

## *Lactobacillus paracasei* NFRI 7415 Reduces Liver Lipid Contents in C57BL/6J Mice Fed a High-fat Diet

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### Abstract

We examined the effects of *Lactobacillus paracasei* NFRI 7415 isolated from Japanese fermented fish (funa-sushi) on the whole-body and fat-tissue weights, plasma lipid concentration and hepatic lipid contents of mice. Male C57BL/6J mice were fed a control diet (CD), high-fat diet (HD) or HD containing *Lb. paracasei* NFRI 7415 ( $10^6$  cfu/g) (HLD) for 10 weeks. The plasma triacylglycerol concentration of the HLD group was lower than that of the HD group. The plasma glucose, leptin and hepatic lipid concentration of the CD and HLD groups were lower than those of the HD group. As a result of measuring the liver lipids and the liver histology, it was observed that the number of lipid droplets in liver cells in the HD group was clearly greater than that in the CD and HLD groups. The total liver lipids in the CD and HLD group were lower than that of the HD group. This suggests that intake of *Lb. paracasei* NFRI 7415 is effective in overcoming the effects of HD to prevent hepatic lipid accumulation.

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*Lactobacillus paracasei*, high-fat diet, liver lipid, mice, probiotics

### Introduction

In recent years, the increase in obesity and diabetes with the westernization of eating habits in Japan has become an important factor in the development of arteriosclerosis and cerebrovascular diseases [1,2]. According to the national health and nutrition survey of 2013 [3], 28.6% of men and 20.3% of women over 20 years old were obese (BMI  $\geq 25$ ). Obesity is one of the major risk factors of coronary heart disease, arteriosclerosis, fatty liver, diabetes B, hypertension and various other diseases [4]. Improving diet is effective for the prevention of lifestyle-related diseases. Functional foods and supplements have received a good deal of attention in this context [5].

Lactic acid bacteria (LAB) have been utilized as a natural health food from ancient times, and the health-promoting effects of LAB are well recognized [6]. Some *Lactobacillus* strains are used in food fermentation, and typical examples are found in the dairy industry for the production of cheese, yogurt, and other fermented milk products [2,7]. Recent studies have indicated that several *Lactobacillus* strains are effective for the prevention of obesity as probiotics. For example, *Lactobacillus plantarum* and *Lactobacillus gasseri* may exert a beneficial effect on the onset of diet-induced obesity by reducing the cell size of white adipose tissues [8,9]. Andersson et al. [10] indicate that *Lactobacillus plantarum* DSM 15313 has anti-diabetic properties in the context of a high-fat diet.

We reported that *Lactobacillus paracasei* NFRI 7415 isolated from traditional Japanese fermented fish (funa-sushi) showed high  $\gamma$ -aminobutyric acid (GABA)-producing ability [11]. GABA has several well-known physiological functions, including neurotransmission, induction of hypotensivity, a diuretic effect, and tranquilizer effects [12,13]. Although we found that *Lb. paracasei* NFRI 7415 is used in the development of functional fermented foods, this phenomenon has not yet been studied in vivo in humans and animals. Our previous study suggests that *Lb. paracasei* NFRI 7415 is beneficial for improving liver damage due to chronic alcohol intake, [14] but to our knowledge no effect of *Lb. paracasei* in the prevention of obesity has been reported.

We speculated that *Lb. paracasei* NFRI 7415 may have improved liver function in the above-mentioned clinical study by somehow reducing hepatic lipid contents. In the present study, we focused on the effect of LAB consumption on obesity, assuming that it would change the lipid metabolism. We investigated body and fat tissue weights, the plasma lipid concentration and hepatic lipid contents in a high-fat diet (HD)-fed C57BL/6J mice.

