Korean J. Food Sci. Ani. Resour.
Vol. 32, No. 1, pp. 40~44(2012)
DOI http://dx.do.org/10.5851/kosfa.2012.32.1.40

ARTICLE

Effects of Mozzarella Cheese Manufactured by *S. macedonicus* LC743 on the Immune Status of Mouse

Seong-A Cho, Kee-Sung Kim, and Sang-Dong Lim*
Korea Food Research Institute, Seongnam 463-746, Korea

Abstract

To develop a new starter culture for Mozzarella cheese, the immunomodulatory action of *Streptococcus macedonicus* LC743 in male C57BL/6 mice was studied. Mice were fed for 7 wk with feed containing 1% Mozzarella cheese made with three kinds of starter cultures from *S. macedonicus* LC743 (G3), FD-DVS TCC-3 (G2) and *S. macedonicus* LC743 : FD-DVS TCC-3(1:1) (G4) and control (feed only, G1), respectively. No significant differences in body weight gain were observed among the various groups of mice. The spleen index and thymus index were observed and no significant differences were found among the groups. The production of TNF-α of *S. macedonicus* LC743 group significantly increased compared to the control group. The production of IL-1β was significantly enhanced by the feeding of *S. macedonicus* LC743 group compared to the control group. In regards to the white blood cell counts, the neutrophil percentages were significantly higher in the G1 group compared to other groups. The lymphocyte percentages were significantly higher in G2, G3 and G4 groups in comparison to the control group. The results of this study may suggest that the supplementation of *S. macedonicus* LC743 can increase the cytokine production activity by the activated macrophages in mice. Based on the result of this study, it could be concluded that *S. macedonicus* LC743 could stimulate the immune functions of mice.

Key words: immunomodulating activity, Mozzarella cheese, *S. macedonicus* LC743, mouse

Introduction

The consumption of Mozzarella cheese has increased rapidly during the last 15 years, primarily because it is one of the ingredients of 'pizza' and other Italian specialities that are widely consumed all over the world. Mozzarella cheese is mainly used for its unique functional properties such as stretchability, meltability free oil formation, and browning. Under industrial conditions, selected thermophilic lactic acid bacteria cultures are used as starters in Mozzarella cheese manufacturing (Oberg *et al.*, 1991). More recently, several trials have been made regarding the production of cheeses with probiotic bacteria as adjuncts. These trials include soft cheeses such as Cottage (Blanchette *et al*., 1996; Riordan and Fitzgerald, 1998), Crescenza (Gobbetti *et al*., 1998), Fresh (Vinderola *et al*., 2000) and Kariesh (Murad *et al*., 1998) or semi-hard cheeses like Cheddar (Dinakar and Mistry, 1994; Furtado *et al*., 1993; Gardiner *et al*., 1998; Gardiner *et al*., 1999; Shaw and White, 1994; Stanton *et al*., 1998), Goat cheese (Gomes and Malcata, 1998) and Canestrato Pugliese (Corbo *et al*., 2001). Probiotic fresh cheese (Argentinean fresh cheese) containing *L. acidophilus* A9, *B. bifidum* A12 and *L. paracasei* A13 showed immunomodulating capacity in mice, providing increased phagocytic activity in the small intestine of peritoneal macrophages, after 2, 5 and 7 d of its ingestion. Moreover, a significant increase in the number of IgA producing cells in the large intestine after 5 d of administration was reported. Interaction of probiotic bacteria as bacterial antigens in the small (Peyer's patches) and large (lymphoid nodules) intestine was also observed (Medici *et al.,* 2004). Phagocytic cells play a central role in protection against microbial infections. In addition, macrophages are concerned in antigen presentation, tissue repair and also play an important role in the regulation of immune responses (Gill, 1998). Cytokines influence the defense system of the host directly or indirectly. Soluble factors such as IL-1, IL-6 and TNF-α released by monocytes have been shown to play key roles in proliferation, activation and differentiation of immune cells (Czarniecki, 1993). Also, IL-1 stimulates T-and B-cell proliferation,

*Corresponding author: Sang-Dong Lim, Korea Food Research Institute, Seongnam 463-746, Korea. Tel: 82-31-780-9082, Fax: 82-31-780-9160, E-mail: limsd@kfri.re.kr

TNF-α has a cytotoxic effect on tumour cells (Gill, 1998). IL-1 is a major product of the stimulated monocyte and is responsible for diverse biological effects. The systemic effects of IL-1 include fever, increased circulating neutrophils, hepatic acute phase proteins, slow wave sleep, elevated insulin levels and hypotension (Dinarello, 1987). TNF-α is also produced by activated macrophages, fibroblasts, and many different types of cells. The regulation of TNF-α production is critical in the maintenance of homeostasis of the immune system and in the prevention and treatment of the immune diseases (Kang *et al*., 1996). IL-6 is an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine. It is secreted by T cells and macrophages to stimulate immune response, e.g. during infection and after trauma, especially burns or other tissue damage leading to inflammation. In terms of host response to a foreign pathogen during infection, IL-6 has been shown, in mice, to be required for resistance against the bacterium, *Streptococcus pneumoniae* (van der Poll *et al.*, 1997).

