

# Effects of live *Lactobacillus paracasei* NFRI 7415 on the intestinal immune system and intestinal microflora of mice

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**Abstract.** This study examined the effect of *Lactobacillus paracasei* NFRI 7415 on the intestinal immune system and intestinal microflora of mice. Two studies were performed. In study 1, ten 5-week-old female BALB/c mice were assigned to a control (CO) group or lactic acid bacteria (LB) group for 4 weeks. The LB group freely ingested water containing *L. paracasei* NFRI 7415 ( $10^6$  cfu/ml). After the feeding period, the intestinal bacterial flora in the feces were investigated. The total counts of anaerobic bacteria in the feces were higher in the CO group than in the LB group, although the total counts of aerobic bacteria were not significantly different between the two groups. No harmful bacteria such as *Enterococci* and *Bacteroides* spp. were detected in the feces of the LB group. A Basic Local Alignment Search Tool (BLAST) search revealed that one of the anaerobic bacteria in the feces of LB group showed 100% homology to *L. paracasei*. In study 2, the effects of *L. paracasei* NFRI 7415 on the gut immune system of BALB/c mice were evaluated *in vitro*. As a result, production of the cytokine IL-12 in Peyers' patch (PP) and mesenteric lymph nodes (MLN) cells, which are gut-associated lymphoid tissues (GALT), was increased by treatment with *L. paracasei* NFRI 7415 in a concentration-dependent manner. These results indicate that oral administration of live *L. paracasei* NFRI 7415 may have the potential to improve intestinal conditions and immune function in humans.

**Keywords:** Lactobacillus, intestinal immune system, intestinal microflora, IL-12.

## INTRODUCTION

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 (Elmadfa *et al.*, 2010; Enomoto *et al.*, 2006). Some *Lactobacillus* strains are used in food fermentation, typically in the dairy industry for the production of cheese, yogurt, and other fermented milk products (Zhao *et al.*, 2012; Kim *et al.*, 2008).

It has been reported that *L. rhamnosus* and *L. acidophilus* have immunostimulation effects and can help prevent respiratory and intestinal-tract infection (Goyal *et al.*, 2013; Perdigon *et al.*, 2002). The cytokine IL-12,

produced by immune cells, works to activate important cell-mediated immunity factors in the early period of infection (Carasi *et al.*, 2015). The production of immunoglobulin A (IgA), which has important roles in the immunization of the intestinal tract, is also expected to have an infection-controlling effect (Kikuchi *et al.*, 2014). Therefore, it is speculated that LAB, which strongly stimulate IL-12 and IgA production in immune cells, would have a defensive action against viral infection.

In a previous work, we reported that *Lactobacillus paracasei* NFRI 7415 isolated from a traditional Japanese fermented fish (funa-sushi) showed high  $\gamma$ -aminobutyric

acid (GABA)-producing ability (Komatsuzaki *et al.*, 2005). The live *L. paracasei* NFRI 7415 is beneficial for improving liver damage due to chronic alcohol intake (Komatsuzaki and Shima, 2012). We showed that administration of this strain reduced hepatic cholesterol concentration in mice fed a high-fat diet (Komatsuzaki *et al.*, 2016). Our data suggest that this strain may be effectively applied as a probiotic Lactobacillus. It is speculated that plasma and liver cholesterol in mice are reduced due to the high fecal cholesterol excretion levels of mice treated with this strain. It is believed, but has not yet been proven, that this strain survives the harsh stomach environment to reach the intestines of mice. In this experiment, to explore the potential probiotic effects of live *L. paracasei* NFRI 7415, we examined the intestinal bacterial flora in the feces of mice after its administration.

Moreover, we focused on the effect of this strain on the intestinal immune system and intestinal microflora of mice. Peyer's patch (PP) cells and mesenteric lymph nodes (MLN) cells from BALB/c mice were isolated from the small intestine, and IL-12 production was investigated.

## MATERIALS AND METHODS

### Preparation of extract

A pre-culture of *L. paracasei* NFRI 7415 was grown to the stationary phase at 37°C for 20 h in de Man, Rogosa, Sharpe (MRS) (Difco Laboratories, Detroit, MI) medium. The medium was separated from cells by centrifugation (5000 rpm for 10 min at 4°C). The cells were washed with phosphate buffered saline (PBS at pH 7.0) containing 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na<sub>2</sub>HPO<sub>4</sub> and 0.24 g of KH<sub>2</sub>PO<sub>4</sub> (per liter), and then the cells were diluted (10<sup>6</sup> cfu/g) with sterilized water. The cell suspension was used in animal experiments.