### Materials and Methods

**Preparation of extract:** A pre-culture of *Lb. paracasei* NFRI 7415 was grown to the stationary phase at 37°C for 20 h in MRS (Difco Laboratories, Detroit, MI) medium. The medium was prepared by mixing a high-fat diet (HD) and sterilized water at a ratio of 1 to 3. The pre-cultures ( $10^6$  cfu/g) were inoculated in the HD at 37°C for 48 h. The medium was immediately freeze-dried and used in the animal experiments.

**Animals and diets:** Eighteen 5-week-old C57BL/6J mice were purchased from Charles River Japan (Yokohama, Japan). All the animals were housed individually in plastic cages in a controlled environment of  $22 \pm 1^\circ\text{C}$  at 50% relative humidity under a 12-h dark/light cycle (19:00-7:00). The animals were randomly divided into three dietary treatment groups with equal mean body weight: the control diet (CD) group (n = 6), the HD group (n = 6), and the freeze-dried medium with *Lb. paracasei* NFRI 7415 (106 cfu/g) and HD blended at a ratio of 1 to 4 (HLD) group (n = 6). The HLD contained approximately 100 mg/kg of live *Lb. paracasei* NFRI 7415, as in other studies. The composition of the diets (CD, HD, and HLD), shown in Table 1 was based on the AIN-93G diet [15]. HD and HLD were

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Table 1: Compositions of the Experimental Diets.

Ingredient	CD <sup>1</sup>	HD <sup>2</sup>	HLD <sup>3</sup>
Casein	20.0	20.0	20.0
L-Cystine	0.3	0.3	0.3
Cornstarch	55.4	24.9	24.9
Sucrose	10.0	10.0	10.0
Soybean oil	4.5	4.5	4.5
Lard	-	30.5	30.5
Cellulose	5.0	5.0	5.0
Mineral mixture <sup>3</sup>	3.5	3.5	3.5
Vitamin mixture <sup>3</sup>	1.0	1.0	1.0
Choline bitartrate	0.25	0.25	0.25
<i>tert</i> -Butylhydroquinone	0.0014	0.0014	0.0014(10 <sup>6</sup> cfu/g)
<i>Lb. paracasei</i> extract	-	-	-
Energy (kcal/g)	3.6	5.2	5.2
Protein energy ratio (%)	22.5	15.3	15.3
Fat energy ratio (%)	11.4	60.4	60.4
Carbohydrate energy ratio (%)	66.1	24.3	24.3

<sup>1</sup> CD: control diet; <sup>2</sup> HD: high-fat diet; <sup>3</sup> HLD: *Lb. paracasei* containing high-fat diet

<sup>4</sup> Mineral mixture (g/kg of mix): CaHPO<sub>4</sub>, 500.0; NaCl, 74.0; K<sub>2</sub>C<sub>20</sub>H<sub>38</sub>O<sub>7</sub>·H<sub>2</sub>O, 220.0; K<sub>2</sub>SO<sub>4</sub>, 52.0; MgO, 24.0; MnSO<sub>4</sub>·5H<sub>2</sub>O, 6.77; FeSO<sub>4</sub>·7H<sub>2</sub>O, 4.95; ZnCO<sub>3</sub>, 1.6; CuCO<sub>3</sub>·Cu(OH)<sub>2</sub>·H<sub>2</sub>O, 0.3; KIO<sub>3</sub>, 0.01; NaSeO<sub>3</sub>, 0.01; CrK(SO<sub>4</sub>)<sub>2</sub>·12H<sub>2</sub>O, 0.55; NaF, 0.06; Sucrose, 115.75.