The objective of this study is to investigate the immunomodulatory potential of oral administration of Mozzarella cheese manufactured by *S. macedonicus* LC743 in mice.

Materials and Methods

Preparation of Mozzarella cheese

Pasteurised milk was used on each occasion for the manufacture of Mozzarella cheese in three (including the control) cheese treatments. The first treatment was the control Mozzarella cheese (cheese C1), with the starter culture of FD-DVS TCC-3 (mixed culture of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, CHR. HANSEN A/S), 2% (v/v). For the second treatment of Mozzarella cheese (cheese C2), 2% (v/v, 2×10^7 cells) of *S. macedonicus* LC743. For the third treatment of Mozzarella cheese (cheese C3), 2×10^7 cells of strain *S. macedonicus* LC743, were incorporated with FD-DVS TCC-3 by the ratio of 1:1.

Animal and feeding procedure

SPF C57BL/6 mice, weighted 20.0±2.0 g, were provided by Koatech (Korea), were randomly divided into four groups (n = 15) G1, G2, G3 and G4. The animal group G1: the control group, fed ad libitum a diet (Table 1) without cheese supplementation, G2: fed ad libitum a diet containing the cheese (1%) made with TCC-3, G3: fed ad libitum a diet containing the cheese (1%) made with *S. macedonicus* LC743 and G4: fed ad libitum a diet containing the cheese (1%) made with TCC-3 and *S. macedonicus* LC743 (1:1). Three animals were housed in a plastic cage with wood chip bedding in an animal room with a 12 h light and 12 h dark condition at room temperature (20±2°C).

Table 1. Formulation of basal diet (AIN-76 purified diet)

Ingredient	Contents (%)
Casein (feed grade CP 85%)	20
Corn starch	15
Sucrose	45
Cellulose (fiber)	5
Tallow	5
Sallower oil	5
DL-methionine	0.3
AIN-vitamin mixture[1)]	1.0
AIN-mineral mixture[2)]	3.5
Choline bitartrate	0.2
Total	100

[1)]Contained per kg mixture; Thiamin·HCl 600 mg, Riboflavin 600 mg, Pyridoxine·HCl 700 mg, Nicotinicacid 3 g, Vit. A 400,000 IU (Retinyl acetate), Vit. E (dL-α-Tocopheryl acetate) 5,000 IU, Vit. D_3 2.5 mg, Vit. K 5.0 mg and sucrose

[2)]Contained per kg mixture; $CaHPO_4$ 500 g, NaCl 74 g, $K_3C_6O_7\cdot H_2O$ 220 g, K_2SO_4 52 g, MgO 24 g, 48 Mn 3.5 g, 17% Fe 6.0 g, 70% Zn 1.6 g, 53% Cu 0.3 g, KIO_3 0.01g, $CrK(SO_4)_2\cdot 12H_2O$ 0.55 g and sucrose

Body weight and spleen, thymus index

Body weight was measured once every week. Seven weeks after experimental diet administration the animals were sacrificed, their body, spleen and thymus weights recorded, and the spleen and thymus index for each individual mouse was calculated as follows:

Spleen index = spleen weight/weight × 100
Thymus index = thymus weight/weight × 100

Collection of serum and peritoneal macrophages

Blood was collected from the mice after 7 wk of feeding trial. Serum was then separated from the blood by centrifugation at 2000 g for 10 min and stored at -20°C until cytokines is assayed. Serum was used to estimate the presence of IL-1β, IL-6 and TNF-α using mouse IL-1β, IL-6 and TNF-α ELISA kits (R&D systems, USA).

Peritoneal macrophages were obtained using RPMI-1640 medium from mouse peritoneal. The macrophages were washed with RPMI-1640 medium and placed in a density of 1×10^6 cells per well in 96 well plate. Cells were incubated at 37°C in 5% CO_2 for 2 h and plated on

confluent monolayer. After 2 h, non-adherent cells was washed and removed and then suspended by RPMI 1640 supplemented with 1% fetal bovine serum (1% FBS/RPMI).

Cytokine assay (IL-1β, IL-6 and TNF-α)

Serum was used to estimate the presence of IL-1β, IL-6 and TNF-α using mouse IL-1β, IL-6 and TNF-α ELISA kits according to the manufacturer's instructions.