### Study 1

#### Animals and diets

Fourteen 5-week-old female BALB/c mice were commercially obtained from Charles River Japan Inc. (Kanagawa, Japan). They were housed individually in plastic cages with paper chip bedding (ALPHA-DriTM, Shepherd Specialty Papers, Inc., Michigan). They were maintained in a room kept at a constant temperature (22 ± 1°C) and 50% relative humidity with a 12-h dark/light cycle (19:00 to 7:00). They were fed a pellet diet (AIN-93G, Oriental Yeast CO., LTD, Tokyo, Japan) with free access to water.

The studies were performed in accordance with the Animal Experimentation Guidelines of the Laboratory

Animal Care Committee of Seitoku University.

### Experimental design

Twelve 5-week-old female BALB/c mice were divided into two equal groups, the control (CO) group and the lactic acid bacteria (LB) group. The latter mice were fed an AIN-93G diet (Reeves *et al.*, 1993) for 4 weeks. Specifically, the mice in the LB group freely ingested water containing *L. paracasei* NFRI 7415 (10<sup>6</sup> cfu/ml). Food intake was recorded every other day, and body weight was measured on alternate days. At the end of the experiment, one-day total feces were collected, and the bacterial flora in the feces was investigated. After the feeding period, the mice were sacrificed humanely under ether anesthesia to collect the liver and perirenal fat tissue. The blood was collected by the portal vein with a heparinized syringe. Whole blood was centrifuged for 15 min at 1400 × g, and plasma was aliquoted into microcentrifuge tubes. Plasma and liver was stored at -80°C until they were analyzed.

### Biochemical assays of plasma and liver

Liver lipids were extracted by the method of Folch *et al.* (1996), plasma triacylglycerol (TG) and total cholesterol (T-cho) concentrations were measured, and liver extracts were likewise analyzed using test kits (Triglyceride E-test Wako, Cholesterol E-test Wako, purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan).

The IgA and IgE levels of plasma in mice were measured using test kits. (IgA: Mouse IgA ELISA Kit, ICL, Inc., GA, USA; IgE: IgE EIA KIT, Yamasa, Ltd., Tokyo, Japan)

### Bacteriological analysis

The methods used for isolating and counting bacteria have been described in detail by Crowther *et al.* (1973) and Iwata *et al.* (1986). Namely, 0.1 g fresh feces was suspended into 1 ml of PBS for 5 min, and the suspension was subjected to centrifugation at 5,000 rpm for 3 min and the supernatant was diluted with physiological salt solution by 10 to 10<sup>8</sup> times. Aerobic and anaerobic bacteria in feces of mice were cultured on agar medium as shown in Table 1. Clostridium medium (14 g peptone, 1.75 g Na<sub>2</sub>HPO<sub>4</sub>, 0.35 g K<sub>2</sub>HPO<sub>4</sub>, 0.04 g Na<sub>2</sub>SO<sub>4</sub>, 0.7 g NaCl, 2.1 g Fructose, 5.25 g Agar) was prepared with 350 ml of distilled water. All media were subjected to autoclave sterilization. After 48 h, the colonies on each plate were counted.

Some colonies on the MRS plate of LB group were sent to BEX CO., LTD (Tokyo, Japan) for identification via PCR using TaKaRa PCR Thermal Cycler Dice® standard

**Table 1.** Bacteriological medium

Aerobes	
General viable bacteria	Nissui Pharmaceutical CO., LTD, Tokyo
Stahylococcus Medium 110	Difco Laboratories, Detroit, MI
m Enterococcus Agar	Difco Laboratories, Detroit, MI
Desoxycholate Agar	Difco Laboratories, Detroit, MI
Anaerobic	
GAM broth	Nissui Pharmaceutical CO., LTD, Tokyo
BL agar	Nissui Pharmaceutical CO., LTD, Tokyo
MRS broth	Difco Laboratories, Detroit, MI
Bacteroides	Nissui Pharmaceutical CO., LTD, Tokyo

(TP650) and primers ITS1F (5'-GTAACAAGGT (T/C) TCCGT-3') and ITS1R (5'-CGTTCTTCATCGATG-3'). The 18S rRNA genes were sequenced using these same primers. The cloned DNA was sequenced using an ABI Prism 3130XI Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with a BigDye Terminator v1.1 Cycle Sequencing Kit. The sequences obtained were used in a BLAST search of the DNA Data Bank of Japan (DDBJ).