<sup>5</sup> Vitamin mixture (g/kg of mix): retinol, 4.8; cholecalciferol, 0.4; thiamine, 24.0; riboflavin, 0.6; pantothenic acid, 0.6; pyridoxine, 0.7; cobalamin, 0.01; menadione, 0.05; nicotinic acid, 3.0; D-calcium pantothenic acid, 1.6; folic acid, 0.2; biotin, 0.02; para-aminobenzoic acid, 5.0; inositol, 10.0; glucose, 949.02.

prepared by adding lard (30.5%) to the AIN-93G, respectively, and substituting cornstarch. The mice were fed the CD, HD, or HLD for 10 weeks. Food intake was recorded daily, and body weight was measured on alternate days. After the feeding period, the mice were fasted for 16 h and sacrificed humanely under ether anesthesia to collect the liver and perirenal fat tissue. The blood was collected by heart puncture with a heparinized syringe. The blood was maintained at 4°C and centrifuged at 1,000 g for 15 min. The plasma and liver were stored at -80°C until analysis.

All procedures were performed in accordance with the Animal Experimentation Guidelines of the Laboratory Animal Care Committee of Seitoku University.

**Biochemical assays of plasma and liver:** Liver lipids were extracted by the method of *Folch et al.* [16] *Triacylglycerol* (TG), total cholesterol (T-cho), and glucose concentrations in plasma and liver extracts were measured using test kits (Triglyceride E-Test Wako, Cholesterol E-Test Wako, Glucose CII-Test Wako), purchased from Wako Pure Chemical Industries, (Osaka, Japan). We also used test kits to assess the activities of leptin (Rat Leptin ELISA Kit, Otsuka Tokyo) in the plasma.

**Liver histology:** Livers of mice from the three dietary treatment groups were compared histologically. Under deep anesthesia with ether, the chest of each mouse from the three groups was opened rapidly, and the vasculature was perfused with 100 ml of a fixative [4% paraformaldehyde in 0.01 M sodium phosphate-buffered saline (PBS: pH 7.4)] at a pressure of 120 mmHg from a 18-gauge cannula inserted into the aorta via an incision in the left ventricle. Immediately after fixative perfusion, the liver was removed, cut into small pieces and

immersed in the same fixative for another 2 h at 4°C. Then, the liver pieces were washed with PBS, dehydrated in an ascending series of ethanol aqueous solutions (50%, 70%, 80%, 90%, and 100%), cleared in xylene, and embedded in paraffin wax. Three μm-thick sections made from paraffin-embedded livers were then subjected to hematoxylin and eosin (H&E) staining by a routine procedure (Meyer's hematoxylin staining followed by eosin Y staining), and examined under a Keyence BZ900 all-in-one microscope (Keyence, Osaka, Japan).

**Statistical analysis:** Values are expressed as means ± SDs. Repeated-measures analysis of variance (ANOVA) was used to evaluate the effects of groups. Differences in mean values between groups were tested by Fisher's multiple-range test. p-values less than 0.05 were considered statistically significant.

## Results

Food intake, body and liver weights. The food intake of the HD and HLD groups were much lower than that in the CD group ( $p < 0.05$ ) (Table 2). However, there was no significant difference in the food intake between the HD and HLD groups. No significant differences in food energy intake, final body and liver weight were observed among the three groups. The perirenal fat tissue weight of the HD and HLD groups were higher than that in CD group ( $p < 0.05$ ).

Table 2: Food Intake, Total Energy Intake, Body Weight, Liver and Perirenal Fat Tissue Weights.

