cholesterol level in rats.

Chapter 3. Effective Components of Lactic Acid-Fermented Soymilk on Lipid Metabolism-Modulation in Rat Liver

The lipid metabolism-modulating effect was higher in fermented soymilk than soymilk as described in chapter 2. However, the difference of lipid metabolism-modulating effects between soymilk and fermented soymilk has not been clarified. Major effective components of soymilk and fermented soymilk on lipid metabolism-modulation have been known as soy protein and isoflavone by previous studies. Lipid metabolism-modulating effect of soy protein is mostly clarified, but that of isoflavone is less obvious. Thus, in this chapter, lipid metabolism-modulating effect of isoflavone was focused. Isoflavone glycosides must be converted to aglycone in small intestine to be absorbed into body. As lactic fermentation of soymilk can convert isoflavone glycoside to isoflavone aglycone [30)59)], it is assumed that the conversion to aglycone enhanced the bioavailability of isoflavone [31)]. Therefore, in this chapter, isoflavone-containig fractions were prepared by using 70 % ethanol from soymilk or fermented soymilk. Many researchers assumed that lipid metabolism-modulation effect of soy foods might exert in coexistence with soy protein and isoflavone. Thus, in this chapter, the relationships between the ratios of isoflavone aglycone in total isoflavone and with or without soy protein in fermented soymilk were investigated to clarify lipid metabolism-modulation effects in rats fed a cholesterol-free diet.

Material and Methods

Diets Soymilk and fermented soymilk were prepared as described in previous chapter. The isoflavone-containing fraction was extracted from soymilk or

fermented soymilk using 70% ethanol and evaporated to remove ethanol, and immediately freeze-dried for animal experiments. The compositions and energy of soymilk, soymilk extract and fermented soymilk extract were shown in Table 3-1. The aglyconized ratios of isoflavones in soymilk, soymilk extracts and fermented soymilk extracts were 2.2%, 2.7% and 71.7%, respectively. The other feed materials were purchased from Clea Japan (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries (Osaka, Japan).

Table 3-1. Composition of Freeze-Dried Soymilk, Freeze-Dried Soymilk extract, Freeze -Dried Fermented Soymilk extract

Component	Soymilk		Soymilk extract		Fermented soymilk extract	
	%	Energy (kcal/100 g)	%	Energy (kcal/100 g)	%	Energy (kcal/100 g)
Water	2.6	-	4.7	-	4.7	-
Protein	42.5	170.0	7.1	28.4	9.6	38.4
Fat	34.6	311.4	13.0	117.0	18.4	165.6
Carbohydrate	6.9	27.6	60.6	242.4	42.1	168.4
Dietary Fiber	8.4	16.8	0.0	0.0	0.0	0.0
Mineral	5.1	-	14.6	-	25.3	-
Energy	-	525.8	-	387.8	-	372.4

Animals Forty two male Sprague-Dawley rats (7 weeks old) were purchased from Nihon SLC (Hamamatsu, Japan) and feeding procedure was carried out as described in previous chapter.

In chapter 1, fermented soymilk containing 10 % soy protein as final concentration enhanced lipid metabolism-modulation effects in rats fed a cholesterol free diet, and then the isoflavone amount was approximately 30 mg/100 g diet. Thus, in

the present study, the concentration of isoflavone in diet was decided as approximately 30 mg/100 g diet, and 4% soymilk and fermented soymilk extracts were administrated without soy protein, respectively, in experiment 1. In addition, to investigate the effect of these extracts with soy protein, the half of isoflavone is supplied as soymilk or fermented soymilk extract and the half of isoflavone is supplied as soymilk. Therefore, 10% soymilk plus 2% SE or 10% soymilk plus 2% FSE was administrated in rats. As described in chapter 1, less than 5% of soy protein scarcely exhibited lipid metabolism-modulating effect in rats fed AIN-93G diet. Thus, lipid metabolisms-modulating effect by ingestion of 2% SE or 2% FSE with 10% soymilk containing only 4% soy protein in diet was investigated.

Experiment 1: Effects of soymilk and fermented soymilk extracts without soy protein on lipid metabolism Eighteen rats were assigned to three groups (n=6) which did not exhibit any significant difference in the body weight and serum total cholesterol concentration from each other. The control (C) group was fed with the AIN-93G diet. The soymilk extract (SE) group was fed with a diet where 4% of the control diet had been replaced with soymilk extract, and the fermented soymilk extract (FSE) group was fed with a diet where 4% of the control diet was replaced with fermented soymilk extract (Table 3-2). The comparison of ratio of soy protein and isoflavone contained in each diet was shown in Table 3-3. The rats in each group were fed the appropriate diet for 5 weeks and provided with *ad libitum* access to the diet and water. The food intake and body weight were measured as described in previous chapter. Analyses of blood and liver were carried out as described in previous chapter.

Experiment 2: *Effects of soymilk extract and fermented soymilk extract with soy protein on lipid metabolism* Twenty four rats were assigned to four groups (n=6)

which did not exhibit any significant difference in the body weight and serum total cholesterol concentration from each other. The control (C) group was fed with the AIN-93G diet. The soymilk (S) group was fed with a diet where 10% of the control diet has been replaced with dried soymilk and the diet contained 4 % soy protein as final concentration. Soymilk and SE group (S+SE) was fed with the diet where 10% of the control diet has been replaced with dried soymilk and 2% of the control diet has been replaced with SE. Soymilk and FSE (S+FSE) group was fed with the diet where 10% of the control diet has been replaced with dried soymilk and 2% of the control diet has been replaced with FSE (Table 3-2). The rats in each group were fed for 5 weeks. The comparison of ratio of soy protein and isoflavone contained in each diet was shown in Table 3-3. For feeding procedure, same method as described in experiment 1 was used.

These animal experiments were performed according to the guidelines of the Animal Use Committee of Mukogawa Women's University.

Table 3-2. Composition of Experimental Diets (Weight %)

Ingredient	Diet group					
	C (AIN-93*)	S	S +SE	S +FSE	SE	FSE
Casein [1]	20.0	15.1	14.9	14.8	19.6	19.3
Cornstarch [1]	39.8	39.0	37.7	37.7	37.3	37.6
Dextrinized cornstarch [1]	13.2	13.1	12.8	13.0	12.5	12.7
Sucrose [1]	10.0	10.0	10.0	10.0	10.0	10.0
Soybean oil [1]	7.0	3.7	3.5	3.4	6.6	6.4
Cellulose [1]	5.0	4.1	4.1	4.1	5.0	5.0
Mineral mix (AIN-93G-MX)	3.5	3.5	3.5	3.5	3.5	3.5
Vitamin mix (AIN-93-VX) [1]	1.0	1.0	1.0	1.0	1.0	1.0
L-Cystine [2]	0.3	0.3	0.3	0.3	0.3	0.3
Choline bitartrate [2]	0.25	0.25	0.25	0.25	0.25	0.25
tert -Butylhydroquinone [2]	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014
Soymilk [3]	0.0	10.0	10.0	10.0	0.0	0.0
Soymilk extract [3]	0.0	0.0	2.0	0.0	4.0	0.0
Fermented soymilk extract [3]	0.0	0.0	0.0	2.0	0.0	4.0
Total	100	100	100	100	100	100

C, control; S, administration of soymilk; S+SE, administration of soymilk and soymilk extract; S+FSE, administration of soymilk and fermented soymilk extract; SE, administration of soymilk extract, and FSE, fermented soymilk extracts

[1] Japan CLEA, Osaka, Japan.

[2] Wako Pure Chemical Industries, Osaka, Japan.

[3] Marusan-Ai, Okazaki, Japan.

*Reeves PG, Nielsen FH, and Fahey GCJr, *J. Nutr* ., **123**, 1939–1951 (1993).

Table 3-3. Ratio of Soy protein and Isoflavone Contained in Each Diet (per 100 g)

Ingredient	Diet group					
	C (AIN-93*)	S	S +SE	S +FSE	SE	FSE
Soy protein (g)	0.0	4.2	4.3	4.4	0.3	0.4
Isoflavone (mg)	0.0	18.4	34.2	32.8	31.8	28.8
Aglyconized isoflavone (mg)	0.0	0.4	0.8	10.8	0.9	20.6
Aglycone ratio (%)	0.0	2.2	2.5	33.0	2.7	71.5

Measurement of the plasma and hepatic metabolic parameters Measurement of the plasma and hepatic metabolic parameters were carried out as described in previous chapter.

Real-time reverse transcription-polymerase chain reaction (RT-PCR) The expression of mRNA was quantitatively measured by real-time RT-PCR as described in previous chapter. Data were normalized to GAPDH RNA expression and the fold was presented as a ratio to the C group.

Isoflavones in the diet Isoflavones was measured as described in previous chapter.

Statistical analysis The results are presented as the mean ± standard error, and were analyzed by Tukey's multiple-comparison test at $p<0.05$. Statistical analyses were performed with SPSS 12.0 J for Windows.

Results

Body Weight, Food Intake, Food Efficiency, Total Energy Uptake and Tissue Weights in the Experiment 1 No significant difference in final body weight, food intake, food efficiency, total energy uptake and liver weight of rats was found among three groups (Table 3-4).

Table 3-4. Initial and Final Body Weights, Food intake, Food Efficiency, and Liver Weights of Rats Fed on Experimental Diets for 5 Weeks in Experiment 1

	C	SE	FSE
Experiment 1			
Initial body weight (g)	270.5 ± 2.3 [a]	272.1 ± 2.3 [a]	273.5 ± 3.3 [a]
Final body weight (g)	422.4 ± 6.5 [a]	435.7 ± 7.9 [a]	429.4 ± 7.5 [a]
Food intake (g/day)	19.8 ± 0.7 [a]	21.2 ± 0.6 [a]	20.2 ± 0.7 [a]
Food efficiency (g b.w. gain/g diet)	0.24 ± 0.01 [a]	0.24 ± 0.01 [a]	0.25 ± 0.01 [a]
Tissue weight (% b.w.)			
Liver	3.4 ± 0.1 [a]	3.5 ± 0.1 [a]	3.5 ± 0.1 [a]

Each value is the means ± SE of 6 rats. [a,b] Means not sharing common superscript differ significantly by Tukey's multiple comparison (p <0.05).

Hepatic lipid profile in the Experiment 1 Although hepatic TG level was slightly decreased in the FSE group, no significant difference in hepatic lipid profile of rats was found among three groups (Fig. 3-1).

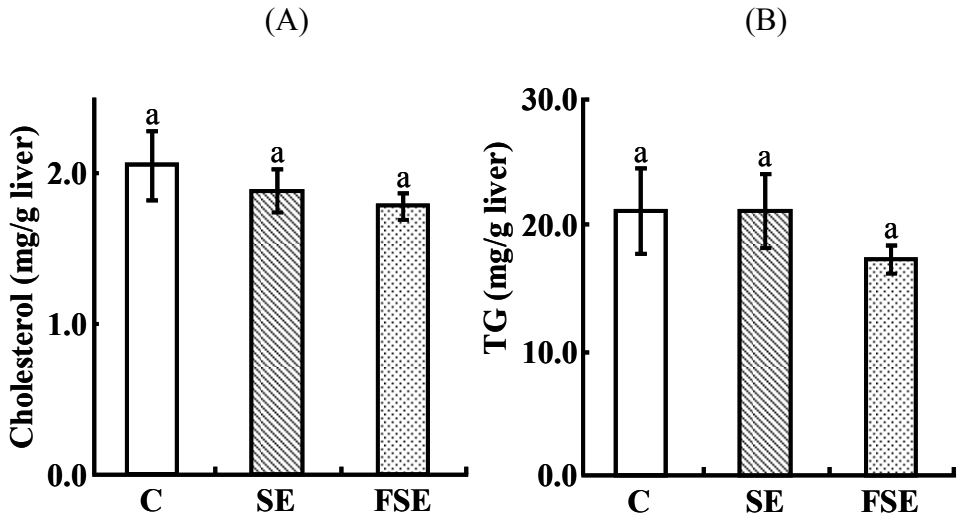

Fig. 3-1. Liver parameters of rats fed on experimental diets for 5 weeks in the experiment 1. (A) hepatic cholesterol; (B) hepatic TG. Each value is the mean ± SE for 6 rats. [a,b] Means not sharing a common superscript differ significantly by Tukey's multiple-comparison test (p<0.05).

Plasma lipid profiles in the Experiment 1 No significant difference in plasma lipid profile of rats was found among three groups (Table 3-5).

