Chapter 4

Immunomodulatory activities of probiotics from traditional Mongolian dairy products: Enhancement of T-helper I immune response and natural killer cell activity

4.1 Introduction

Some lactic acid bacteria (LAB) and bifidobacteria strains are known as probiotics. According to the definition of probiotic bacteria adopted by the joint Food and Agriculture Organization of the United Nations/World Health Organization working group (WHO), they are living microorganisms that confer a health benefit on the host when administered in adequate amounts (WHO 2001). Health benefits of probiotics have been reported for intestinal disorder, decreasing serum cholesterol, antimutagenicity and immunomodulatory functions (Yasui & Ohwaki 1991; Kirjavainen *et al*. 1999; Kimoto *et al*. 2002; Matsumoto & Benno 2004; Sullivan & Nord 2005).

The immunomodulatory effects of probiotics have been focused in past several decades. LAB are reported to activate T-helper (Th) 1 cells and natural killer (NK) cells (Kimoto *et al*. 2004; Baken *et al*. 2006; Kimura *et al*. 2006; Takeda *et al*. 2006; Koizumi *et al*. 2008). Th1 cells play a crucial role in host defense against infections by bacteria, parasites and viruses (Xu *et al*. 2004; Barber *et al*. 2005). Furthermore, Th1 cytokines including interleukin (IL)-12 and interferon (IFN)-γ suppress Th2-dependent allergic diseases (Hessel *et al*. 2005). Therefore, positive regulation of Th1 immunity is a promising strategy for preventing disease. NK cells are crucial for not only host defense but also for antitumor immunity (Papamichail *et al*. 2004). NK activity correlates with health status. Healthy individuals can have high

cytotoxic activity until late senescence and a cytotoxic capacity similar to younger counterparts (Myśliwska *et al*. 1992; Myśliwski *et al*. 1993). In contrast to other innate immunity cells, NK cells are more involved in maintaining innate and adaptive immune responses during aging (Mocchegiani & Malavolta 2004). Therefore, the augmentation of NK cell activity contributes to immunostimulation and a healthy state.

In the previous report, 10 LAB strains from traditional Mongolian dairy products were isolated and characterized them *in vitro* as probiotics (Takeda *et al*. 2011b). In this study, to evaluate the immunomodulatory activity of the LABs, their effects on Th1 cytokine inductive activity using a mice macrophage cell line and mouse spleen cell culture methods were assessed. Moreover, mouse NK activity was investigated by administering the strains and measuring the cytotoxicity of mouse spleen cells toward YAC-1 cells. I found 4 strains that activated Th1 cytokine production and a *Lactobacillus* (*L*.) *plantarum* 06CC2 strain that could affect Th1 cytokine production and NK activity. This study provided information for better understanding the immunomodulatory activities of the 10 LAB strains after ingestion *in vivo*.

4.2 Materials and methods

4.2.1 Lactic acid bacteria

Ten LAB strains with probiotic potential were isolated from Mongolian dairy products (Table 4-1). The strains were cultured at 37˚C for 24 h in Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany) as described previously (Takeda *et al*. 2011b). They were harvested by centrifugation at 10,000 × *g* for 5 min, washed twice with phosphate-buffered saline (PBS), and boiled for 1 h. For co-culture with the macrophage cell line and mice spleen cells, boiled LABs were washed with PBS and suspended in Roswell

Park Memorial Institute (RPMI)-1640 medium. For oral administration to mice, boiled LABs were lyophilized and the powder suspended in distilled water.

Table 4-1 Species and origin of LAB strains

Strain	Origin
L. plantarum 05AM23	Alcoholic fermented mare milk (Airag)[a]
L. plantarum 06TCa8	Fermented camel milk (Tarag)[b]
L. paracasei ssp. *paracasei* 06TCa19	Fermented camel milk (Tarag)
L. paracasei ssp. *paracasei* 06TCa22	Fermented camel milk (Tarag)
L. paracasei ssp. *tolerans* 06TCa39	Fermented camel milk (Tarag)
L. plantarum 06TCa40	Fermented camel milk (Tarag)
L. paracasei ssp. *paracasei* 06TCa43	Fermented camel milk (Tarag)
L. plantarum 06CC2	Cow milk cheese (Aaruul)[C]
L. delbrueckii ssp. *lactis* 06TC3	Fermented cow milk (Tarag)
L. plantarum 06CC9	Cow milk cheese (Aaruul)

[a] Airag is fermented horse milk.