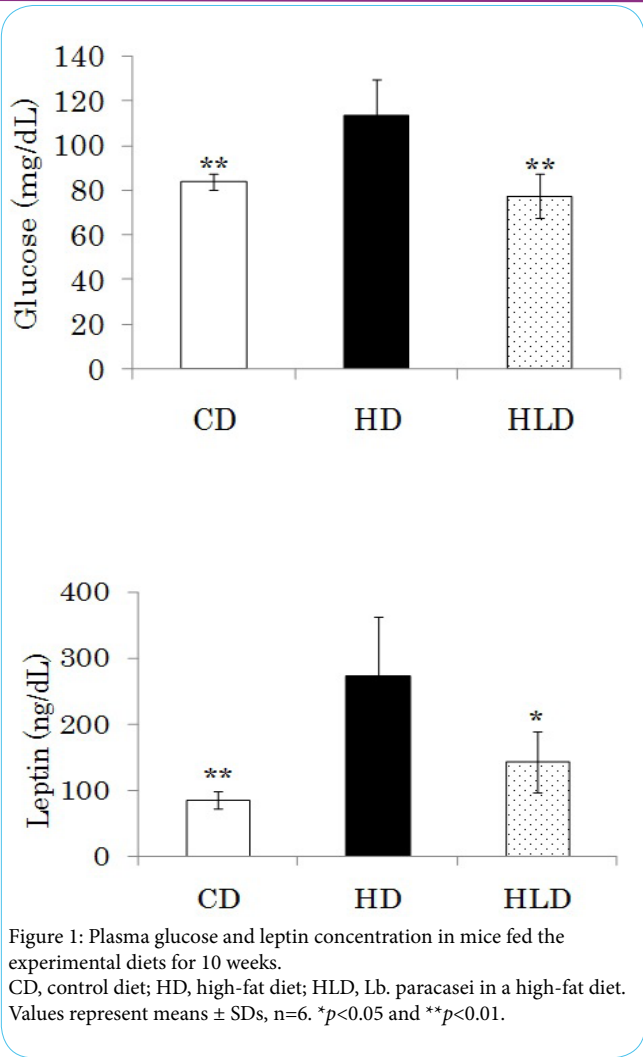
Group	CD <sup>1</sup>	HD <sup>2</sup>	HLD <sup>3</sup>
Food intake (g)	242 ± 19.6 <sup>a</sup>	178 ± 13.5 <sup>b</sup>	193 ± 36.8 <sup>b</sup>
Total energy intake (Kcal)	871 ± 70.6	926 ± 70.2	1006 ± 191
Total energy intake (Kcal/day)	12.4 ± 1.01	13.2 ± 1.00	14.4 ± 2.74
Final body weight (g)	31.2 ± 2.24	32.2 ± 3.39	32.2 ± 1.95
Liver weight (g/100g B.W.)	4.32 ± 0.28	4.16 ± 1.18	3.93 ± 0.35
Perirenal fat tissue weight (g/100g B.W.)	1.06 ± 0.20 <sup>b</sup>	1.69 ± 0.31 <sup>a</sup>	1.58 ± 0.17 <sup>a</sup>
Plasma lipids			
Triacylglycerol (mg/dL)	105 ± 46.6 <sup>b</sup>	246 ± 107 <sup>a</sup>	143 ± 46.5 <sup>b</sup>
T-Cholesterol (mg/dL)	75.1 ± 4.85	80.9 ± 9.32	82.3 ± 5.34

<sup>1</sup>CD: control diet; <sup>2</sup>HD: high-fat diet; <sup>3</sup>HLD: *Lb. paracasei* in a high-fat diet

Values represent means ± SD, n = 5. Within a row, values not sharing a common superscript letter are significantly different at  $p < 0.05$ . T-Cholesterol, total cholesterol.

Plasma lipid profiles. During the dietary treatment period, no significant differences in plasma T-cholesterol concentration were observed among the three groups (Table 2). The plasma TG of the CD and HLD group were lower than that of the HD group ( $p < 0.05$ ). The plasma glucose and leptin levels of the CD and HLD groups were lower than those of the HD group ( $p < 0.05$ ) (Figure 1).

Liver histology and liver lipids. Panels stained with H&E are shown at 600× in Figure 2. The micrograph of the liver tissue sections shows that many massive lipid droplets were present in the HD group hepatic cells, with fewer and smaller lipid droplets in the HLD group.