Table 3-5. Plasma Parameters of Rats Fed on Experimental Diets for 5 Weeks in Expriment 1

	C	SE	FSE
Total cholesterol (mmol/L)			
0W	1.66 ± 0.07 [a]	1.65 ± 0.16 [a]	1.68 ± 0.19 [a]
1W	1.41 ± 0.07 [a]	1.25 ± 0.13 [a]	1.28 ± 0.19 [a]
2W	1.47 ± 0.06 [a]	1.25 ± 0.14 [a]	1.29 ± 0.17 [a]
3W	1.72 ± 0.07 [a]	1.52 ± 0.17 [a]	1.53 ± 0.17 [a]
4W	1.92 ± 0.08 [a]	1.66 ± 0.17 [a]	1.67 ± 0.22 [a]
5W	2.08 ± 0.07 [a]	1.74 ± 0.16 [a]	1.75 ± 0.23 [a]
Triglyceride (mmol/L)			
0W	0.67 ± 0.05 [a]	0.72 ± 0.07 [a]	0.79 ± 0.13 [a]
1W	0.91 ± 0.10 [a]	0.95 ± 0.13 [a]	1.11 ± 0.21 [a]
2W	0.88 ± 0.13 [a]	0.95 ± 0.16 [a]	1.11 ± 0.15 [a]
3W	1.12 ± 0.15 [a]	1.10 ± 0.23 [a]	1.12 ± 0.23 [a]
4W	0.81 ± 0.05 [a]	1.02 ± 0.25 [a]	0.99 ± 0.15 [a]
5W	1.28 ± 0.27 [a]	1.25 ± 0.26 [a]	1.39 ± 0.22 [a]

Each value is the means ± SE of 6 rats. [a,b] Means not sharing common superscript differ significantly by Tukey's multiple comparison (p <0.05).

Real time PCR analysis in the Experiment 1 The gene expression of LXRα was significantly increased in the SE and FSE groups compared with the C group. No significant difference in fatty acid synthesis-related genes, SREBP-1, ACC and FAS, was found among three groups. The expressions of CYP7a1 and SREBP-2 in the FSE group were significantly up-regulated compared with those in the C group. In the other hand, no significant difference in cholesterol synthesis-related gene, HMGCR, was found among three groups.

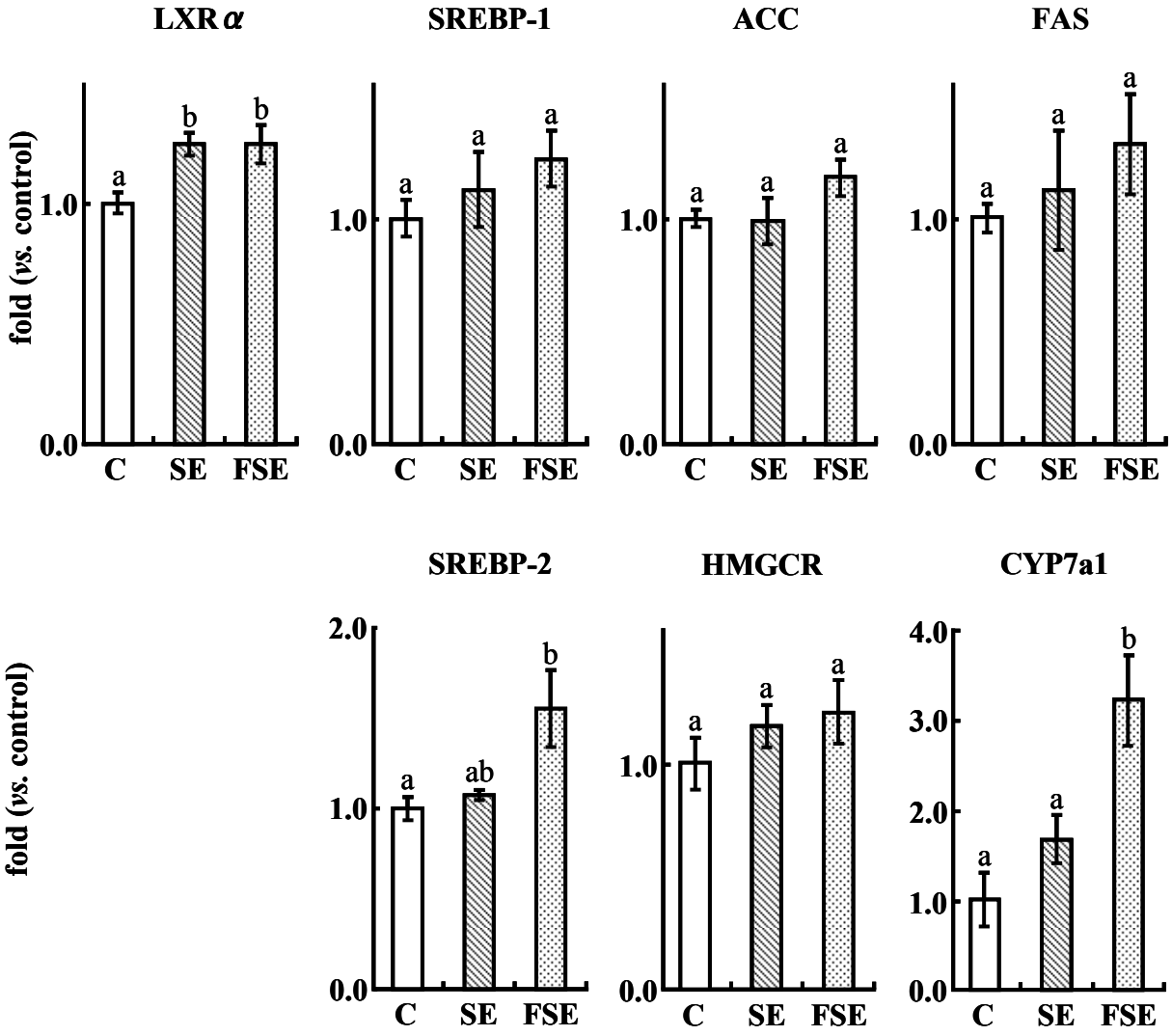

Fig. 3-2. Expression of lipid metabolism-related genes in the liver of rats fed experimental diets for 5 weeks in the experiment 1. The expression of mRNA was quantitatively measured by real-time RT-PCR. Each value is the mean ± SE for 6 rats. The data were normalized to GAPDH RNA expression and are presented as a ratio to the C value. [a,b] Means not sharing a common superscript differ significantly by Tukey's multiple-comparison test ($p<0.05$)

Body Weight, Food Intake, Food Efficiency, Total Energy Intake and Tissue Weights in the Experiment 2 No significant difference in final body weight, food intake, food efficiency, total energy intake and liver weight of rats was found among four groups in the experiment 2.

Table 3-6. Initial and Final Body Weights, Food intake, Food Efficiency, and Liver Weights of Rats Fed Experimental Diets for 5 Weeks in Experiment 2

	C	S	S+SE	S+FSE
Experiment 1				
Initial body weight (g)	261.7 ± 4.0 [a]	261.3 ± 5.7 [a]	260.6 ± 3.9 [a]	259.8 ± 3.9 [a]
Final body weight (g)	426.4 ± 12.9 [a]	424.0 ± 16.5 [a]	410.8 ± 13.3 [a]	392.7 ± 7.9 [a]
Food intake (g/day)	20.5 ± 0.6 [a]	20.9 ± 1.0 [a]	21.0 ± 0.6 [a]	19.4 ± 0.6 [a]
Food efficiency (g b.w. gain/g diet)	0.26 ± 0.01 [a]	0.25 ± 0.01 [a]	0.23 ± 0.01 [a]	0.22 ± 0.01 [a]
Tissue weight (% b.w.)				
Liver	3.7 ± 0.1 [a]	3.6 ± 0.1 [a]	3.6 ± 0.1 [a]	3.5 ± 0.1 [a]

Each value is the means ± SE of 6 rats. [a,b] Means not sharing common superscript differ significantly by Tukey's multiple comparison (p <0.05).

Hepatic Lipid Profile in the Experiment 2 The hepatic cholesterol and TG levels were significantly decreased in the S, S+SE and S+FSE groups compared with the C group. No significant difference in the levels was found among the S, S+SE and S+FSE groups (Fig. 3-3).

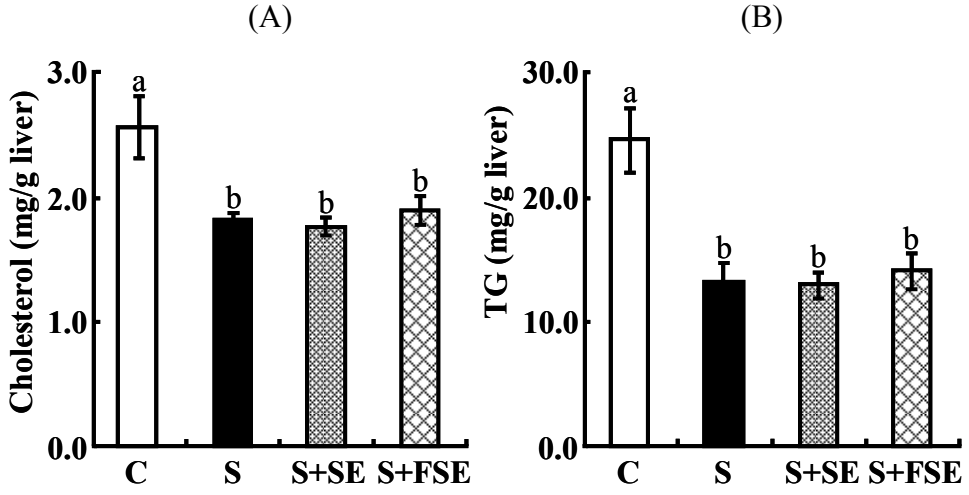

Fig. 3-3. Liver parameters of rats fed on the experimental diets for 5 weeks. (A) hepatic cholesterol; (B) hepatic TG. Each value is the mean ± SE for 6 rats. [a,b] Means not sharing a common superscript differ significantly by Tukey's multiple-comparison test (p<0.05).

Plasma Lipid Profiles in the Experiment 2 Although a significant difference in plasma lipid profile was scarcely found among four groups (Table 3-7), the S+FSE group significantly lowered TG level compared with the C group after 4 weeks.

Table 3-7. Plasma Parameters of Rats Fed on Experimental Diets for 5 Weeks in Expriment 2

	C	S	S+SE	S+FSE
Total cholesterol (mmol/L)				
0W	1.91 ± 0.13 [a]	1.92 ± 0.12 [a]	1.89 ± 0.19 [a]	1.91 ± 0.14 [a]
1W	1.58 ± 0.11 [a]	1.51 ± 0.10 [a]	1.39 ± 0.09 [a]	1.37 ± 0.11 [a]
2W	1.47 ± 0.08 [a]	1.36 ± 0.12 [a]	1.24 ± 0.09 [a]	1.19 ± 0.10 [a]
3W	1.52 ± 0.11 [a]	1.61 ± 0.13 [a]	1.43 ± 0.12 [a]	1.37 ± 0.11 [a]
4W	1.68 ± 0.14 [a]	1.71 ± 0.13 [a]	1.48 ± 0.14 [a]	1.46 ± 0.12 [a]
5W	1.70 ± 0.14 [a]	1.78 ± 0.16 [a]	1.50 ± 0.13 [a]	1.55 ± 0.09 [a]
Triglyceride (mmol/L)				
0W	0.72 ± 0.07 [a]	0.71 ± 0.13 [a]	0.74 ± 0.10 [a]	0.70 ± 0.09 [a]
1W	1.00 ± 0.09 [a]	0.94 ± 0.12 [a]	0.93 ± 0.13 [a]	0.78 ± 0.11 [a]
2W	0.95 ± 0.17 [a]	0.89 ± 0.07 [a]	0.79 ± 0.02 [a]	0.61 ± 0.06 [a]
3W	1.10 ± 0.13 [a]	0.89 ± 0.11 [a]	0.95 ± 0.11 [a]	0.79 ± 0.14 [a]
4W	1.16 ± 0.09 [a]	1.08 ± 0.08 [a]	1.17 ± 0.10 [a]	0.72 ± 0.05 [b]
5W	1.38 ± 0.21 [a]	1.10 ± 0.12 [a]	1.13 ± 0.18 [a]	0.85 ± 0.10 [a]

Each value is the means ± SE of 6 rats. [ab] Means not sharing common superscript differ significantly by Tukey's multiple comparison (p <0.05).