[b] Tarag is a traditional Mongolian yoghurt.

[C] Aaruul is a traditional Mongolian hard cheese.

4.2.2 Cells

J774.1 cells (RIKEN, Ibaraki, Japan) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/mL penicillin and 100 μg/mL streptomycin. J774.1 cells were used for an immune activation assay of macrophages. YAC-1 cells (RIKEN, Ibaraki, Japan) were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin and used for NK cell activity assays.

4.2.3 Mice

Specific-pathogen-free female BALB/c mice (6-weeks-old, 17–19 g) were obtained from Kyudo Animal Laboratory, Kumamoto, Japan. Mice were housed at seven per cage in specific pathogen-free conditions under a 12 h light/dark cycle at 23 ± 2˚C. Mice were given a standard pellet diet (Labo MR Stock, Nosan Corporation, Kanagawa, Japan) and given water *ad libitum*. Acclimation was for 5 to 7 days before experiments. Experimental protocols were approved by the Animal Experiment Committee of University of Miyazaki, Japan, and animal experimentation guidelines of the university were followed in animal studies (Permission No. 2010-014).

4.2.4 Macrophage cell line cultures with lactic acid bacteria

Previously described methods were used with modifications (Kimoto *et al*. 2004). One-milliliter aliquots of J774.1 at 5×10^5 cells/mL were seeded into 24-well plates and incubated at 37˚C for 24 h in 5% CO_2-95% air. After incubation, the culture medium was exchanged and aliquots (100 μL) of heat-killed LAB at 1×10^9 CFU/mL with cell culture medium were transferred into wells to stimulate cells. Lipopolysaccharide (LPS) from *Escherichia coli* strain O55:H5 (Sigma-Aldrich Japan Corporation, Tokyo, Japan) was used at 10 μg/mL, as the positive control for cell stimulation. After incubation for 48 h, co-cultured

medium was collected and stored at -80˚C for determination of IL-12p40 and TNF-α levels by ELISA kit (eBioscience, Santiago, Calif, USA), according to the manufacturer's instructions.

4.2.5 Spleen cell cultures with lactic acid bacteria

Spleen cells were isolated as single-cell suspensions from the spleens of female BALB/c mice. After depletion of erythrocytes by treatment with 0.144 M NH_4Cl in 0.017 M Tris-HCl buffer (pH 7.65), cells suspended in RPMI-1640 with 2% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 1×10^6 cells/mL were seeded into 24-well plates. Co-culturing and sample collection was above, and tests were for IL-12 and IFN-γ levels by ELISA kit (eBioscience, Santiago, Calif, USA), according to the manufacturer's instructions.

4.2.6 Oral administration of heat-killed lactic acid bacteria in mice

Lyophilized LAB powder was suspended in distilled water at 10% (w/v) and 0.2 ml per mouse was orally administered by gavage twice daily. The maximum dose was 20 mg/mouse of LAB, deduced from the maximum dose of supplemental foods such as yoghurt for humans and corresponding to a maximum level to avoid diarrhea in mice. As a control, mice were given an equal volume of distilled water. Oral administration was carried out for 14 days after acclimation.

4.2.7 Natural killer cell activity in spleen cells

The effect of oral administration of the LABs on splenic NK cell activity in mice was examined using a cytotoxicity assay kit (CytoTox96 Non-Radioactive Cytotoxicity Assay, Promega corporation, Tokyo, Japan) based on the modified method of Urbanowicz *et al*. (2009). Mice (5 to 6 mice per group) were housed and administered LAB as described above.