The total lipids of the CD, HD and HLD groups were  $85.7 \pm 13.2$  mg/g, and  $129 \pm 23.4$  mg/g,  $100 \pm 8.35$  mg/g liver weight, respectively (Figure 3). Total liver lipids was markedly higher in the HD group than in the CD group ( $p < 0.01$ ), and total liver lipids of the HLD group was significantly lower than that of the HD group ( $p < 0.05$ ). The liver TG and T-cho concentrations of the CD and HLD groups were lower than those of the HD group ( $p < 0.05$ ) (Figure 3).

### Discussion

In the current study, we investigated whether *Lb. paracasei* NFRI7415 influences body and fat tissue weight, plasma lipid concentration and hepatic lipid contents in HD-fed C57BL/6J mice. No significant differences in food energy intake, final body and liver weights were observed among the three groups (Table 2). However, the perirenal fat tissue weights of the HD and HLD were higher than that in CD group ( $p < 0.05$ ). The HD and HLD in this study both contained 30.5% lard and 4.5% soybean oil (Table 1). Many studies have demonstrated that intake of a high-fat diet tends to lead to a higher body mass index and body fat content than intake of a low-fat diet [17-19]. Excessive intake of animal fat causes increased adipose tissue deposition because perirenal fat-tissue weights are affected by the amount of dietary fat [18]. The present results (Table 2) were consistent with those studies.

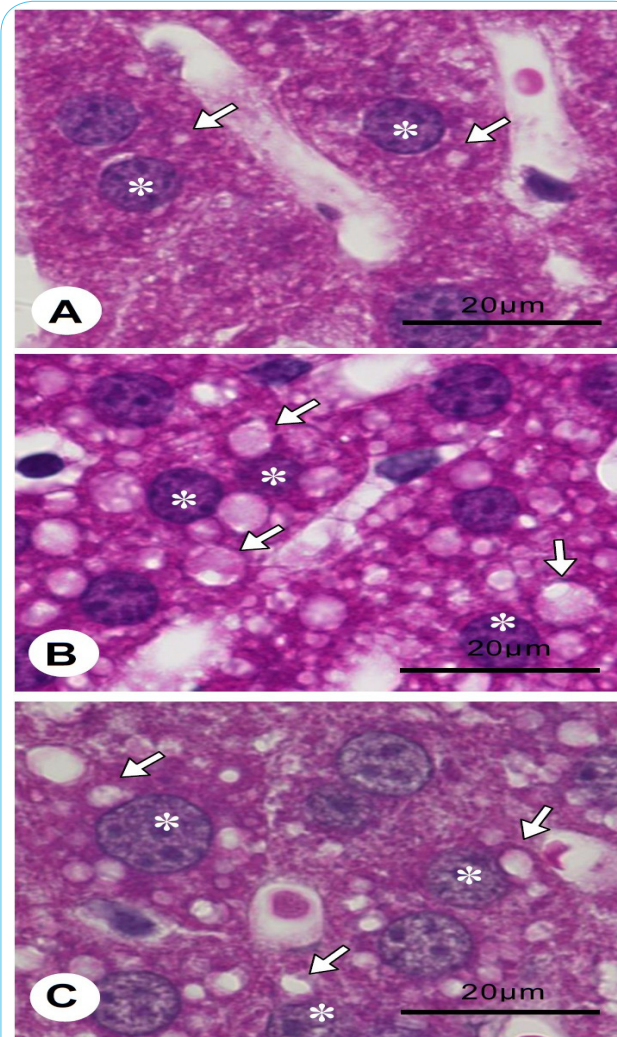
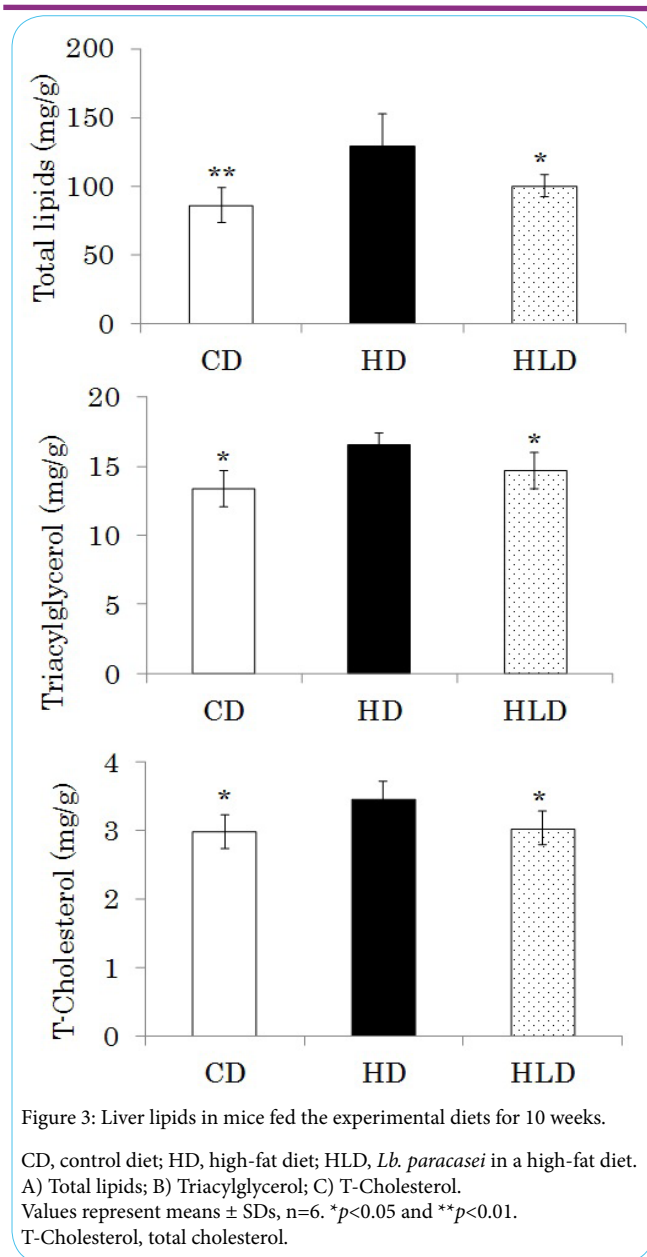