Real Time PCR Analysis in the Experiment 2 The gene expressions of LXRα, SREBP-1, ACC, FAS, SREBP-2 and HMGCR were not affected in the S, S+SE and S+FSE groups (Fig. 3-4). The expressions of CYP7a1 in the S+SE and S+FSE groups were significantly increased compared with those in the C group.

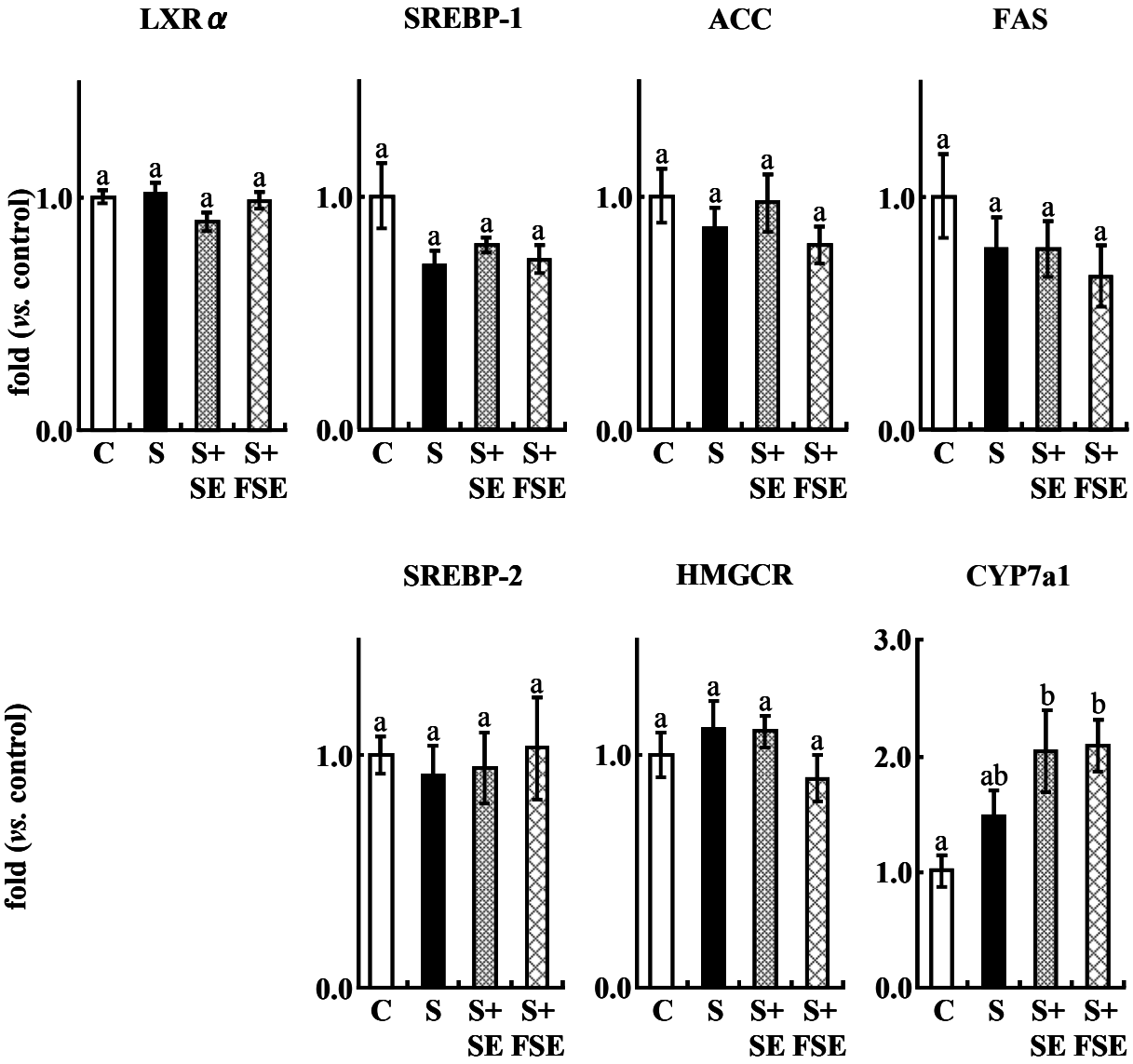

Fig. 3-4. Expression of lipid metabolism-related genes in the liver of rats fed on experimental diets for 5 weeks in the experiment 2. The expression of mRNA was quantitatively measured by real-time RT-PCR. Each value is the mean ± SE for 6 rats. The data were normalized to GAPDH RNA expression and are presented as a ratio to the C value. [a,b] Means not sharing a common superscript differ significantly by Tukey's multiple-comparison test (p<0.05)

Discussion

In this chapter, effects of isoflavone aglycone in lactic fermented soymilk on hepatic lipid-metabolism modulation were investigated. As it was reported that the hypocholesteromic mechanism of dietary soy protein involves interaction cooperated

with isoflavone [60] the relationship between the ratio of isoflavone aglycone in total isoflavone and with or without soy protein were compared.

The hepatic cholesterol and TG levels were not significantly decreased in the SE and FSE groups of experiment 1 (Fig. 3-1), the hepatic cholesterol and TG levels in the S, S+SE and S+FSE groups in the experiment 2 were significantly decreased compared with the C group (Fig. 3-3). It was reported that soy proteins and soy peptides decreased hepatic cholesterol and TG level by inhibiting absorption in intestines [5)-23)34)-39)57)-58)61)]. However, it has never been reported that less than 5 % soy protein of diet improved lipid metabolism as shown in the present study. The administration of 0.1 % isoflavone aglycone of diet to rats fed a 0.3% cholesterol diet reduced the levels of hepatic and plasma lipid, cholesterol and TG [62]. But the administration of 0.01% to 0.03% isoflavone of diet by using isoflavone-rich fermented soybean extract to male rat fed an AIN-76 diet did not affect the hepatic and plasma lipid levels [63]. Although the isoflavone aglycone concentration of diet used in the present study was 0.02%, significant effect of isoflavone aglycone on lipid level-lowering was found in coexistence with soymilk.

Hepatic lipid profile was not significantly changed by ingestion of SE alone or FSE alone, but hepatic gene expression was remarkably exchanged by these ethanol extracts. The expression of LXRα was significantly increased in the SE and FSE groups in the experiment 1 (Fig. 3-2). The liver X receptor α (LXRα) regulates genes involved in cholesterol and fatty acid synthesis [64]. The genes controlled by LXRα, SREBP-1 and SREBP-2 [41], were significantly or slightly up-regulated in the SE and FSE groups (Fig. 3-2). Isoflavone-rich fermented soybean extract slightly increased the gene expression of SREBP-1 and SREBP-2 [63]. Therefore, it seems that isoflavones

promote fatty acid synthesis-related and cholesterol synthesis-related gene expressions by up-regulation of SREBP-1 and SREBP-2 in liver, respectively. The up-regulation of LXR gene by isoflavone coexisted in soy protein was reported [65] whereas the gene expressions of SREBP-1 and SREBP-2 were decreased by soy protein [44)55)]. Thus, in the present study, the gene expression of LXRα did not changed in the S, S+SE and S+FSE groups, and the gene expressions of SREBP-1 and FAS were also not affected in the S, S+SE and S+FSE groups (Fig. 3-4). SE and FSE did not indicate synergistic effect with soy protein on gene expression of SREBP-1 and FAS. As two folds level of isoflavone aglycone and soy protein in a diet were ingested by rats in chapter 2 compared with that of the present study, the expression of LXRα was up-regulated and FAS was not down-regulated by fermented soymilk ingestion in rats fed a cholesterol-free diet. However, in the present study, the expression of FAS was down-regulated by the ingestion of S+FSE because the effect of isoflavone aglycone content was lower than that of soy protein.

In cholesterol metabolism, CYP7a1, the rate limiting enzyme in the formation of bile acid from cholesterol [42] controlled by LXRα [66], was increased as shown in the fermented soymilk group fed a cholesterol-free diet and high cholesterol diet in chapter 2. In the present study, the expression of CYP7a1 was significantly increased in the FSE groups (Fig. 3-2) and both of the S+SE and S+FSE groups also significantly increased the expression of CYP7a1 (Fig. 3-4). The gene expression of LXRα controlling CYP7a1 expression were up-regulated in the experiment 1, but did not affected in the experiment 2. Farnesoid X receptor (FXR) is known to inhibit CYP7a1 [66]. The down-regulation of FXR is known to reduce the gene expression of small heterodimer partner (SHP) [67]. In the present study, the gene expression of SHP was

decreased in the SE and FSE groups in the experiment 1 (data not shown). Thus, the gene expression of CYP7a1 seems to promote by FXR and SHP. It is reported that administration of 0.1% isoflavone glycoside or aglycone up-regulated CYP7a1 expression in rats fed a 0.3% cholesterol diet [62]. Genistein or daizein coexisted with casein up-regulated CYP7a1 expression in rats fed an AIN-93G diet [44]. Isoflavone derived from kudzu roots had hypocholesterolemic activity mediated by increased CYP7a1 gene expression [53]. On the other hand, a soy protein isolated (SPI) after removing isoflavone by ethanol washing lowered CYP7a1 expression than untreated SPI [54)68]. Isoflavone-poor soy protein similarly reduced CYP7a1 gene expression [55]. These results indicated that the expression of CYP7a1 depends on isoflavone, especially aglyconized isoflavone and it was not affected by soy protein. In spite of up-regulation of CYP7a1, the difference of plasma and hepatic TC levels between the SE and FSE groups was not found. The gene expressions of HMGCR, cholesterol synthesis-accelerating factor [69)70], were inclined to be increased in the FSE groups compared with the C group. The gene expression of SREBP-2 was significantly increased in the FSE group (Fig.3-2). The up-regulations of SREBP-2 and HMGCR involving in cholesterol synthesis seem to compensate the cholesterol loss through cholesterol catabolism induced by up-regulation of CYP7a1 in the experiment 1. On the other hand, the gene expressions of SREBP-2 and HMGCR in the experiment 2 were not affected in the S+FSE group because of the existence of soy protein in a diet (Fig. 3-4).

In this chapter, it was assumed that aglyconized isoflavone coexsisted with soy protein markedly reduced hepatic lipid. Therefore, it is suggested that fermented soymilk containing both of aglyconaized isoflavone and soy protein enhanced lipid

metabolism-modulation. As the lactic acid fermentation of soymilk converts isoflavone glycoside to aglycone, soy proteins may be converted to soy peptides and saponin glycoside may be converted to aglycone. Thus, saponin and soy peptides also could attribute to lipid metabolism-modulating effect [24)-27)57)-58)71)]. It is assumed that isoflavone is concerned to the up-regulation of CYP7a1, cholesterol catabolism-related enzyme and soy protein reduces the gene expression of fatty acid synthesis-related enzymes. These results indicate that isoflavones and soy proteins have different roles on lipid metabolism from each other and isoflavone aglycone enhances lipid metabolism-modulation by coexistence with soy protein.

Chapter 4. Hypocholesterolemic Effects of Lactic Acid-Fermented Soymilk on Rats Fed a High Cholesterol Diet

In previous chapter, the effect of fermented soymilk on lipid metabolism-modulation in rats fed an AIN-93G diet, normal diet, was described and the mechanism was partially clarified. Recently, hyperlipidemia induced by cholesterol-rich diet is a severe problem. Thus, in this chapter, hypocholesterolemic effect of fermented soymilk on rats fed a high cholesterol diet was investigated.

Materials and Methods

Diets The fermented soymilk was prepared as described in previous chapter. The compositions and energy of fermented soymilk are shown in Table 4-1. Total isoflavone content in aglycon equivalent was approximately 500 μmol/100 g of dried fermented soymilk. The other feed materials were purchased from Clea Japan (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries (Osaka, Japan).

Table 4-1. Composition of Freeze-Dried Fermented Soymilk

Component	Fermented soymilk	
	%	Energy (kcal/100 g)
Water	4.9	-
Protein	42.9	171.6
Fat	36.8	331.2
Carbohydrate	2.6	10.4
Dietary Fiber	7.2	14.4
Mineral	5.6	-
Energy	-	527.6

Animals Twenty four male Sprague-Dawley rats (7 weeks old) were purchased from Nihon SLC (Hamamatsu, Japan) and feeding procedure was carried out as described in previous chapter. The rats were assigned to three groups (n = 8) which did not exhibit any significant difference in the body weight and serum total cholesterol concentration from each other.