## Study 2

### Cell preparation and culture conditions

Cells preparation was performed by the procedure of Aoki-Yoshida *et al.* (2016) Cells were cultured in RPMI1640 (Nissui Phamaceutical, Tokyo, Japan) containing 10% fetal calf serum (FCS; GIBCO, Grand Island, NY, USA), 2 g/L NaHCO<sub>3</sub>, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol, and 300 mg/L L-glutamine at 37°C in 5% CO<sub>2</sub> in air.

Peyer's patch (PP) cells and mesenteric lymph nodes (MLN) cells from BALB/c mice were isolated from the small intestines as follows: PP cells and MLN cells were removed from the small intestines and washed with Roswell Park Memorial Institute medium (RPMI) (+). The PP cells were crushed in RPMI (+) treated with 1 mg/ml collagenase IV (Shigma Aldrich, St Louis, MO, USA) in 50 ml tube with gentle stirring at 37°C in air for 60 min. After collagenase treatment, the preparation was filtered with gauze and the cells were washed with PBS followed by centrifugation at 4°C, 1300 rpm for 5 min. The supernatant was suspended with 1 ml of RPMI (+). MLN cells were crushed in 5 ml of RPMI (+) and filtered with gauze. Then, the cell suspension was centrifuged at 4°C, 1300 rpm for 5 min. The supernatant was suspended with 1 ml of RPMI (+).

### Cytokine analysis

Isolated PP cells (1 × 10<sup>5</sup> cells/well) and MLN cells (1 ×

10<sup>5</sup> cells/well) were cultured with *L. paracasei* NFRI 7415 (10<sup>2</sup>, 10<sup>3</sup> and 10<sup>4</sup> cfu/ml) in 96-well flat-bottomed plates at 37°C in 5% CO<sub>2</sub> in air. After incubation for 2 days, culture supernatants were collected.

Determination of the IL-12p70 level in the culture supernatants was performed by a sandwich enzyme-linked immunosorbent assay (ELISA). The mouse IL-12p70 ELISA Ready-SET-Go! (eBioscience, Inc. San Diego, CA, USA) was used according to the manufacturer's instructions.

### Statistical analysis

Values are expressed as means ± SDs. Student's t-test was used for all pair-wise comparisons. Differences in values between groups were tested by Scheffe's multiple-range test. *P*-values less than 0.05 were considered statistically significant.

## RESULTS AND DISCUSSION

### Study 1

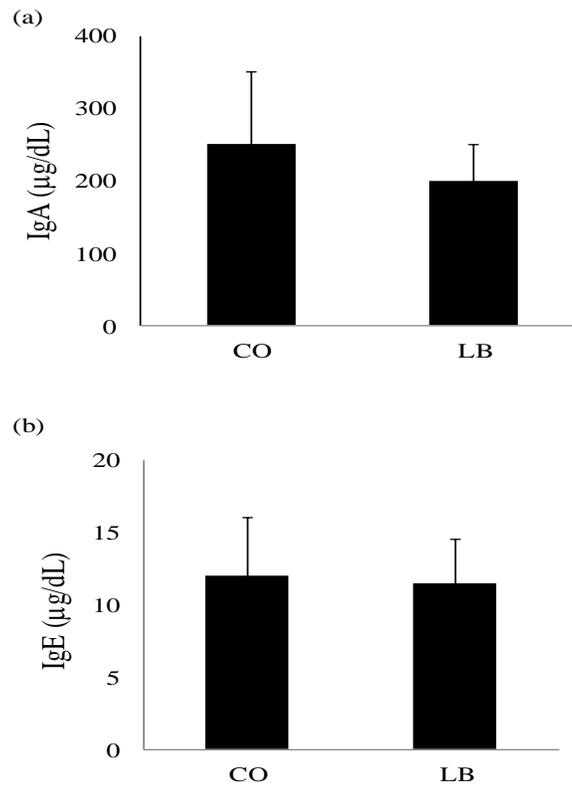
One major aim of study 1 was to explore the potential probiotic effects of live LAB. As a result of the calculations, it was found that the LB group had consumed an average of 7.06 ml/day of water containing LAB, and hence they had ingested approximately 1.0 × 10<sup>8</sup> cfu/day of the *Lactobacillus* bacteria during the dietary treatment period. The required number of LAB to exert a probiotic effect varies depending on the type. Various studies have reported LAB administrations to mice of 10<sup>6</sup>-10<sup>10</sup> cfu/day (Kim *et al.*, 2016; Xu *et al.*, 2011; Perdigon *et al.*, 2002).