Spleen cells as effector cells were prepared from mice given LAB and erythrocytes were depleted from the spleen cells. YAC-1 cells were used as the target cells for NK cell activity. The effector (1×10^5 cells/well) and target cells (1×10^4 cells/well) were mixed at a ratio of 10:1, plated in quadruplicate on 96-well U-bottomed plates, and incubated for 4 h at 37˚C in a humidified atmosphere containing 5% CO_2. After incubation, cultured mixtures in the 96-well plates were centrifuged, and supernatants were collected and incubated with substrate solutions from the kit in 96-well plates for 30 min at room temperature to detect lactate dehydrogenase (LDH) activity. Absorbance was measured at 490 nm by a microplate reader (Bio-rad laboratories corporation, Tokyo, Japan). The percentage of specific release was calculated according to the following equation:

Specific lytic activity (%) = [(A_{490} of effector and target cell mix – A_{490} of spontaneous effector LDH release – A_{490} of spontaneous target LDH release)/(A_{490} of maximum target LDH release – A_{490} of spontaneous target LDH release)] × 100

4.2.8 Gene expression in spleen cells

IFN-γ and IL-12p40 mRNA in spleen cells was examined by real-time reverse transcriptase (RT) - PCR with an Applied Biosystems 7300 real-time PCR system (Applied Biosystems Japan, Tokyo, Japan), and data evaluated by normalizing results to mouse β-actin. Mice (5 to 6 per group) were housed and administered *L. plantarum* 06CC2 as described above. After preparation of spleen cells from mice give the 06CC2 strain, RNA was isolated from collected cells using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Isolated RNA was transcribed into cDNA by ReverTra Ace α (Toyobo Co. Ltd, Osaka, Japan) using an oligo-dT(20) primer according to the manufacturer's instructions. PCR amplification for IFN-γ and IL-12p40 used TaqMan Gene

Expression Master Mix (Life Technologies Japan, Tokyo, Japan) with IFN-γ (TaqMan Gene Expression Assays Mm01168134_m1, Life Technologies Japan) or IL-12p40 specific primers (TaqMan Gene Expression Assays Mm00434174_m1, Life Technologies Japan). For β-actin, the PCR amplification used Power SYBR Green PCR Master Mix (Life Technologies Japan) with the β-actin primers (forward: 5'- TGACAGGATGCAGAAGGAGA -3', reverse: 5'- GCTGGAAGGTGGACAGTGAG -3'). Samples were measured by real-time PCR with initial denaturation at 50˚C for 2 min and 95˚C for 10 min, followed by 45 cycles of 95˚C for 15 s and 60˚C for 60 s. Following the β-actin amplification reaction, melting curve analysis was conducted to determine the specificity of PCR reactions by with 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 s. Relative gene quantities were determined using the comparative *Ct* method and normalized by β-actin.

4.2.9 Statistical analysis

The results of defecation frequency and intestinal bacterial enumeration were analyzed statistically with one-way analysis of variance (ANOVA), followed by the Tukey test and a two-tailed paired Student's *t*-test. A *P*-value of less than 0.05 was defined as statistically significant.