Table 4-2. Composition of Experimental Diets (Weight %)

Ingredient	Diet group			
	AIN-93*	HC	F-5	F-10
Casein [1]	20.0	20.0	14.2	8.4
Cornstarch [1]	39.8	38.8	38.1	36.5
Dextrinized cornstarch [1]	13.2	13.2	13	12.5
Sucrose [1]	10.0	10.0	10.0	10.0
Soybean oil [1]	7.0	7.0	2.8	0.0
Cellulose [1]	5.0	5.0	4.1	3.2
Mineral mix (AIN-93G-MX) [1]	3.5	3.5	3.5	3.5
Vitamin mix (AIN-93-VX) [1]	1.0	1.0	1.0	1.0
L-Cystine [2]	0.3	0.3	0.3	0.3
Choline bitartrate [2]	0.25	0.25	0.25	0.25
tert -Butylhydroquinone [2]	0.0014	0.0014	0.0014	0.0014
Cholesterol [2]	0.0	1.0	1.0	1.0
Fermented soymilk [3]	0.0	0.0	11.7	23.4
Total	100	100	100	100

HC, high cholesterol diet; F-5, 11.7% of the high cholesterol diet was replaced with dried fermented soymilk to 5% soy protein as final concentration; F-10, 23.4% of the high cholesterol diet was replaced with fermented soymilk to 10% soy protein as final concentration;

[1] Japan CLEA, Osaka, Japan.

[2] Wako Pure Chemical Industries, Osaka, Japan.

[3] Marusan-Ai, Okazaki, Japan.

*Reeves PG, Nielsen FH, and Fahey GCJr, *J. Nutr* ., **123**, 1939–1951 (1993).

The high cholesterol (HC) group was fed with the AIN-93G diet containing 1% cholesterol (control diet). The 5% soy protein containing-fermented soymilk (F-5)

group was fed with a diet in where 11.7% of the high cholesterol diet had been replaced with dried fermented soymilk, and the 10% soy protein containing-fermented soymilk (F-10) group was fed with a diet where 23.4% of the high cholesterol diet had been replaced with fermented soymilk (Table 4-2). The rats in each group were fed the appropriate diet for 5 weeks and provided with *ad libitum* access to the diet and water. The food intake and body weight were measured as described in previous chapter. Analyses of blood and liver were carried out as described in previous chapter.

Measurement of the Plasma and Liver Metabolic Parameters Measurement of the plasma and hepatic metabolic parameters were carried out as described in previous chapter.

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) The expression of mRNA was quantitatively measured by real-time RT-PCR as described in previous chapter. Data were normalized to GAPDH RNA expression, and the fold was presented as a ratio to the HC group.

Statistical Analysis The results are presented as the mean ± standard error. Tukey's multiple comparison was used for blood analysis at each feeding time, hepatic lipid parameters and fat mass increased between initial and end points of feeding period. Student's *t*-test was used for the real time RT-PCR data of hepatic gene expression. The statistical analyses were performed with SPSS 12.0 J for Windows.

Results

Body Weight, Food Intake, Food Efficiency, Total Energy Intake and Tissue Weights No significant difference in final body weight, food intake, food efficiency and total energy intake were found among the three groups (Table 4-3). The liver weight of the F-10 group after 5 weeks was significantly decreased compared with that of the HC group.

Table 4-3. Initial and Final Body Weights, Food intake, Food Efficiency, and Liver Weights of Rats Fed Experimental Diets for 5 Weeks

	HC	F-5	F-10
Initial body weight (g)	271.7 ± 3.5 [a]	269.5 ± 3.4 [a]	266.2 ± 2.9 [a]
Final body weight (g)	422.1 ± 11.6 [a]	401.1 ± 15.2 [a]	394.9 ± 13.0 [a]
Food intake (g/day)	19.8 ± 0.7 [a]	19.1 ± 0.7 [a]	18.3 ± 0.7 [a]
Food efficiency (g b.w. gain/g diet)	0.23 ± 0.01 [a]	0.20 0.02 [a]	0.21 0.01 [a]
Tissue weight (% b.w.)			
Liver	4.6 ± 0.1 [a]	4.1 ± 0.1 [ab]	3.6 ± 0.1 [b]

Each value is the means ± SE of 6 rats. [a,b] Means not sharing common superscript differ significantly by Tukey's multiple comparison (p <0.05).

Hepatic Lipid Profile Hepatic cholesterol and TG levels were higher with a high cholesterol diet compared with those on cholesterol-free diet ingestion in chapter 2 and were increased to 13.7-fold and 4.6-fold *vs*. those on a cholesterol-free diet, respectively. The hepatic cholesterol, TG and total lipid levels in the F-5 and F-10 groups were significantly decreased in a dose dependent manner *vs.* those of the HC group, respectively (Fig.4-1A,B,C). Furthermore, the hepatic cholesterol, TG and total lipid levels of the F-10 group were significantly decreased compared with those of the F-5 group.

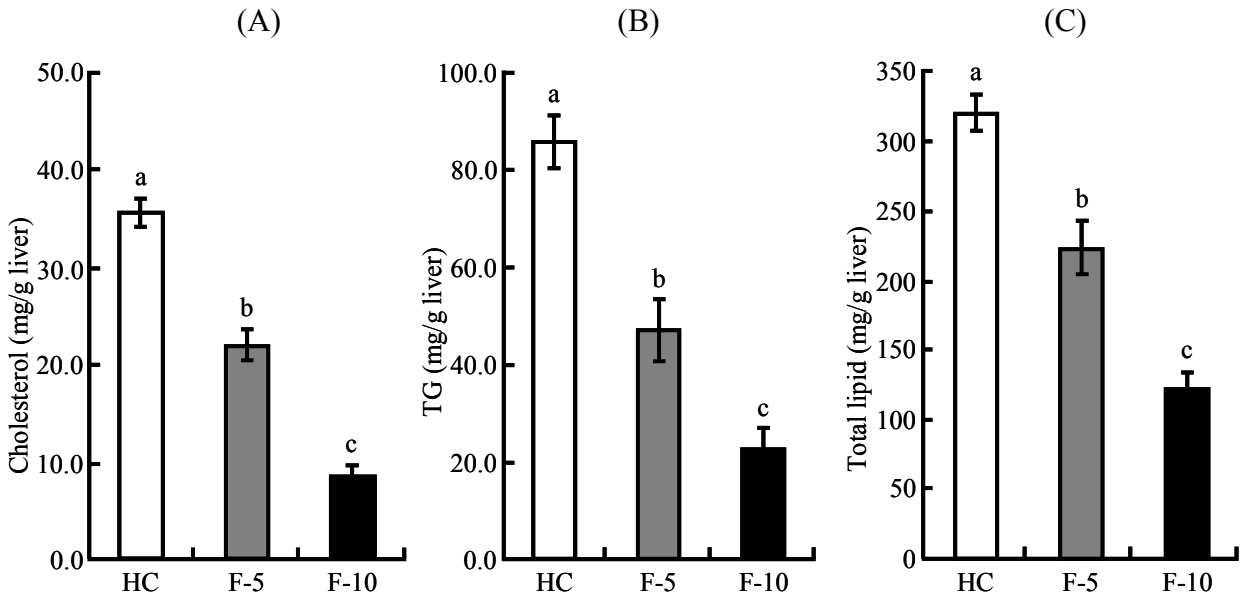

Fig.4-1. Liver parameters of rats fed on the experimental diets for 5 weeks. (A) hepatic cholesterol; (B) hepatic TG; (C) hepatic total lipid. Each value is the mean ± SE for 8 rats. [a,b,c] Means not sharing a common superscript differ significantly by Tukey's multiple-comparison test ($p < 0.05$).

Plasma Lipid Profiles Plasma TC and non-HDL-C levels in the HC group fed a high cholesterol diet were higher compared with those on a cholesterol-free diet in chapter 2. Plasma TG level was slightly lowered by a high cholesterol diet. Plasma TC level was decreased in a dose dependent manner by ingestion of fermented soymilk (Fig.4-2A). The F-10 group showed a significantly lower TC level from 1 week to 5 weeks. As shown in Fig.4-2B, plasma non-HDL-C was temporally decreased in a dose dependent manner by ingestion of fermented soymilk from 3 weeks to 4 weeks. The F-10 group displayed significantly decreased non-HDL-C compared with the HC group from 1 week to 5 weeks. The HDL-C/TC ratio was significantly increased in the F-10 group compared with the HC group (data not shown). Plasma TG level showed temporally significant decrease in the F-10 group from 2 weeks to 4 weeks (Fig 4-2C).

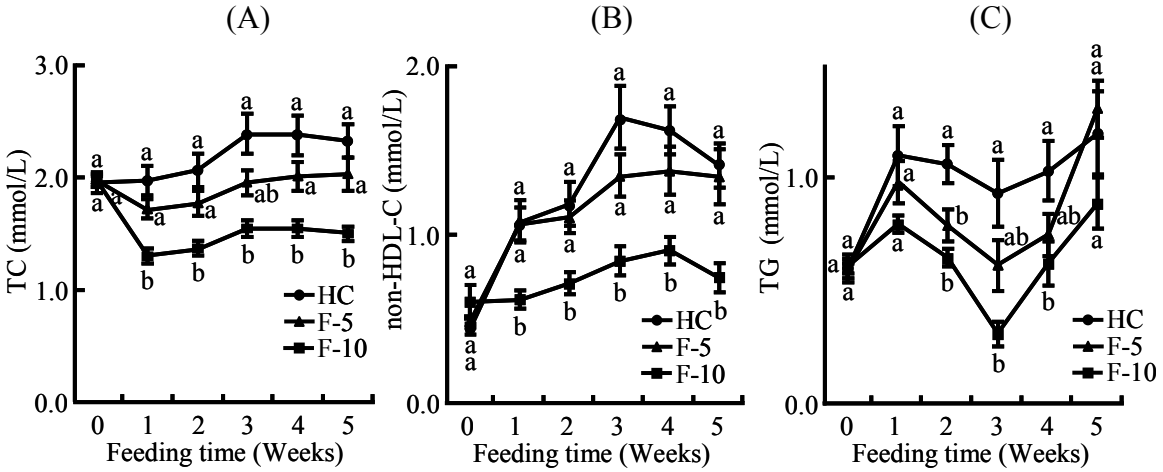

Fig. 4-2. Plasma parameters of rats fed on the experimental diets for 5 weeks. (A) plasma TC level; (B) non-HDL-C; (C) plasma TG level. Each value is the mean ± SE for 8 rats. [a,b] Means not sharing a common superscript differ significantly by Tukey's multiple-comparison test ($p < 0.05$).

Real Time PCR Analysis With regard to the change of hepatic lipid profile by the ingestion of fermented soymilk, the gene expression of hepatic lipid metabolism in the only F-5 group was compared with those of the HC group because the gene expression from livers of the F-10 group was not obtained (Fig. 4-3). The gene expression of LXRα was scarcely changed in the F-5 group (1.1 fold). In cholesterol metabolism, the expression of CYP7a1 of the F-5 group was significantly up-regulated to 2.0 fold compared with that of the HC group. In contrast, the expression of SREBP-2 was significantly decreased to 0.7 fold in the F-5 group compared with the HC group. Although the gene expressions of SREBP-1 and FAS in fatty acid synthesis-related metabolism were down-regulated 0.8 and 0.7 fold, respectively, significant change of those expressions was not found in the F-5 group.