Table 2 shows the body weight, food intake, liver weight, plasma lipids and liver lipids in mice fed the experimental diets for 4 weeks. No significant difference in body weight, food intake, plasma T-cho and liver lipids was observed between the two groups. However, the liver weight and plasma TG in the LB group was lower

**Table 2.** Body weight, food intake, plasma lipids and liver lipids in mice fed the experimental diets for 4 weeks.

Group	CO <sup>1</sup>	LB <sup>2</sup>
Body weight (g)	18.4 ± 1.08	18.3 ± 1.34
Total food intake (g)	92.8 ± 5.69	102 ± 3.96
Liver weight (g/100 g B.W.)	0.88 ± 0.08	0.86 ± 0.12*
Plasma triacylglycerol (mg/dl)	89.7 ± 10.4	55.5 ± 11.0*
Plasma T-cholesterol (mg/dl)	77.6 ± 8.99	68.4 ± 15.9
Liver lipids		
Total lipids (mg/g)	77.5 ± 24.2	78.1 ± 18.2
Triacylglycerol (mg/g)	8.59 ± 2.60	9.66 ± 3.72
T-Cholesterol (mg/g)	4.30 ± 0.62	4.11 ± 0.78

<sup>1</sup> CO, control group; <sup>2</sup> LB, Lactic acid bacteria (LB) group. Values are means ± SDs, n = 6. Student's t-test; significantly different at  $p < 0.05$ . T-Cholesterol, total cholesterol.



**Figure 1.** Plasma IgA and plasma IgE level concentration of mice fed experimental diets. CO, control group; LB, Lactic acid bacteria group. Each value is mean ± SD; n = 6.

than that in the CO group ( $p < 0.05$ ). Previously, we examined the effects of live *L. paracasei* NFRI 7415 on the fecal cholesterol excretion of BALB/c mice over a 4 weeks administration periods (Komatsuzaki *et al.*, 2014). Female BALB/c mice were fed a control diet (CO) and control diet + *L. paracasei* NFRI 7415 (LB). The plasma TG and T-cho concentrations of the LB group were found

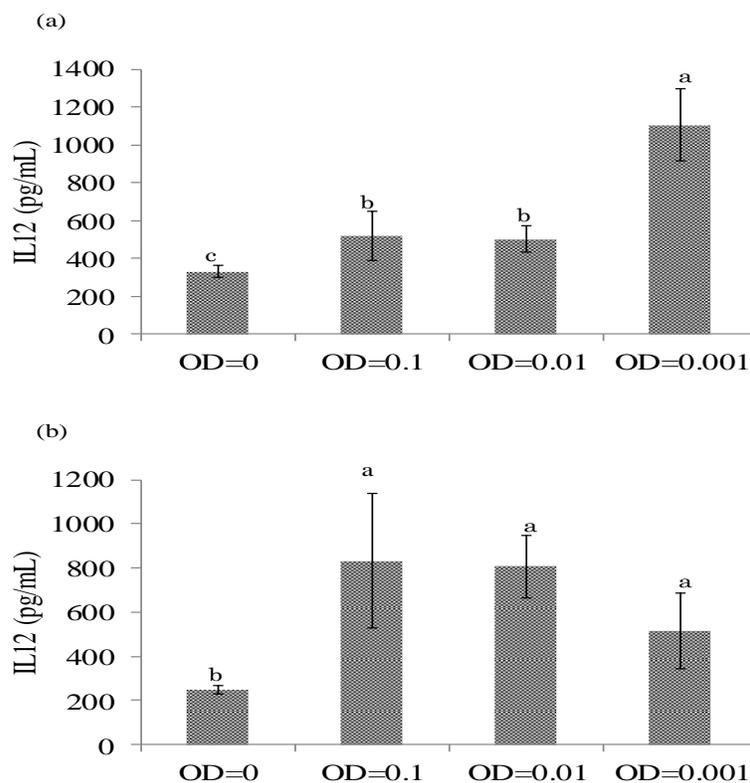
to be lower than that of the CO group in that experiment. It was found from these experiments that the parameters of mouse hepatic lipids were not influenced by this strain under control diets.