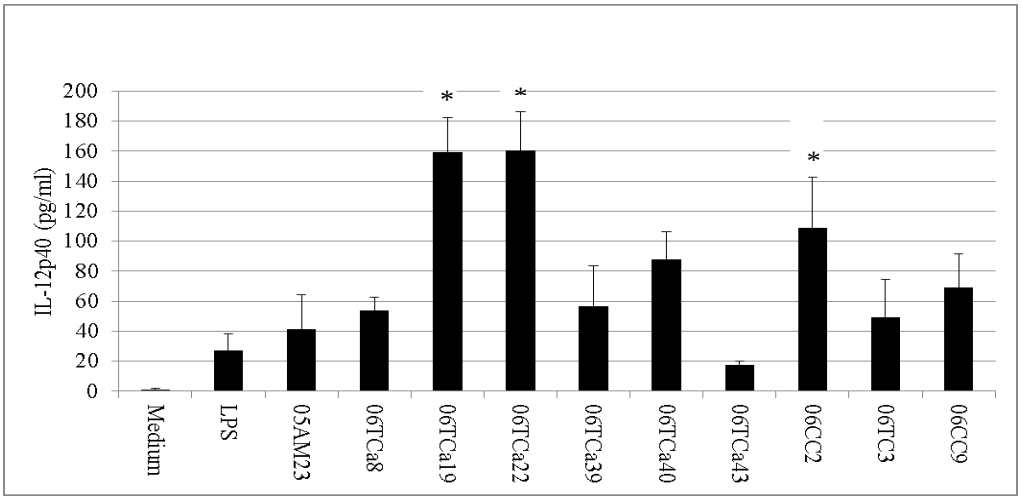
4.3 Results

4.3.1 Effects of lactic acid bacteria on cytokine production in mice macrophages

Previous reports evaluated IL-12p40, IL-6 and TNF-α levels released from a mouse macrophage cell line affected by LAB immunomodulation (Kimoto *et al*. 2004). In this study, to evaluate the antigen-presenting function and activation of macrophages by LAB, IL-12p40 and TNF-α from J774.1 cells were measured after co-culture with 10 LAB strains or with

LPS as the positive control (Figures 4-1A and B). The levels of IL-12p40 and TNF-α released from J774.1 cells were elevated by LPS compared to the blank (medium). The IL-12p40 levels in 06TCa19, 06TCa22 and 06CC2 strains were significantly elevated compared with that of LPS by one-way ANOVA followed by Tukey test ($P < 0.05$). The TNF-α in 06TCa22, 06TCa40 and 06CC2 were also significantly elevated compared with that of LPS by one-way ANOVA followed by Tukey test ($P < 0.05$). Among of them, strains 06TCa22 and 06CC2 significantly elevated IL-12p40 and TNF-α levels compared to LPS. In addition, although I measured IL-12 quantity in this examination, it was not detected at all.

(A)



(B)