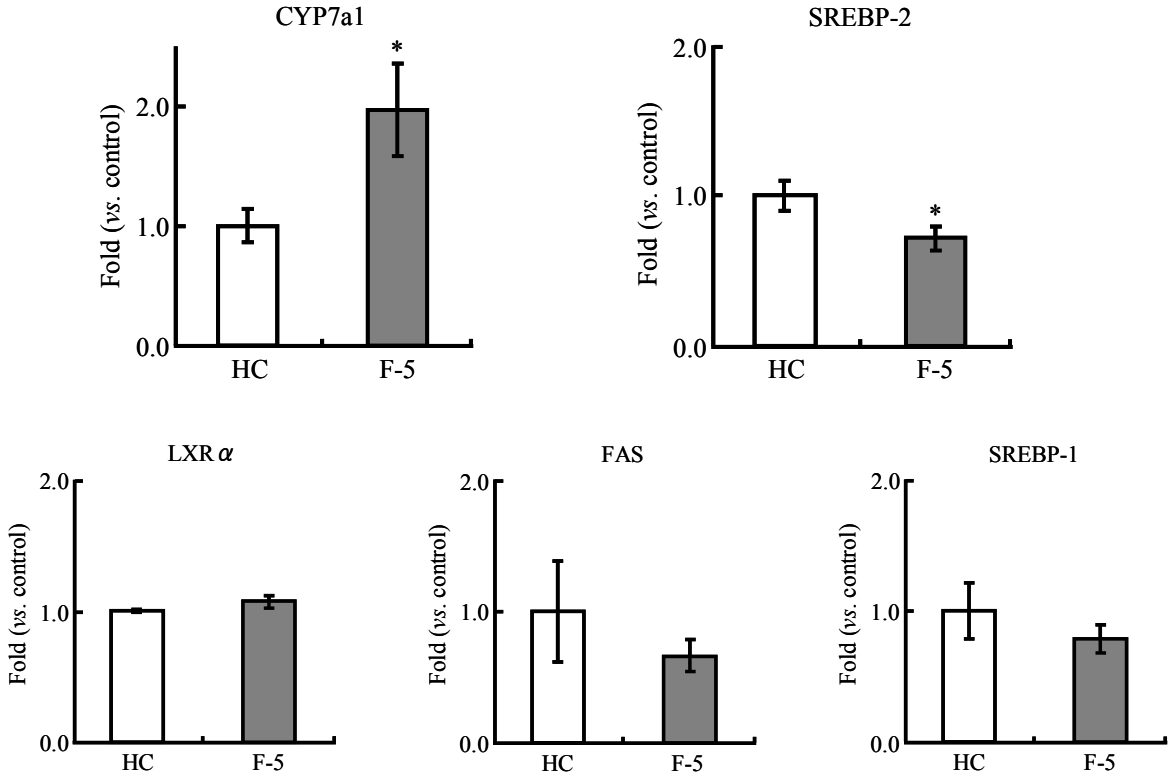

Fig. 4-3. Expression of lipid metabolism-related genes in the liver of rats fed experimental diets for 5 weeks. The expression of mRNA was quantitatively measured by real-time RT-PCR. Each value is the mean ± SE for 8 rats. The data were normalized to GAPDH RNA expression and are presented as a ratio to the HC value. Statistically significant compared with the control group (* $p < 0.05$; Student's t-test).

Discussion

The effects of lactic acid-fermented soymilk on lipid metabolism in rats fed an AIN-93G diet have been known [32)59)]. In chapter 1, it was found that fermented soymilk diet containing 10% soy protein as final concentration was necessary to significantly enhance the lipid metabolism modulation in rats fed a cholesterol-free diet. Thus, in the present study, we examined the dose response of the lipid metabolism-modulation effect by using different concentrations of fermented soymilk in rats fed a high cholesterol diet. Furthermore, the effect of lipid metabolism modulation on rats fed a high cholesterol diet was compared with that on rats fed a cholesterol-free diet in chapter 2. The consumption of fermented soymilk resulted in

reduction of liver weight (Table 4-3) and decreased hepatic cholesterol and TG levels in a dose dependent manner (Fig. 4-1A,B). The hepatic total lipid level was also decreased in a dose dependent manner in the presence of fermented soymilk (Fig.4-1C). Although the HC group fed a high cholesterol diet showed higher levels of hepatic cholesterol, TG and total lipid compared with those of the AIN-93G diet group in chapter 2, the levels in the F-5 and F-10 groups were decreased by fermented soymilk intake. Especially, hepatic total lipid and TG levels in the F-10 group were decreased to the same ones as those of rats fed an AIN-93G diet in chapter 2, respectively, and the hepatic cholesterol level in the F-10 group was decreased to lower one than that of fed an AIN-93G diet in chapter 2. These data indicated that the reduction of hepatic cholesterol and TG levels by fermented soymilk intake leads to prevention of hepatic lipid accumulation or fatty liver. Plasma TG level in the F-10 group was continuously decreased from 1 week to 5 weeks (Fig. 4-2C). In contrast, plasma TC level increased by high cholesterol diet was decreased in the dose dependent manner of fermented soymilk. Although the lowering of plasma TC level in the F-5 group did not show a significant difference, the TC level was decreased to the same one as that of the AIN-93G diet group in chapter 2. The TC level in the F-10 group showed lower one than that of the AIN-93G diet group. Additionally, although LDL-C was directly measured, the ingestion of fermented soymilk inhibited the increase of plasma non-HDL-C as shown in the F-10 group (Fig.4-2B) and the level was the same as that of the AIN-93G diet group in chapter 2. LDL-C is the major cause of onset of atherosclerosis [1)]. Therefore, results of this study suggest that the consumption of fermented soymilk may reduce atherosclerosis.

The hepatic cholesterol and TG levels were affected by ingestion of fermented

soymilk diet containing more than 5% soy protein as shown in Fig.4-1. In contrast, the fermented soymilk diet containing 10% soy protein was essential to obtain the significant reduction of plasma TC level and non-HDL-C level (Fig. 4-2). Although 20% soy protein in the diet has been often used to investigate the improvement of lipid metabolism in rats [37)38)39)], the soy protein concentration of fermented soymilk diets used in the present study was 5% or 10%. It was assumed that a relatively low concentration of soy protein in fermented soymilk diet exhibits a hypocholesterolemic effect. Lowering of cholesterol levels have been reported by using functional substances such as soy protein [72)] soy peptide [57)58)], isoflavone [59)] and saponin [24)]. As soymilk includes many kinds of functional substances, cholesterol-lowering effects of fermented soymilk may be caused by not only soy protein or peptide but also by isoflavone and saponin. Although the combination of plant sterol ester with soy protein increased the fecal excretion of bile acid [11)], in the present study, the significant difference in bile acid level of feces was not found between the control group and the fermented soymilk groups. However, as the administration of fermented soymilk diet containing 5% soy protein significantly affected hepatic lipid profiles in rats, the gene expressions of lipid metabolisms in the F-5 group were examined compared with those of the HC group (Fig. 4-3). The gene expression of LXRα was scarcely changed in the F-5 group and the changes in expressions of SREBP-1 and FAS [41)], the fatty acid synthesis-related genes controlled by LXRα, were also not statistically significant (Fig. 4-3). In contrast, CYP7a1, the rate-limiting enzyme in the formation of bile acid from cholesterol [42)] controlled by LXRα [66)], was increased as shown in the fermented soymilk group fed a cholesterol-free diet in chapter 2. In the present study, in spite of almost invariable expression of LXRα, the expression of CYP7a1 was significantly

up-regulated in the F-5 group (Fig.4-3). Therefore, it seems that other gene than LXRα strongly controlled the expression of CYP7a1. Farnesoid X receptor (FXR) and pregnane X receptor (PXR) is known to inhibit CYP7a1 [66]. The enhancement of CYP7a1 expression was likely to be involved in suppressing FXR and PXR. In addition, the expression of SREBP-2, cholesterol synthesis-accelerating factor [69], was decreased in the F-5 group. In this chapter, protein levels of SREBP-2 and CYP7a1 were not determined. However, if the down-regulation of SREBP-2 and up-regulation of CYP7a1 reflect the amount of protein and enzymes related to cholesterol metabolism, the fermented soymilk might decrease the excess pool of cholesterol incorporated through a high cholesterol diet. The abundance of SREBP-2 and CYP7a1 must be examined to confirm changes of cholesterol synthesis and catabolism.

Chapter 5. Effects of Isoflavone Aglycone Ratio in Lactic Acid-Fermented Soymilk on Hepatic Lipid Metabolism in Rats Fed a High Cholesterol Diet

In previous chapters, it was demonstrated that fermented soymilk has a lipid metabolism-modulating effect in rats fed AIN-93G diet and high cholesterol diet, respectively. Furthermore, isoflavone and soy protein have different roles on lipid metabolism from each other and isoflavone aglycone enhances lipid metabolism-modulation by coexistence with soy protein. Thus, we investigated the effect of isoflavone aglycone ratio in lactic acid-fermented soymilk on hepatic lipid metabolism. Two kinds of fermented soymilks were prepared using 2 strains as follows. *Lactobacillus delbrueckii* subsp. *delbrueckii* strain of TUA4408L had high conversion ability of isoflavone glycone to aglycone and *Lactobacillus delbrueckii* subsp. *delbrueckii* strain of TUA4404L had low conversion ability of isoflavone glycone to aglycone. Therefore, in the present chapter, soymilk and two kinds of different isoflavone aglycone ratio of fermented soymilk on lipid metabolism were investigated in rats fed a high cholesterol diet to clarify lipid metabolism-modulating effect of isoflavone aglycone.

Materials and Methods

Diets The fermented soymilk was prepared by lactic acid fermentation by the *Lactobacillus delbrueckii* subsp. *delbrueckii* strain of TUA4408L or TUA-4404L for 15 h and then immediately freeze-dried for the animal experiments. Soymilk was also freeze-dried for the animal experiments. The compositions and energy of soymilk

and fermented soymilk were shown in Table 5-1. The other feed materials were purchased from Clea Japan (Tokyo, Japan), Nacarai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries (Osaka, Japan).

Table 5-1. Composition of Freeze-dried Soymilk and Fermented Soymilk

Component	Soymilk		Fermented Soymilk TUA4404L		Fermented Soymilk TUA4408L	
	%	Energy (kcal/100 g)	%	Energy (kcal/100 g)	%	Energy (kcal/100 g)
Water	1.7	-	1.6	-	1.9	-
Protein	42.8	171.2	43.0	172.0	42.7	170.8
Fat	34.3	308.7	39.1	351.9	39.1	351.9
Carbohydrate	7.1	28.4	2.7	10.8	2.7	10.8
Dietary fiber	8.7	17.4	7.6	15.2	7.6	15.2
Mineral	5.4	-	5.9	-	5.9	-
Total energy	-	525.7	-	549.9	-	548.7

Fermented Soymilk TUA4404L, fermented soymilk by lactic acid fermentation using *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4404L;
Fermented Soymilk TUA4408L, fermented soymilk by lactic acid fermentation using *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4408L

Animals Thirty two male Sprague-Dawley rats (7 weeks old) were purchased from Nihon SLC (Hamamatsu, Japan) and feeding procedure was carried out as described in previous chapters. The rats were assigned four groups (n=8). The high cholesterol (HC) group was fed with the AIN-93G diet containing 1% cholesterol. The soymilk (S) group was fed with a diet where 23.2% of the high cholesterol diet had been replaced with dried soymilk so that 10% soy protein was contained as the final concentration. The fermented soymilk produced by TUA4404L (F4404) group was fed with a diet where 23.2% of the high cholesterol diet had been replaced with

dried fermented soymilk produced by TUA4404L so that 10% soy protein was contained as the final concentration, and the fermented soymilk TUA4408L (F4408) group was fed with a diet where 23.2% of the high cholesterol diet had been replaced with dried fermented soymilk TUA4408L so that 10% soy protein was contained as the final concentration (Table 5-2). The rats in each group were fed the appropriate diet for 5 weeks and provided with *ad libitum* access to the diet and water. The food intake and body weight were measured as described in previous chapter. Analyses of blood and liver were carried out as described in previous chapter. These animal experiments were performed according to the guidelines from the Animal Use Committee of Mukogawa Women's University.

Table 5-2. Composition of the Experimental Diets (weight %)

Ingredient	Diet group				
	AIN-93*	HC	S	F4404	F4408
Casein [1]	20.0	38.8	37.1	36.9	36.8
Cornstarch [1]	39.8	20.0	8.4	8.4	8.5
Dextrinized cornstarch [1]	13.2	13.2	12.3	12.3	12.3
Sucrose [1]	10.0	10.0	10.0	10.0	10.0
Soybean oil [1]	7.0	7.0	0.0	0.0	0.0
Cellulose [1]	5.0	5.0	2.9	3.1	3.1
Mineral mix (AIN-93G-MX) [1]	3.5	3.5	3.5	3.5	3.5
Vitamin mix (AIN-93-VX) [1]	1.0	1.0	1.0	1.0	1.0
L-Cystine [2]	0.3	0.3	0.3	0.3	0.3
Choline bitartrate [3]	0.25	0.25	0.25	0.25	0.25
tert -Butylhydroquinone [2]	0.0014	0.0014	0.0014	0.0014	0.0014
Cholesterol [2]	—	1.0	1.0	1.0	1.0
Soymilk [4]	—	—	23.3	—	—
Fermented soymilk 4404 [4]	—	—		23.3	—
Fermented soymilk 4408 [4]	—	—	—	—	23.3
Total	100	100	100	100	100

HC, high cholesterol diet; S, high cholesterl diet containg soymilk
F4404, high cholesterol diet containing fermented soymilk by lactic acid fermentation using *Lactobacillus delbrueckii* subsp. d*elbrueckii* TUA4404L;
F4408, high cholesterol diet containing fermented soymilk by lactic acid fermentation using *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4408L
[1] Clea Japan, Osaka, Japan
[2] Wako Pure Chemical Industries, Osaka, Japan
[3] Nacalai tesque, Kyoto, Japan
[4] Marusan-Ai, Okazaki, Japan
*Reeves, PG., Nielsen, FH. and Fahey, JrGC., *J. Nutr* ., **123**, 1939–1951 (1993)

Measurement of the Plasma and Liver Metabolic Parameters Plasma TC, HDL-C and TG were enzymatically measured by using commercial kits as described in previous chapters. The measurement of hepatic metabolic parameters was carried out

as described in previous chapter.