There were no significant differences in plasma IgA and IgE levels between the two groups (Figure 1). Findings from animal studies showed that dietary supplementation

**Table 3.** Intestinal bacterial flora in the feces of mice.

log CFU/g fece	CO <sup>1</sup>	LB <sup>2</sup>
Aerobic bacteria	5.66 ± 0.92	6.14 ± 3.00
<i>Staphylococcus</i> spp.	ND	ND
Enterococci	2.86 ± 2.67	ND**
<i>E. coli</i> group	1.39 ± 2.59	ND**
Anaerobic bacteria	9.31 ± 0.29	7.13 ± 2.90 *
Bifidobacteria	9.33 ± 0.30	8.51 ± 0.55
Lactic acid bacteria	8.59 ± 0.65	8.63 ± 0.52
Bacteroides	9.31 ± 0.29	ND **
Clostridium	9.38 ± 0.29	9.30 ± 0.81

<sup>1</sup>CO, control group; <sup>2</sup> LB, Lactic acid bacteria (LB) group. Values are means ± SD, n = 6. Student's t-test; \*significantly different at  $p < 0.05$ . \*\*significantly different at  $p < 0.01$ .



**Figure 2.** Production of IL-12 induced by *Lactobacillus paracasei* NFRI 7415. (a) Peyer's patch (PP) cells were cultured with LAB (OD 0, OD 0.1, OD 0.01, OD 0.001). (b) Mesenteric lymph intestine (MLN) cells were cultured with LAB (OD 0, OD 0.1, OD 0.01, OD 0.001). The level of IL-12 in supernatants was measured using ELISA. Values represent mean ± SD, n = 3. Within a row, values not sharing a common superscript letter are significantly different at  $p < 0.05$ .

with LAB were efficacious against symptoms of infection and atopic dermatitis of mice (Kim *et al.*, 2016). On the other hand, there are reports of no effect when changing the kind of strain or quantity of LAB. Further studies using disease model of mice with *L. paracasei* NFRI 7415 are

needed.

No significant differences in aerobic bacteria, *Staphylococcus* spp., Bifidobacteria, lactic acid bacteria or Clostridium were observed between the two groups (Table 3). No Enterococci, *E. coli* or Bacteroides were

detected in the LB group. A BLAST search revealed that one of the anaerobic bacteria in the feces of LB group showed 100% homology to *L. paracasei*.

More than 100 trillion intestinal bacteria inhabit the intestinal flora in the mammalian bowels, and more than 100 types of Bifidobacteria and anaerobic bacteria form intestinal flora (Mitsuoka *et al.*, 1990). Over 99% of the bacteria in the gut of the intestinal flora of mammals are anaerobes. Among them, *Streptococcus* spp. and *Bacteroides* spp. are known as human carcinogens; further, it has been shown that the number of anaerobic bacteria exceeds that of aerobic bacteria in the feces of cancer patients (Shinohara, 1990). In this study, *Bacteroides* were not detected in the feces of the LB group, but *L. paracasei* were detected in the feces of the LB group. Therefore, it is thought that ingesting live *L. paracasei* affects the formation of bacterial flora in mice.

## Study 2

The effects of live *L. paracasei* NFRI 7415 on the gut immune system of BALB/c mice were evaluated. PP cells or MLN cells prepared from mice were cultured with live *L. paracasei* NFRI 7415. Production of the cytokine IL-12 in the PP and the MLN cells, which comprise gut-associated lymphoid tissues (GALT), were increased by treatment with live *L. paracasei* NFRI 7415 in a concentration-dependent manner *in vitro*. The culture supernatant was recovered and the amount of IL-12 was measured. No clear effect on the MLN cells was found, but IL-12 production increased when PP cells were cultured with LAB (OD=0.001) ( $p < 0.05$ ) (Figure 2). It has been reported that LAB act on human immunity to prevent respiratory and intestinal-tract infection. Because IL-12 produced by immune cells activates cell-mediated immunity, it has been expected that this strain, which strongly stimulates IL-12 production from immune cells, would show defensive action against viral infection (Carasi *et al.*, 2015). The probiotic *Lactobacillus casei* is characterized by a potential to strongly induce IL-12 production and thereby strongly induce a T helper type 1 cellular response; this proinflammatory response is properly controlled in PP cells (Chiba *et al.*, 2009). *L. casei* has an optimum temperature of 37°C, and appears to thrive in stomach acid and therefore reaches the intestine (Sutula *et al.*, 2012). *L. paracasei* NFRI 7415 is viable in temperatures ranging from 25-37°C, with the temperature most suitable for cell proliferation being 37°C (Komatsuzaki *et al.*, 2005). It was found from the result of Study 1 that this strain also manages to reach the intestine of mice of the LB group. It is suggested that, like *L. casei* Shirota, this strain strongly induces IL-12 production.

In summary, our results demonstrate for the first time that live *L. paracasei* NFRI 7415 reaches the intestinal tract of mice and improves enteric bacterial flora. Moreover, oral administration of live *L. paracasei* NFRI

7415 may have the potential to improve intestinal conditions and immune functions in humans.

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