Figure 2: Comparison of the size and number liver lipid droplets (A)-(C) H&E staining of liver sections. (A) Liver section from a normal mouse fed with control diet (CD). Lipid droplets (arrows) in hepatocytes (asterisk) are typically small in size (about 2  $\mu$ m in diameter), and a couple of lipid droplets (2-3 pieces) are contained in a cell. SI: Sinusoid. (B) Liver section from a mouse fed with high fat-diet (HD). Lipid droplets (arrows) in hepatocytes (asterisk) are larger than that of normal liver (5-6  $\mu$ m in diameter). Further, an increased number of droplets in cells (usually 5-7 per cell) is observed. SI: Sinusoid. (C) Liver section from a mouse fed with HD containing *Lb. paracasei*. The size of lipid droplets (arrows) in hepatocytes (asterisk) is smaller than that of animals fed HD (about 3  $\mu$ m in diameter). The number of lipid droplets in each hepatocyte is also reduced (usually 2-3 per cell) in comparison to that of animals fed with HD.

Andersson et al. [10] reported the protective effects of a strain of *Lactobacillus plantarum* on obesity and early diabetes in, to high-fat diet (HFD) fed C57BL/6J mice fed a high-fat diet for 20 weeks. Despite *L. plantarum* decreasing the plasma glucose level in a high-fat diet fed mice, the energy intake, body fat content and plasma cholesterol were not different compared to the control group. In other studies, *Enterococcus*, *Lactobacillus* and *Bifidobacterium* were also not observed lower plasma cholesterol in C57BL/6J mice fed a high-fat diet [2,20]. In this study, there was no significant difference in the energy intake and plasma T-cho concentration between the HD and HLD groups (Table 2). Therefore, it was supposed that *Lb. paracasei* NFRI 7415 did not have an influence on plasma lipids in mice fed





a high-fat diet. Several studies have indicated that LAB effectively reduced the cholesterol concentration of plasma in animals fed a high-fat diet because LAB enhanced dietary cholesterol excretion into feces by direct cholesterol assimilation [21,22]. Further studies are needed to investigate whether *Lb. paracasei* NFRI 7415 can reduce the plasma T-cho concentration in hypercholesterolemia-induced mice.