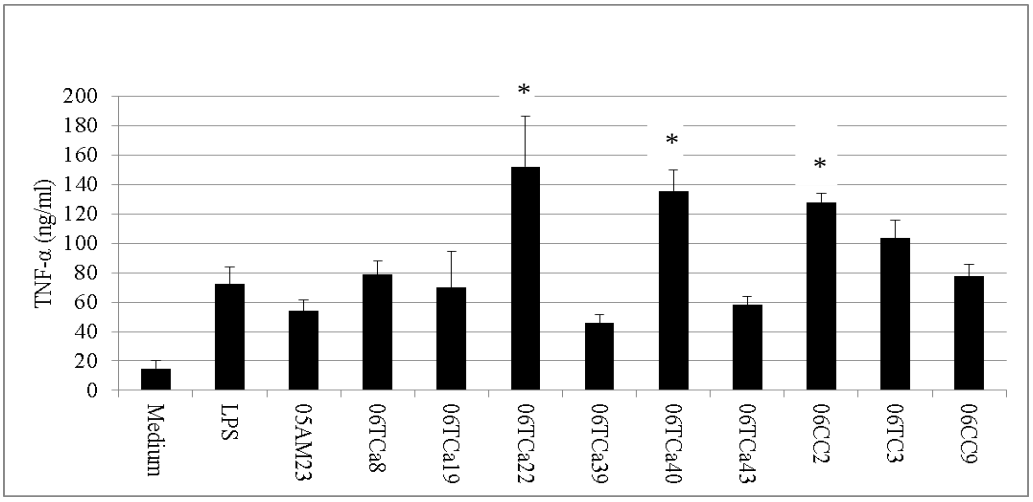


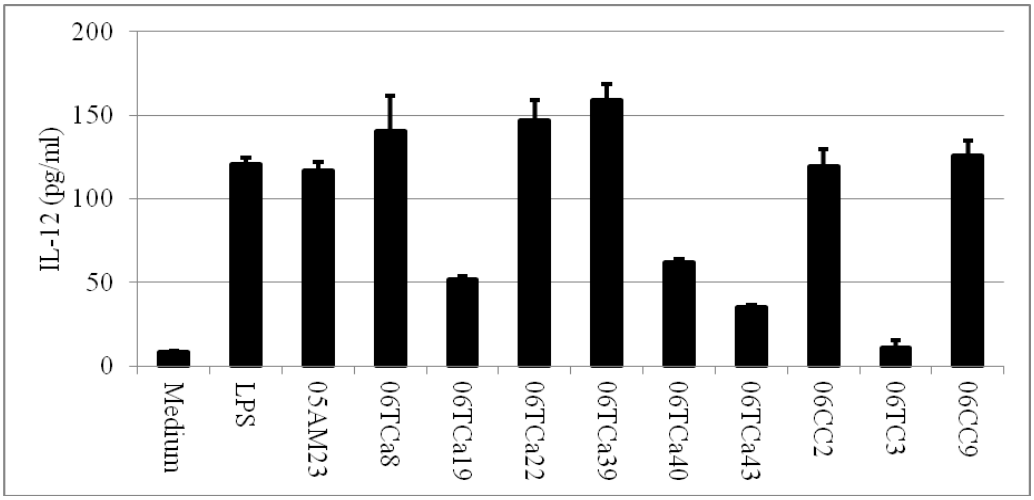
Figure 4-1 Effects of LABs on cytokines production in J774.1 cells.

(A) shows IL-12p40 levels and (B) shows TNF-α levels in J774.1 cells cultures with LAB for 48 h. The samples were measured by ELISA. Data are representative of three separate experiments. Bars indicate standard deviation. The asterisk shows the significant differences compared with LPS as the positive control by one-way ANOVA, followed by the Tukey test ($P < 0.05$).

4.3.2 Effects of lactic acid bacteria on Th1 cytokine production in mouse spleen cells

The effect on Th1 cytokine induction by the 10 probiotic LAB strains was demonstrated using the mouse spleen cell culture method. Figures 4-2A and B show IL-12 and IFN-γ levels released from mouse spleen cells after co-culture. Cytokines were induced from spleen cells by LPS as a positive control, but not by medium as a negative control. Several LAB strains induced IL-12 and IFN-γ. Of the 10 LABs, 6 strains (05AM23, 06TCa8, 06TCa22, 06TCa39, 06CC2 and 06CC9) induced IL-12, and 4 strains (06TCa22, 06TCa39, 06CC2 and 06CC9) induced IFN-γ to levels greater than or equal to LPS. However, differences in IL-12 and IFN-γ levels between the LAB strains and LPS was not significant by one-way ANOVA, followed by Tukey test.

(A)



(B)

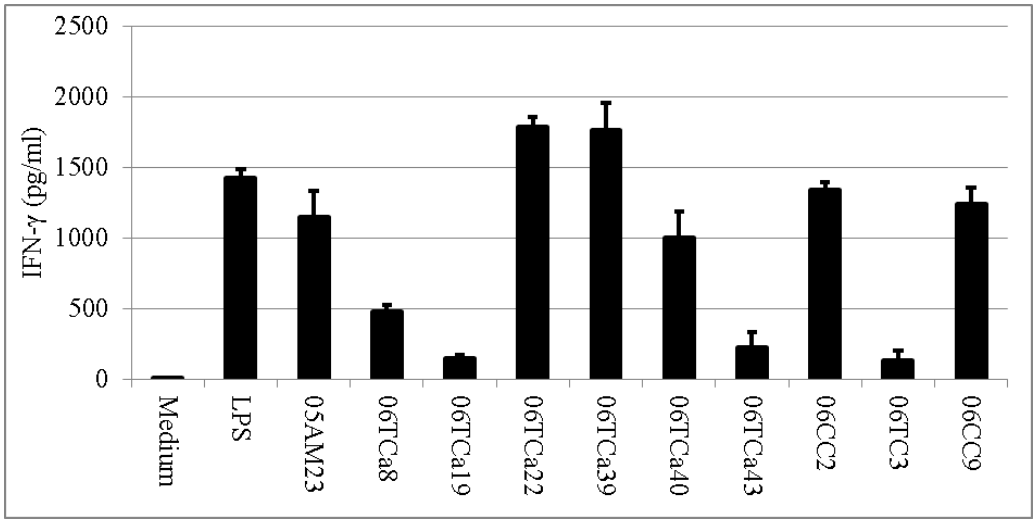


Figure 4-2 Effects of LABs on Th1 cytokines production in mice spleen cell.