Isoflavones in the diet Isoflavone was measured as decribed in previous chapter.

Statistical analysis The results are presented as the mean ± standard error, and were analyzed by Tukey's multiple-comparison test at $p<0.05$. Statistical analyses were performed with SPSS 12.0 J for Windows.

Results and Discussion

Different ratio of isoflavone aglycones of soymilk and two kinds of fermented soymilks Isoflavone amount of soymilk, fermented soymilk TUA4404L and fermented soymilk TUA4408L were compared (Table 5-3). The isoflavone aglycone, genistein and daidzein, were higher in fermented soymilk produced by soymilk TUA4408L than soymilk and that produced by TUA4404L. On the other hand, amounts of glycone form, daidzin and genistin, were higher in soymilk and fermented soymilk by TUA4404L than fermented soymilk by TUA4408L. As glycitein and glycitin did scarcely occur in soymilk, no difference in glycitein and glycitin contents was found among soymilk, fermented soymilks by TUA4404L and by TUA4408L. Isoflavone aglycone ratio of soymilk, fermented soymilk by TUA4404L and fermented soymilk by TUA4408L were 2.2%, 3.0%, and 73.5%, respectively.

These results indicated that *Lactobacillus delbrueckii* subsp. *delbrueckii* strain of TUA4404L has low conversion ability of isoflavone glycone to aglycone, whereas, stain of TUA4408L has high conversion ability of isoflavone glycone to aglycone.

Table 5-3. Isoflavone Level of Soymilk and Fermented Soymilk (μmol/100 g of dried sample)

	Soymilk	Fermented Soymilk TUA4404L	Fermented Soymilk TUA4408L
Genistein	5.6	7.4	196.9
Daidzein	5.5	7.1	143.2
Genistin	290.1	276.8	70.3
Daidzin	201.4	188.8	52.3
Total Isoflavones	502.6	480.1	462.7
Aglycone ratio (%)	2.2	3.0	73.5

Fermented Soymilk TUA4404L, fermented soymilk by lactic acid fermentation using *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4404L;
Fermented Soymilk TUA4408L, fermented soymilk by lactic acid fermentation using *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4408L

Body weight, food intake, food efficiency and tissue weights No significant differences in the final body weight, food intake and food efficiency were found among the four groups (Table 5-4). The liver weights were decreased in the S, F4404 and F4408 groups compared with the HC group.

Table 5-4. Initial and Final Body Weights, Food Intake, Food Efficiency, and Liver Weights of Rats Fed on the Experimental Diets for 5 Weeks

	HC	S	F4404	F4408
Initial body weight (g)	254.2 ± 4.1 [a]	251.9 ± 2.7 [a]	253.2 ± 3.2 [a]	250.7 ± 2.8 [a]
Final body weight (g)	429.8 ± 12.6 [a]	402.4 ± 4.3 [a]	411.2 ± 12.7 [a]	411.0 ± 8.8 [a]
Food intake (g/d)	21.0 ± 0.8 [a]	19.1 ± 0.3 [a]	19.7 ± 0.6 [a]	20.2 ± 0.3 [a]
Food efficiency (g b.w. gain/g diet)	0.26 ± 0.00 [a]	0.24 ± 0.00 [a]	0.25 ± 0.01 [a]	0.25 ± 0.01 [a]
Tissue weight (% b.w.)				
Liver	4.9 ± 0.1 [a]	3.9 ± 0.1 [b]	3.8 ± 0.1 [b]	4.1 ± 0.1 [b]

Each value is the mean ± SE for 8 rats. [a,b] Means not sharing a common superscript differ significantly by Tukey's multiple-comparison test (p <0.05).

Plasma lipid level The plasma TC level was significantly decreased in the F4404 and F4408 groups compared with the HC group from 1 week to 5 weeks (Table 5-5). The S group significantly decreased in plasma TC level *vs.* the HC group after 1, 2 and 4 weeks.

Additionally, the TC level of the F4408 group was decreased *vs.* that of the S group in 1 week feeding. Additionally, non-HDL-C was significantly decreased in the F4408 group compared with the HC group from 1 week to 5 weeks. Lowering effect of non-HDL-C was strongest in the F4408 group, followed by the F4404 and S groups. These results indicated that the reduction of plasma TC and non-HDL-C levels depended on isoflavone aglycone ratio of soymilk or fermented soymilk. The difference of plasma TG level was not found among 4 groups for 5 week (Table 5-5).

Table 5-5. Plasma Parameters of Rats Fed on the Experimental Diets for 5 Weeks

	HC	S	F4404	F4408
Total cholesterol (mmol/l)				
0W	1.53 ± 0.05 [a]	1.55 ± 0.10 [a]	1.55 ± 0.15 [a]	1.55 ± 0.08 [a]
1W	2.01 ± 0.08 [a]	1.54 ± 0.06 [b]	1.43 ± 0.06 [bc]	1.26 ± 0.04 [c]
2W	1.83 ± 0.12 [a]	1.40 ± 0.05 [b]	1.38 ± 0.07 [b]	1.20 ± 0.05 [b]
3W	1.95 ± 0.15 [a]	1.61 ± 0.07 [ab]	1.54 ± 0.09 [b]	1.29 ± 0.05 [b]
4W	1.96 ± 0.11 [a]	1.59 ± 0.07 [b]	1.49 ± 0.11 [b]	1.32 ± 0.05 [b]
5W	2.19 ± 0.15 [a]	1.78 ± 0.07 [ab]	1.62 ± 0.13 [b]	1.51 ± 0.06 [b]
non-HDL-C (mmol/l)				
0W	0.17 ± 0.03 [a]	0.18 ± 0.05 [a]	0.18 ± 0.06 [a]	0.14 ± 0.04 [a]
1W	1.18 ± 0.10 [a]	0.85 ± 0.05 [b]	0.78 ± 0.05 [b]	0.65 ± 0.05 [b]
2W	1.08 ± 0.11 [a]	0.70 ± 0.03 [b]	0.62 ± 0.08 [b]	0.56 ± 0.05 [b]
3W	1.22 ± 0.14 [a]	0.92 ± 0.05 [ab]	0.83 ± 0.06 [b]	0.67 ± 0.05 [b]
4W	1.02 ± 0.09 [a]	0.83 ± 0.05 [ab]	0.76 ± 0.08 [ab]	0.56 ± 0.08 [b]
5W	1.39 ± 0.14 [a]	1.07 ± 0.06 [ab]	0.90 ± 0.06 [b]	0.81 ± 0.07 [b]
Triglyceride (mmol/l)				
0W	0.96 ± 0.09 [a]	0.95 ± 0.10 [a]	0.96 ± 0.11 [a]	0.96 ± 0.08 [a]
1W	1.49 ± 0.34 [a]	1.20 ± 0.08 [a]	1.09 ± 0.13 [a]	1.06 ± 0.06 [a]
2W	1.77 ± 0.16 [a]	1.41 ± 0.11 [a]	1.47 ± 0.17 [a]	1.24 ± 0.15 [a]
3W	1.57 ± 0.34 [a]	1.49 ± 0.11 [a]	1.36 ± 0.15 [a]	1.11 ± 0.08 [a]
4W	1.98 ± 0.31 [a]	1.85 ± 0.13 [a]	1.90 ± 0.20 [a]	1.44 ± 0.09 [a]
5W	1.82 ± 0.24 [a]	1.68 ± 0.06 [a]	1.49 ± 0.20 [a]	1.45 ± 0.10 [a]

Each value is the mean ± SE for 8 rats. [a,b,c] Means not sharing a common superscript differ significantly by Tukey's multiple-comparison test (p <0.05).

Hepatic lipid level A hepatic lipid level was significantly decreased in the S, F4404 and F4408 groups compared with the HC group. Additionally, hepatic cholesterol and TG level was also significantly decreased in the S, F4404 and F4408 groups compared with the HC group. No significant difference was found in hepatic lipid, cholesterol and TG levels among the S, F4404 and F4408 groups.

Table 5-6. Liver Parameters of Rats Fed on the Experimental Diets for 5 Weeks

	HC	S	F4404	F4408
Hepatic lipid (mg/g)	309.2 ± 27.0 [a]	178.7 ± 32.6 [b]	173.4 ± 21.3 [b]	168.7 ± 29.8 [b]
Cholesterol (mg/g)	28.3 ± 0.7 [a]	13.5 ± 1.3 [b]	11.0 ± 1.1 [b]	11.8 ± 1.5 [b]
Triglyceride (mg/g)	85.1 ± 4.8 [a]	43.4 ± 3.3 [b]	41.8 ± 3.0 [b]	42.7 ± 4.6 [b]

Each value is the mean ± SE for 8 rats. [a,b] Means not sharing a common superscript differ significantly by Tukey's multiple-comparison test ($p < 0.05$).

The lipid metabolism-modulating effect of soy foods seems to depend on mainly soy protein. It was suggested that isoflavone converted from glycones to aglycones by lactic acid-fermentation promoted the absorption into intestine and increased the concentration in liver and plasma. Because the ability lowering hepatic lipid, cholesterol and TG levels reached a limit in the S, F4404 and F4408 group, there was no difference in the levels among the 3 groups. On the other hand, the ability decreasing the plasma TC and non-HDL-C levels was found to be most effective in fermented soymilk by TUA4408L. Therefore, it was assumed that fermented soymilk containing high ratio of isoflavone aglycone promoted lipid metabolism-modulating effects lowering plasma TC and non-HDL-C levels.

Chapter 6. Effects of Isoflavone Aglycone Ratio in Lactic Acid-Fermented Soymilk on Hepatic Lipid Metabolism in Rats by Lipid Loading Test

In the chapter 5, the higher ratio of isoflavone aglycone in lactic acid-fermented soymilk obviously showed the effect of hepatic lipid metabolism-modulation in rats fed a high cholesterol diet. In daily diet, obesity was not induced only by excess cholesterol but also by excess fat. Thus, in the present chapter, the effect of isoflavone aglycone ratio in lactic acid-fermented soymilk on hepatic lipid metabolism was investigated in rats by lipid loading test.

Materials and Methods

Diet The two kind of fermented soymilk were prepared as described in previous chapter. The compositions and energy of soymilk and fermented soymilk are shown in Table 6-1. The other feed materials were purchased from Clea Japan (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries (Osaka, Japan).

Table 6-1. Composition of Freeze-dried Fermented Soymilk

Component	Fermented Soymilk TUA4404L		Fermented Soymilk TUA4408L	
	%	Energy (kcal/100 g)	%	Energy (kcal/100 g)
Water	1.9	-	2.4	-
Protein	45.7	182.8	44.7	178.8
Fat	37.0	333.0	37.3	335.7
Carbohydrate	2.6	10.4	2.6	10.4
Dietary fiber	7.2	14.4	7.3	14.6
Mineral	5.6	-	5.7	-
Total energy	-	540.6	-	539.5

Fermented Soymilk TUA4404L, fermented soymilk by lactic acid fermentation using *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4404L;
Fermented Soymilk TUA4408L, fermented soymilk by lactic acid fermentation using *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4408L

Animals Eighteen male Sprague-Dawley rats (7 weeks old) were purchased from Nihon SLC (Hamamatsu, Japan) and feeding procedure was carried out as described in previous chapters.