Leptin is a cytokine hormone secreted by adipocytes. It affects food intake and energy consumption through its receptors in the hypothalamus. Leptin is produced by adipose tissues, and the plasma leptin concentration is linearly related to fat mass and BMI [23-25]. The plasma leptin levels of the CD and HLD group were much lower than that in the HD group (Figure 1). Although no significant differences in the perirenal fat-tissue weight were observed between the HD and HLD groups, the plasma TG of the HLD group was decreased (Table 1). It appears that *Lb. paracasei* NFRI 7415 was involved in an obesity-preventing action in mice fed the HLD in this study.

To investigate the effects of *Lb. paracasei* NFRI 7415 on the liver in C57BL/6J mice fed the HLD, we measured the liver lipids and the liver histology. Figure 2 shows that the number of liver cells in the HD group was clearly greater than those in the CD and HLD groups. At the same time, the total liver lipids in the CD and HLD group were lower than that of the HD group (Figure 3) ( $p$ <0.05). This suggests that intake of *Lb. paracasei* NFRI 7415 is effective at preventing hepatic lipid accumulation in a HD setting. The nuclei in the hepatic cells of the HD group appear to be blackened and moribund (Figure 2). Heterochromatin is the chromatin structure in the domain, and its gene expression is inhibited [26]. In this study, it is supposed that the chromatin in the liver cells of the HD group is in an inactive state. A previous study found that adipose-tissue, expression of the energy consumption gene (lysine-specific demethylase-1) in mice fed a high-fat diet was reduced compared with that of mice on a normal diet. [27]. In the future, we need to investigate the gene expression control mechanisms of the liver by excess intake of fat to determine whether intake of *Lb. paracasei* NFRI 7415 is involved in the regulation of the lipid metabolism.

Previous studies have reported that *Lb. paracasei* NFRI 7415 can remove cholesterol from the plasma and liver of rats fed an ethanol-containing diet [14]. The present study clearly showed that the *Lb. paracasei* NFRI 7415 administration reduced hepatic T-cho concentration. The cholesterol-reducing effect by LAB is known to occur through high cholesterol adsorption on the cell body, and increased bile acid adsorption ability [28,29]. Hepatic cholesterol contents have been shown to be significantly lowered by LABs such as *Lactobacillus gasseri* and *Pediococcus pentosaceus* [2,30]. In another study on rodents fed a high-fat diet, the hepatic cholesterol content was significantly lowered by *P. pentosaceus*, and lipid metabolism-related genes such as cluster of differentiation 36 (CD36) and stearoyl-CoA desaturase 1 (SCD1) showed decreased expression [2]. It is necessary to investigate the effect of this strain on mRNA expression in the liver in mice fed HD. We predict that *Lb. paracasei* NFRI 7415 will be shown to have cholesterol adsorption capability ability in the intestinal tract. Further work is in progress to elucidate the mechanism of cholesterol-reducing function of *Lb. paracasei* NFRI 7415; specifically, the fecal cholesterol excretion levels of mice treated with this LAB is under investigation.

In conclusion, the present investigation shows that *Lb. paracasei* NFRI 7415 reduces the content of liver lipids in C57BL/6J mice fed a high-fat diet. Our data suggest that this strain may be effectively applied as a probiotics *lactobacillus*.

### Conflicts of Interests

The authors have no conflicts of interest to reports.

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