(A) shows IL-12 levels and (B) shows IFN-γ levels in mice spleen cells cultures with LAB for 24 h. The samples were measured by ELISA. The experiments were performed in triplicate. Bars indicate standard deviation.

4.3.3 Effect of oral administration of lactic acid bacteria on NK cell activity in spleen cells

The effect of the 10 probiotic LAB strains on NK cell activity in spleen cells of mice was investigated. LABs were administered for 14 days. Mice body weights were observed and no losses were observed during LAB administration. Figure 4-3 shows the NK cell activity of mice given the 10 LABs in a 10:1 ratio of effector/target cells. According to the result, the NK cell activity in splenocyte of mice administered the 06CC2 strain was the highest of all strains and showed significant differences to the control by one-way ANOVA, followed by Tukey test ($P < 0.05$).

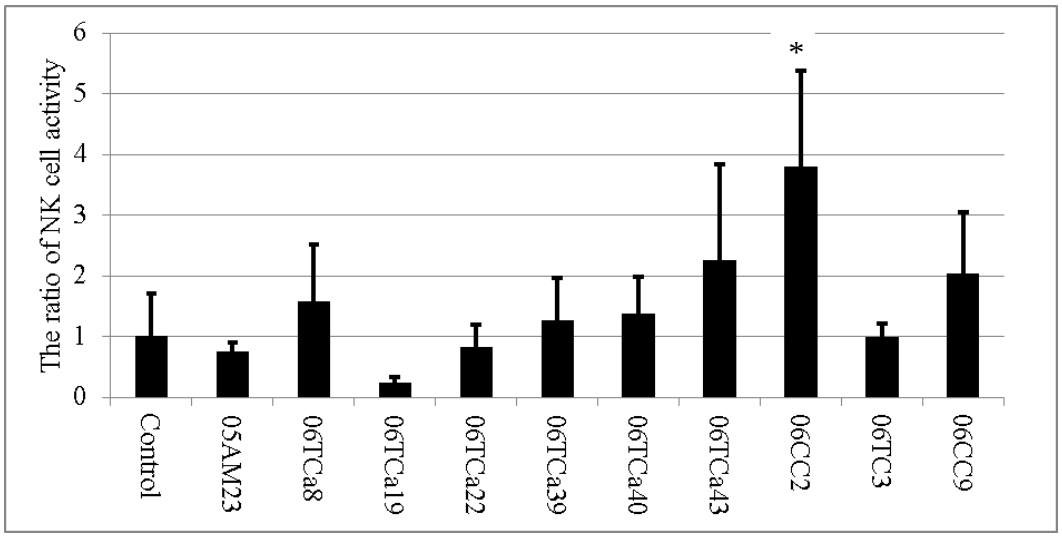


Figure 4-3 Effects of administration of LABs on NK cell activity in mice spleen cell

Lyophilized LAB powder was suspended in distilled water at 10% (w/v) and 0.2 ml per mouse was orally administered by gavage twice daily for 14 days after acclimation. Spleen cells as effector cells were prepared from mice and erythrocytes were depleted from the spleen cells. YAC-1 cells were used as the target cells for NK cell activity. The effector and target cells were mixed at a ratio of 10:1 and incubated for 4 h at 37˚C in a humidified atmosphere containing 5% CO_2. After incubation, the supernatants in cultured mixtures collected and detect lactate dehydrogenase (LDH) activity. The percentage of specific release from the target cells was calculated. The ratio of NK activity was calculated by the each control percentage values in dividing experiments. Five to 6 mice were prepared in each group. Bars indicate standard deviation. The asterisk shows the significant differences compared with the control by one-way ANOVA, followed by the Tukey test ($P < 0.05$).