Table 6-2. Composition of the Experimental Diets (weight %)

Ingredient	Diet group			
	AIN-93*	LL	F4404	F4408
Casein [1]	20.0	20.0	8.4	8.4
Cornstarch [1]	39.8	32.6	31.3	31.0
Dextrinized cornstarch [1]	13.2	10.9	10.4	10.3
Sucrose [1]	10.0	10.0	10.0	10.0
Soybean oil [1]	7.0	7.0	0.0	0.0
Cellulose [1]	5.0	5.0	3.3	3.3
Mineral mix (AIN-93G-MX) [1]	3.5	3.5	3.5	3.5
Vitamin mix (AIN-93-VX) [1]	1.0	1.0	1.0	1.0
L-Cystine [2]	0.3	0.3	0.3	0.3
Choline bitartrate [3]	0.25	0.25	0.25	0.25
tert -Butylhydroquinone [2]	0.0014	0.0014	0.0014	0.0014
Cholesterol [2]	—	0.125	0.125	0.125
Beef tarrow [1]	—	9.4	9.4	9.4
Fermented soymilk 4404 [4]	—	—	22.0	—
Fermented soymilk 4408 [4]	—	—	—	22.4
Total	100	100	100	100

LL, lipid loading diet;
F4404, lipid loading diet containing fermented soymilk by lactic acid fermentation using *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4404L;
F4408, lipid loading diet containing fermented soymilk by lactic acid fermentation using *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4408L
[1] Clea Japan, Osaka, Japan
[2] Wako Pure Chemical Industries, Osaka, Japan
[3] Nacalai tesque, Kyoto, Japan
[4] Marusan-Ai, Okazaki, Japan
*Reeves, PG., Nielsen, FH. and Fahey, JrGC., *J. Nutr* ., **123**, 1939–1951 (1993)

The rats were assigned three groups (n=6) which did not exhibit any significant difference in the body weight and serum total cholesterol concentration from each other. The lipid loading test (LL) group was fed with the AIN-93G diet containing

15% fat and 0.125% cholesterol. The fermented soymilk TUA4404L (F4404) group was fed with a diet where 22.0% of the lipid loading diet had been replaced with dried fermented soymilk produced by TUA4404L so that 10% soy protein was contained as the final concentration, and the fermented soymilk TUA4408L (F4408) group was fed with a diet where 22.4% of the lipid loading diet had been replaced with dried fermented soymilk produced by TUA4408L so that 10% soy protein was contained as the final concentration (Table 6-2). The rats in each group were fed the appropriate diet for 5 weeks and provided with *ad libitum* access to the diet and water. The food intake and body weight were measured as described in previous chapter. Analyses of blood and liver were carried out as described in previous chapter.

These animal experiments were performed according to the guidelines from the Animal Use Committee of Mukogawa Women's University.

Measurement of the plasma and liver metabolic parameters Plasma total cholesterol (TC) and triglyceride (TG) were enzymatically measured by using commercial kits as described in previous chapters. The measurement of hepatic metabolic parameters was carried out as described in previous chapter. The hepatic water was measured by the method of atomosphelic heat dry. The hepatic sugar was measured by the phenol-sulfuric acid method [73)]

Real-time reverse transcription-polymerase chain reaction (RT-PCR) The expression of mRNA was quantitatively measured by real-time RT-PCR as described in previous chapter. Data were normalized to GAPDH RNA expression, and the fold was presented as a ratio to the LL group.

Isoflavones in the diet and liver Isoflavone was measured as described in previous chapter.

Statistical analysis The results are presented as the mean ± standard error, and were analyzed by Tukey's multiple-comparison test at $p<0.05$. Statistical analyses were performed with SPSS 12.0 J for Windows.

Results and Discussion

Different ratio of Isoflavone aglycone of two kinds of fermented soymilks. Isoflavone amounts of fermented soymilk TUA4404L and TUA4408L were compared (Table 6-3). The isoflavone aglycone, genistein and daidzein, were higher in fermented soymilk produced by TUA4408L than that produced by TUA4404L. On the other hand, amounts of glycone form, daidzin and genistin, amounts were higher in fermented soymilk by TUA4404L than that by TUA4408L. Additionally, As glycitein and glycitin did scarcely occur in soymilk, no difference in glycitein and glycitin contents was found between fermented soymilks by TUA4404L and by TUA4408L (data not shown). Isoflavone aglycone ratio of soymilk, fermented soymilks by TUA4404L and by TUA4408L were 2%, 35%, and 93%, respectively. These results indicated that the conversion ratio of isoflavone from glycone to aglycone in *Lactobacillus delbrueckii* subsp. *delbrueckii* strain of TUA4404L was low, whereas that of strain of TUA4408L was high.

Table 6-3. Isoflavone Level of Fermented Soymilk (μmol/100 g of dried sample)

	Soymilk	Fermented Soymilk TUA4404L	Fermented Soymilk TUA4408L
Genistein	5.6	99.2	254.6
Daidzein	5.5	72.8	191.2
Genistin	290.0	129.1	13.0
Daidzin	201.4	193.9	19.7
Total Isoflavones	502.5	495.0	478.5
Aglycone ratio (%)	2.2	34.7	93.2

Fermented Soymilk TUA4404L, fermented soymilk by lactic acid fermentation using *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4404L;
Fermented Soymilk TUA4408L, fermented soymilk by lactic acid fermentation using *Lactobacillus delbrueckii* subsp. *delbrueckii* TUA4408L

Body weight, food intake, food efficiency and tissue weights No significant differences in the final body weight, food intake and food efficiency were found among the three groups (Table 6-4). The liver weight of the F4408 group was significantly lower than that of the LL and F4404 group.

Table 6-4. Initial and Final Body Weights, Food Intake, Food Efficiency, and Liver Weights of Rats Fed on the Experimental Diets for 5 Weeks

	LL	F4404	F4408
Initial body weight (g)	274.6 ± 6.1 [a]	269.9 ± 3.7 [a]	267.8 ± 4.4 [a]
Final body weight (g)	440.8 ± 10.9 [a]	429.7 ± 3.7 [a]	424.8 ± 12.6 [a]
Food intake (g/d)	19.0 ± 0.5 [a]	18.7 ± 0.3 [a]	18.8 ± 0.4 [a]
Food efficiency (g b.w. gain/g diet)	0.28 ± 0.01 [a]	0.28 ± 0.01 [a]	0.27 ± 0.01 [a]
Liver weight (% b.w.)	4.1 ± 0.1 [a]	3.7 ± 0.1 [ab]	3.3 ± 0.1 [b]
Liver weight (g)	18.6 ± 0.7 [a]	16.7 ± 0.7 [a]	14.7 ± 0.8 [b]

Each value is the mean ± SE for 6 rats. [a,b] Means not sharing a common superscript differ significantly by Tukey's multiple-comparison test (p <0.05).

Plasma lipid level The plasma TC level indicated a similar pattern in both of the F4404 and F4408 groups (Table 6-5). In feeding for 1 week, plasma TC level was significantly decreased in the F4404 and F4408 groups compared with the LL group. In feeding for 5 weeks, plasma TC level was tended to decrease in the F4408 group *vs.* the LL group (p<0.1). Moreover, plasma TG level was also significantly decreased in the F4408 group compared with the LL group in 1 week. Additionally, in feeding for 5 weeks, the F4408 group was tended to decrease plasma TG *vs.* the LL and F4404 groups.

By these results, it was assumed that the effect decreasing plasma TG level was stronger in fermented soymilk by TUA4408L than in that by TUA4404L.

Table 6-5. Plasma Parameters of Rats Fed on the Experimental Diets for 5 Weeks

	LL	F4404	F4408
Total cholesterol (mmol/L)			
0W	1.71 ± 0.12 [a]	1.63 ± 0.13 [a]	1.80 ± 0.14 [a]
1W	1.48 ± 0.07 [a]	1.19 ± 0.05 [b]	1.21 ± 0.08 [b]
5W	1.72 ± 0.11 [a]	1.44 ± 0.11 [a]	1.34 ± 0.12 [a]
Triglyceride (mmol/L)			
0W	1.16 ± 0.17 [a]	1.08 ± 0.08 [a]	1.07 ± 0.06 [a]
1W	2.49 ± 0.35 [a]	1.64 ± 0.11 [ab]	1.33 ± 0.24 [b]
5W	2.82 ± 0.33 [a]	2.76 ± 0.19 [a]	1.83 ± 0.29 [a]

Each value is the mean ± SE for 6 rats. [a,b] Means not sharing a common superscript differ significantly by Tukey's multiple-comparison test (p <0.05).

Hepatic lipid level, hepatic water level and hepatic sugar Although hepatic lipid levels were suppressed in the F4404 and F4408 groups compared with the LL group (Table 6-6). Additionally, hepatic cholesterol and TG levels were significantly

decreased in the F4404 and F4408 groups compared with the LL group. Hepatic water level was also significantly decreased in the F4408 group *vs.* the LL group. Hepatic sugar levels did not exhibit significant difference among three groups. It was found that the decrease of liver weight in the F4408 group was caused by decreasing 2.3 g of lipid and 1.8 g of water compared with that in the LL group (Table 6-7). As the F4408 group remarkably decreased hepatic lipid, cholesterol, TG and water levels, the liver weight was decreased (Table 6-4). These results indicated that high aglycone ratio of isoflavones in fermented soymilk was suitable for prevention of fatty liver.

Table 6-6. Liver Parameters of Rats Fed on the Experimental Diets for 5 Weeks

	LL	F4404	F4408
Crude lipid (g/liver)	4.6 ± 0.3 [a]	3.0 ± 0.3 [b]	2.3 ± 0.3 [b]
Hepatic lipid (mg/g)	248.3 ± 11.7 [a]	180.5 ± 14.4 [b]	157.5 ± 22.2 [b]
Cholesterol (mg/liver)	187.7 ± 11.5 [a]	82.0 ± 14.2 [b]	58.2 ± 10.9 [b]
Cholesterol (mg/g)	10.1 ± 0.6 [a]	4.8 ± 0.7 [b]	3.9 ± 0.7 [b]
Triglyceride (mg/liver)	1786.2 ± 174.4 [a]	804.8 ± 171.9 [b]	606.6 ± 116.6 [b]
Triglyceride (mg/g)	95.8 ± 8.6 [a]	47.2 ± 8.6 [b]	40.3 ± 6.7 [b]

Each value is the mean ± SE for 6 rats. [a,b] Means not sharing a common superscript differ significantly by Tukey's multiple-comparison test ($p < 0.05$).

Table 6-7. Hepatic Sugar and Water of Rats Fed on the Experimental Diets for 5 Weeks

	LL	F4404	F4408
Water (g/liver)	11.4 ± 0.5 [a]	10.7 ± 0.4 [ab]	9.6 ± 0.5 [b]
Water (mg/g)	0.61 ± 0.01 [a]	0.64 ± 0.01 [ab]	0.65 ± 0.01 [b]
Sugar (g/liver)	1.4 ± 0.1 [a]	1.4 ± 0.1 [a]	1.2 ± 0.1 [a]
Sugar (mg/g)	77.4 ± 2.1 [a]	82.9 ± 0.1 [a]	77.1 ± 3.6 [a]

Each value is the mean ± SE for 6 rats. [a,b] Means not sharing a common superscript differ significantly by Tukey's multiple-comparison test ($p < 0.05$).

Hepatic isoflavone level Hepatic isoflavone concentration was shown in Table 6-8. No significant difference in hepatic isoflavone concentration was shown between the F4404 and F4408 groups. However, genistein concentration of the F4408 group was1.4-folds compared with the F4404 group. Additionally, equol was found in the F4404 and F4408 groups. Daizein is converted to equol by enterobacteria [30)74)75)]. These results indicated that hepatic isoflavone level in the F4408 group increased *vs.* the F4404 group, and then the intestinal absorption of isoflavone might be promoted by increased aglycone ratio of isoflavone [31)].

Table 6-8. Hepatic Levels of Isoflavones in Rats Fed on Experimental Diets for 5 Weeks

(nmol/g)	F4404	F4408
Genistein	2.2 ± 0.9	3.1 ± 1.0
Daidzein	3.3 ± 0.7	3.8 ± 0.7
Equol	4.5 ± 1.2	5.7 ± 0.7
Total Isoflavones	10.0 ± 1.3	12.6 ± 1.9

Real-time PCR analysis The lipid metabolism modulating gene expression in liver was shown in Fig 6-1. CYP7a1, the rate limiting enzyme in the formation of bile acid from cholesterol [42)], was tended to increase in the F4408 group compared with the LL group. The gene expressions of SREBP-2, cholesterol synthesis-accelerating factor [69)] was inclined to be increased in the F4408 group *vs.* the LL group. Decreasing of intracellular cholesterol increased SREBP-2 gene expression [76)]. Additionally, isoflavone promoted SREBP-2 gene expression [77)]. As LL group accumulated cholesterol, the gene expression of SREBP-2 was suppressed. On the other hand, the F4408 group fed a diet containing high isoflavone aglycone ratio (93%) promoted

cholesterol catabolism in liver, and the gene expression of SREBP-2 in the F4408 group was increased *vs*. the LL group.