4.3.4 Effects of oral administration of 06CC2 on IL-12p40 and IFN-γ mRNA expressions in spleen cells

To investigate the mechanism for enhanced NK cell activity by the 06CC2 strain, mRNA of IL-12p40 and IFN-γ in spleen cells of mice given 06CC2 were measured by real-time RT-PCR (Fig. 4-4). IL-12 and IFN-γ are known Th1 cytokines. IL-12p40 is part of both IL-12 and IL-23 and is specifically expressed in macrophages and dendritic cells. The administration of the 06CC2 strain significantly augmented the mRNA gene expression of IFN-γ in spleen cells compared to the control by two-tailed paired Student's *t*-test ($P < 0.05$). Although the gene expression of IL-12p40 also seemed to be augmented, differences were not significant.

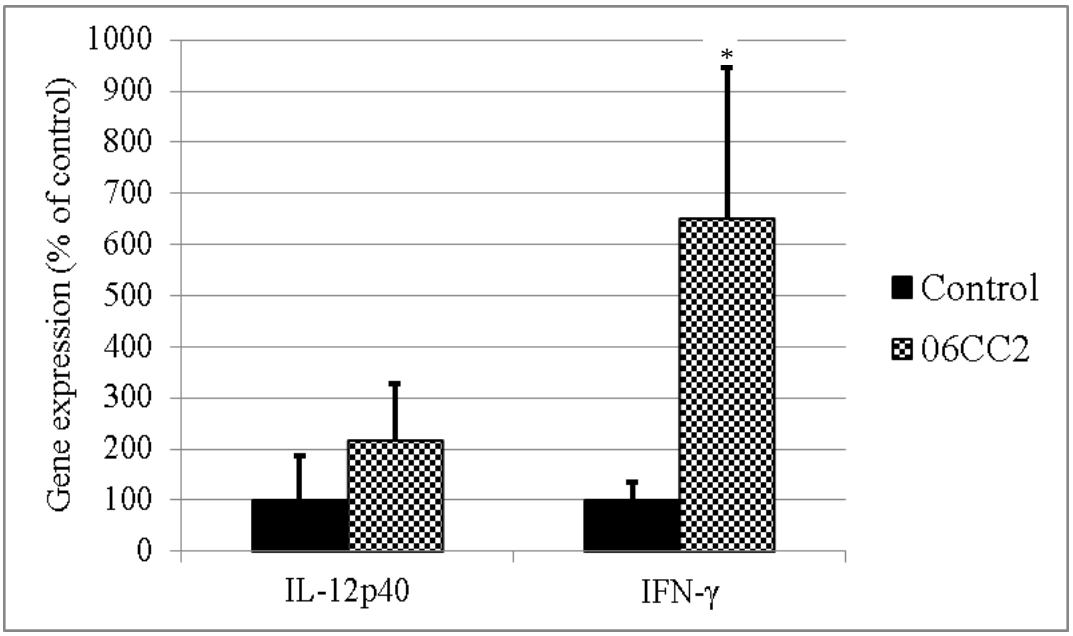


Figure 4-4 Effects of administration of 06CC2 strain on mRNA expressions of IL-12p40 and IFN-γ in mouse spleen cells

Effects of administration of 06CC2 on expression of IL-12p40 and IFN-γ in mouse spleen cells (5-6 mice per group). mRNA was measured by real-time RT-PCR and normalized to mouse β-actin mRNA. Gene expression levels are expressed as percentages of the control group. Bars indicate standard deviation. Closed columns show the control group. Squared columns show the 06CC2 group. The asterisk indicates statistical significance versus the control group by Student's-t test ($P < 0.05$).

4.4 Discussion

Many studies have focused on LAB immunomodulation functions in several decades (Yaeshima 2006). In the present study, the immunomodulatory activity of 10 LABs from Mongolian dairy products was investigated by using mouse macrophage cell line and spleen cells culture methods. According to those results, 4 probiotic LAB strains (06TCa22, 06TCa39, 06CC2 and 06CC9 strains) enhanced Th1 cytokine production (Figs. 4-1 and 4-2).

Previously, the cell wall composition of LAB was reported to play crucial role for affecting immunocytes (Tejada-Simon & Pestka 1999; Shida *et al*. 2011). On the other hand, because the LAB cell wall composition is highly similar in related species, new methods were proposed to identify species and compare analyses of LAB cell wall compositions (Lortal *et al*. 1997; Lonigro *et al*. 2009). According to those reports, LAB strains in the same species are appeared to have similar immunomodulation activity. In this study, two LAB groups with several strains in the same species (5 *L. plantarum* and 3 *L. paracasei paracasei* strains) were subjected to co-culturing with mouse spleen cells and macrophage cell lines. I observed no trends in Th1 cytokine or TNF-α production from mouse spleen cells or macrophage cell lines, even using the same LAB species (Figs. 4-1A, B and Figs. 4-2A, B). Thus, other materials in addition to the LAB cell wall might exist to affect immunocytes.

Kimura *et al*. (2006) demonstrated that 4 of 51 LAB strains from Mongolian fermented milk enhanced IL-12 and IFN-γ production using the mouse spleen cell culture method. In the study reported here, 4 probiotic LAB strains (06TCa22, 06TCa39, 06CC2 and 06CC9 strains) with this ability were found (Figs. 4-2A and 4-2B). The probiotic LAB *L. paracasei* KW3110 strain improved the Th1/Th2 imbalance by inducing IL-12 in Th2-polarized murine splenocytes *in vitro*, and oral administration of KW3110 in a mouse allergy model directed the Th1/Th2 balance toward Th1 through the maturation of antigen-presenting cells and

inhibition of serum immunoglobulin E elevation (Fujiwara *et al*. 2004). The KW3110 strain was effective against atopic dermatitis-like skin lesions in mice and cedar pollinosis in human (Wakabayashi *et al*. 2008; Yonekura *et al*. 2009). The 4 strains (06TCa22, 06TCa39, 06CC2 and 06CC9 strains) demonstrated in this study to enhance the production of Th1 cytokines are expected to affect allergy symptoms in the further study.

In another study, it was demonstrated that oral administration of the 06CC2 strain augmented NK cell activity in mouse spleen cells (Fig. 4-3). The expression of IL-12p40 and IFN-γ was elevated in the spleen cells compared to controls (Fig. 4-4). Previously, Takeda *et al*. (2006) reported that the ability of *L. casei* Shirota strain to enhance NK cell activity in human peripheral blood and induce IL-12 production *in vitro* was closely correlated. Koizumi *et al*. (2008) found that NK cells and NKT cells were responsible for producing IFN-γ after stimulation with *L. pentosus* S-PT84 strain *in vitro*. Chiba *et al*. (2010) found that *L. casei* Shirota strain promoted the development of Th1 cells through weak induction of IL-12 in Peyer's patch cells, which constitute the intestinal immune organ. Shida *et al*. (2011) proposed a mechanism for stimulation of Th1 and NK cells by *L. casei* Shirota strain, specifically reaction through recognition by macrophage and dendric cells in Peyer's patch cells. By their previous descriptions, oral administration of 06CC2 strain might also activate NK cells through enhancement of IL-12p40 gene expression followed by IL-12 production in the mouse spleen. IFN-γ gene expression in spleen cells including NK cells stimulated by administration of 06CC2 was enhanced and followed by enhancement of IFN-γ production. Moreover, the 06CC2 strain demonstrated to induce TNF-α in macrophage cell line and Th1 cytokine quantities in macrophage cell line and mouse spleen cells. Therefore, macrophages and Th1 related cells might be activated and stimulated by administration of the 06CC2 strain in Peyer's patches. This might reflect augmented NK cell activity in spleen cells, although the reaction mechanism from the intestine to the spleen is unclear. Further study is needed to

investigate this.

4.5 Conclusion

In this study, the immunomodulatory activity of 10 probiotic strains isolated from traditional Mongolian dairy products was assessed for enhancement of Th1 cytokine production in a mouse macrophage cell line and spleen cells and for mouse NK activity after administering the strains to mice. The results of co-culture with J774.1 cells and mouse spleen cells showed that 4 of the 10 strains promoted Th1 cytokine production. Furthermore, the administration of the 06CC2 strain gave the highest level of mouse NK cell activity of the 10 strains. Therefore, the 06CC2 strain is expected to have superior immunomodulatory activity in the host.