The expression of fatty acid synthesis-related gene, SREBP-1, was decreased in the F4408 group *vs.* the LL group. The gene expression of FAS controlled by SREBP-1 tended to decreased in the F4404 and F4408 groups *vs.* the LL group ($p<0.1$). Thus, it was suggested that the F4408 group decreased the gene expressions of SREBP-1 and FAS, and therefore, the hepatic TG level in the F4408 group was dramatically decreased. As the F4408 group showed significant change of hepatic gene expression

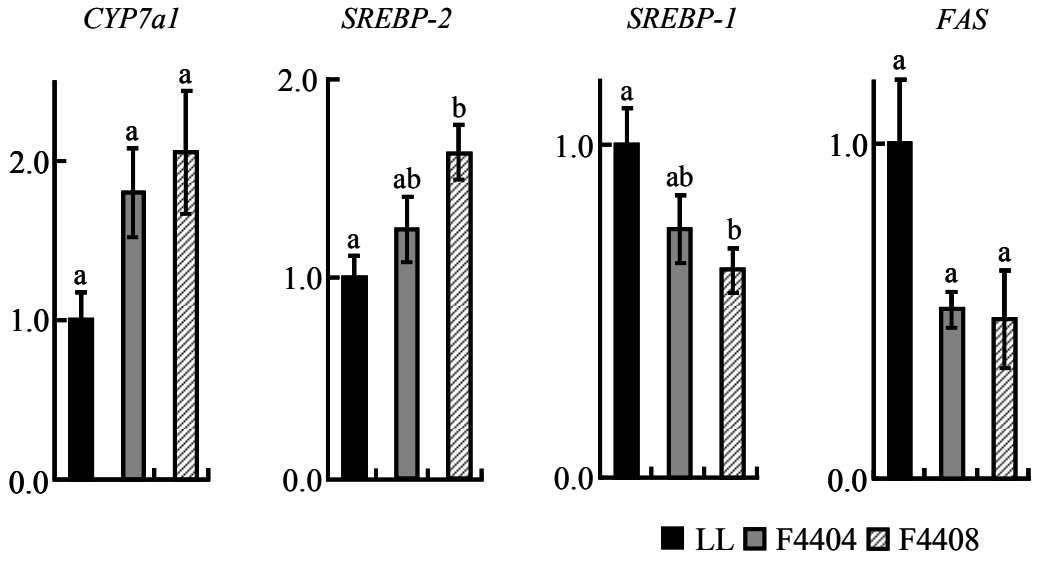

Fig. 6-1. Real-time PCR data for fatty acid synthesis and catabolism-related genes in the liver of rats fed on the experimental diets for 5 weeks. The data were normalized to GAPDH RNA expression and are presented as a ratio to the LL value. Each value is the mean±SE for 6 rats. [a,b] Means not sharing a common superscript differed significantly by Tukey's multiple-comparison test ($p<0.05$).

compared with the LL group, lipid metabolism-modulating effect of fermented soymilk was stronger by TUA4408 than by TUA4404. Aglycone of isoflavone promoted lipid metabolism-modulating effect [59) 68)78)].

The lipid metabolism modulating effect of soy foods depends on mainly soy protein. Isoflavones converted from glycone to aglycone by lactic acid-fermentation seem to promote the intestinal absorption and increase hepatic concentration of isoflavone. Therefore, it was suggested that fermented soymilk containing high ratio of isoflavone aglycone promoted lipid metabolism-modulating effects. Fermented soymilk containing high ratio of isoflavone aglycone might prevent fatty liver.

Conclusion

The ingestion of fermented soymilk containing 10% soy protein induced the up-regulation of hepatic CYP7al and then reduced the plasma cholesterol level in rats compared with that of soymilk. Furthermore, it was suggested that isoflavone up-regulated cholesterol catabolism and cholesterol synthesis-related gene expression, whereas soy protein reduced the expression of fatty acid synthesis-related gene. These results indicated that isoflavones and soy proteins have different roles on lipid metabolism from each other and isoflavone aglycone enhances lipid metabolism-modulation by coexistence with soy protein.

Recently, hyperlipidemia, severe problem, is known to be induced by cholesterol-rich diet. Thus, when hypocholesterolemic effect of fermented soymilk on rats fed a high cholesterol diet was investigated, the fermented soymilk induced the down- regulation of SREBP-2 and up-regulation of CYP7a1 to modulate the cholesterol metabolism in rats fed a high cholesterol diet. The different isoflavone aglycone ratios in lactic acid-fermented soymilk differently affected the hepatic lipid metabolism in rats fed a high cholesterol diet and lipid loading test. When fermented soymilks containing the different aglycone ratio of isoflavones were ingested by rats , it was found that the lipid metabolism-modulation function was stronger in fermented soymilk including higher aglycone ratio of isoflavone than in that including lower aglycone ratio of isoflavone. Therefore, it was suggested that the aglycone ratio of isoflavone in fermented soymilk affected lipid metabolism. Isoflavone converted from glycone to aglycone by lactic acid-fermentation seems to promote the intestinal absorption and increase hepatic concentration of isoflavone. Thus, fermented soymilk containing high

ratio of isoflavone aglycone might promote lipid metabolism-modulating effects.

Fermented soymilk exhibited lipid metabolism-modulating effect in all rats fed an AIN-93G diet, high cholesterol diet and lipid loading test. Moreover, aglyconized isoflavone coexsisted with soy protein markedly reduced hepatic lipid. Higher ratio of isoflavone aglycone in fermented soymilk had stronger lipid metabolism-modulating effect *vs.* lower ratio of isoflavone aglycone in fermented soymilk, Therefore, fermented soymilk containing both of aglyconaized isoflavone and soy protein seems to enhance lipid metabolism-modulation. Fermented soymilk may have primary prevention effect of hyperlipidemia induced by excessive eating and underexercising.

It was concluded that fermented soymilk rich in soy protein and isoflavone aglycone is a suitable food for hepatic lipid metabolism-modulation. The intake of fermented soymilk might contribute to prevent metabolic syndrome such as fatty liver.

Summary

Excessive eating and underexercising has been caused hyperlipidemia in recent years. Soy food, especially soymilk, has been expected to prevent hyperlipidemia. In the thesis, The author focused on new physiological effects of soymilk and lactic acid-fermented soymilk on lipid metabolism-modulation in liver. The effects of lactic acid-fermented soymilk containing okara on the plasma and hepatic lipid profiles, and expression of the lipid metabolism-related genes in rats were previously reported [32]. Additionally, the dose of soymilk for improving lipid metabolism was also reported [33]. However, the dose of fermented soymilk for improving lipid metabolism has not been solved. Therefore, the dose of fermented soymilk for improving lipid metabolism was found in the chapter 1. The administration of amount of fermented soymilk corresponding to more than 10% soy protein in diet was necessary to keep a significant physiological effect during about 1 month.

In the chapter 2, the different effects between soymilk and fermented soymilk on lipid metabolism and lipid metabolism-related gene expression were clarified through the regulation of hepatic gene expression induced by the ingestion of lactic acid-fermented soymilk. Soymilk and fermented soymilk down-regulated the expression of hepatic fatty acid synthesis-related gene to reduce the hepatic TG level in rats. Hepatic cholesterol level of the soymilk and fermented soymilk groups were also decreased *vs.* that of C group. Especially, the ingestion of fermented soymilk induced the up-regulation of hepatic CYP7al and then reduced the plasma cholesterol level in rats compared with that of soymilk. Therefore, the bioactive components produced by lactic acid fermentation, isoflavone and others, seemed to induce the up-regulation of

hepatic CYP7a1 to reduce the plasma cholesterol level in rats.

Although the lipid metabolism-modulating effect of soy protein has mostly been clarified, that of isoflavone is still less obvious. Therefore, the author noted isoflavone as bioactive component on lipid metabolism-modulating effect. In the chapter 3, the relationships between the ratio of isoflavone aglycone in total isoflavone and with or without soy protein in fermented soymilk were investigated to clarify lipid metabolism-modulation effects in rats fed a cholesterol-free diet. Thus, the aglyconized isoflavone coexsisted with soy protein markedly reduced hepatic lipid level. Therefore, it was assumed that both of aglyconaized isoflavone and soy protein enhanced lipid metabolism-modulation in rats ingested fermented soymilk. Moreover, it was suggested that isoflavone not only the up-regulated of CYP7a1 expression but also increased cholesterol synthesis–related gene expression. On the other hand, soy protein reduced the expression of fatty acid synthesis-related gene. These results indicated that isoflavones and soy proteins have different roles on lipid metabolism from each other and isoflavone aglycone enhances lipid metabolism-modulation by coexistence with soy protein.

Recently, hyperlipidemia is known to be induced by cholesterol-rich diet is a severe problem. Thus, in the chapter 4, hypocholesterolemic effect of fermented soymilk on rats fed a high cholesterol diet was investigated. It was assumed that the fermented soymilk induced the down-regulation of SREBP-2 and up-regulation of CYP7a1 to modulate the cholesterol metabolism in rats fed a high cholesterol diet.

In the chapter 5 and 6, The author investigated the effect of aglycone ratio of isoflavone in fermented soymilk on lipid metabolism. The different isoflavone aglycone ratios in lactic acid-fermented soymilk differently affected the hepatic lipid metabolism

in rats fed a high cholesterol diet and lipid loading test. It was found that the modulation function was stronger in fermented soymilk including high aglycone ratio of isoflavone than in that including low aglycone ratio of isoflavone. Isoflavone converted from glycone to aglycone by lactic acid-fermentation seems to promote the intestinal absorption and increase hepatic concentration of isoflavone. Therefore, it was suggested that fermented soymilk containing high ratio of isoflavone aglycone promoted lipid metabolism-modulating effects.

In the present studies, fermented soymilk exhibited lipid metabolism-modulating effect in all rats fed an AIN-93G diet, high cholesterol diet and lipid loading test. Additionally, aglyconized isoflavone coexsisted with soy protein markedly reduced hepatic lipid. Therefore, it is suggested that fermented soymilk containing both of aglyconaized isoflavone and soy protein enhanced lipid metabolism-modulation, because higher ratio of isoflavone aglycone in fermented soymilk had stronger lipid metabolism-modulating effect *vs.* lower ratio of isoflavone aglycone in fermented soymilk, Thus, it is assumed that fermented soymilk has primary prevention effect of hyperlipidemia induced by excessive eating and underexercising.

Hence, it is concluded that fermented soymilk containing abundant soy protein and isoflavone aglycone is suitable for hepatic lipid metabolism-modulating food. The intake of fermented soymilk might contribute to prevent metabolic syndrome such as fatty liver.

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Presented paper list

1. Kobayashi, M., Harada, T., Takagi, N., Tsuzuki, K., Sugawara, M. and Fukuda, M., Effects of lactic acid-fermented soymilk on lipid metabolism-related gene expression in rat liver. *Biosci. Biotechnol. Biochem*., **76**, 19–24 (2012).
   (Described in Chapter 2)

2. Kobayashi, M., Hirahata, R., Egusa, S. and Fukuda, M., Hypocholesterolemic Effects of Lactic Acid-Fermented Soymilk on Rats Fed a High Cholesterol Diet. *Nutrients.,* **4**, 1304–1316 (2012).
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3. Kobayashi, M., Sakakibara, R., Egusa,S. and Fukuda, M., Effects of Isoflavone Aglycone Ratio in Lactic Acid- Fermented Soymilk on Hepatic Lipid Metabolism in Rats Fed a High Fat and High Cholesterol Diet (in Japanese). *Nippon Shokuhin Kagaku Kogaku Kaishi*., **2013***, 60,* 509-515.
   (Described in Chapter 6)

4. Harada, T., Tanaka, M., Tsuzuki, K., Sugawara, M., Takagi, N. and Fukuda, M. Effective Dosage of Lactic Fermented Soymilk for Improving Lipid Metabolism in Rats (in Japanese). *Nippon Shokuhin Kagaku Kogaku Kaishi*., **2010**, *57*, 175–179.
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