The peritoneal macrophages were suspended in 900 uL of RPMI 1640 supplemented with 1% fetal bovine serum (1% FBS/RPMI) and added 100 μL of FBS each well and incubated at 37ºC in 5% CO_2 for 48 h. After incubation, the culture supernatants (100 μL) were used to estimate the presence of IL-1β, IL-6 and TNF-α using mouse IL-1β, IL-6 and TNF-α ELISA kits according to the manufacturer's instructions.

Differential white blood cell count and tissue preparation

White blood cells (WBC) were enumerated on a cell counter. Differential WBC counts were performed on Wright's-stained smears.

All organs were fixed in 10% formalin, embedded in paraffin, and sectioned at 2-3 μm. Sections were stained with hematoxylin and eosin and examined in the light microscope.

Results and Discussion

Body weight and spleen, thymus index

The mice were fed a diet containing cheese made with *S. macedonicus* LC743, FD-DVS TCC-3, LC743 and FD-DVS TCC-3 (1:1) and a diet without cheese supplementation everyday for seven weeks. For the basic data, body weight and physiological parameters such as spleen index and thymus index were observed. No significant differences of body weight gain were recorded in various groups of animals (Fig. 1). The spleen index was higher the G1 (control group) than the other groups. The thymus index was higher the G3 treated with *S. macedonicus* LC743 than the other groups. But, the spleen index and the thymus index were observed no significant differences (Fig. 2).

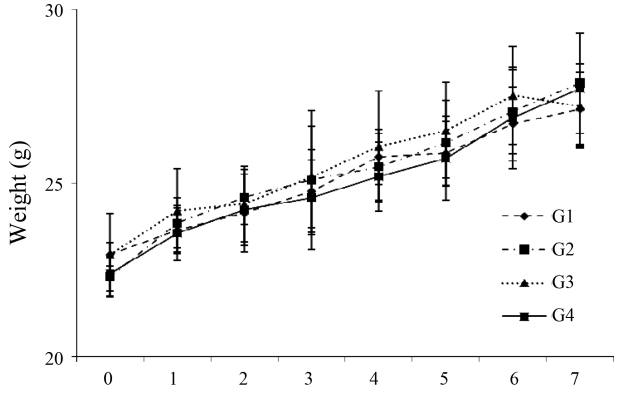

Fig. 1. Body weight changes of C57BL/6 mice fed experimental diets for 7 wk. G1, Control group without cheese supplementation; G2, Diet group containing the cheese (1%) made with TCC-3 (Chr. Hansen A/S); G3, Diet group containing the cheese (1%) made with *S. macedonicus* LC743; G4, Diet group containing the cheese (1%) made with TCC-3 and *S. macedonicus* LC743

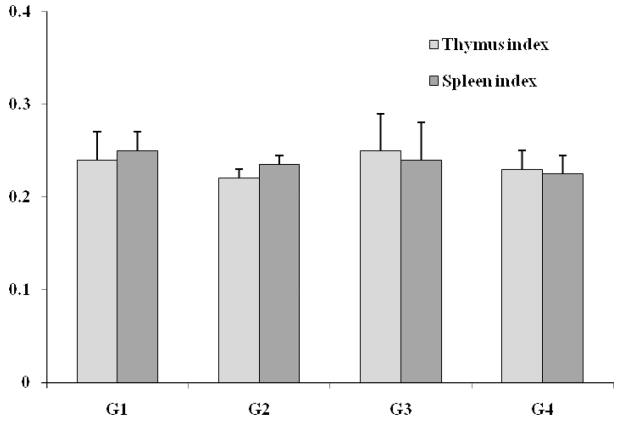

Fig. 2. Lymphatic organ index changes of C57BL/6 mice fed experimental diets for 7 wk. G1, Control group without cheese supplementation; G2, Diet group containing the cheese (1%) made with TCC-3 (Chr. Hansen A/S); G3, Diet group containing the cheese (1%) made with *S. macedonicus* LC743; G4, Diet group containing the cheese (1%) made with TCC-3 and *S. macedonicus* LC743

Cytokine assay (IL-1β, IL-6 and TNF-α)

The macrophages phagocytose the infected cells, preluding the production of antibodies, cytokines and chemokines to modulate immune responses (Morrissette *et al*., 1999). IL-1β, IL-6 and TNF-α are excellent indicators of macrophage activation and play an important role in killing of tumor cells (Mantovani *et al.* 1992). Fig. 3 showed that IL-1β, IL-6 and TNF-α production by mice peritoneal macrophages treated with cheese containing *S. macedonicus* LC743, TCC-3, *S. macedonicus* LC743 + TCC-3 (1:1) and without cheese supplement. The IL-1β level was significantly higher in G2, G3 and G4 treated with cheese supplement than control group. The TNF-α level was significantly higher in G3 and G4 treated with cheeses containing *S. macedonicus* 743 and *S. macedonicus* LC743 + TCC-3 (1:1) than G1 group. The IL-6 level was signifi-

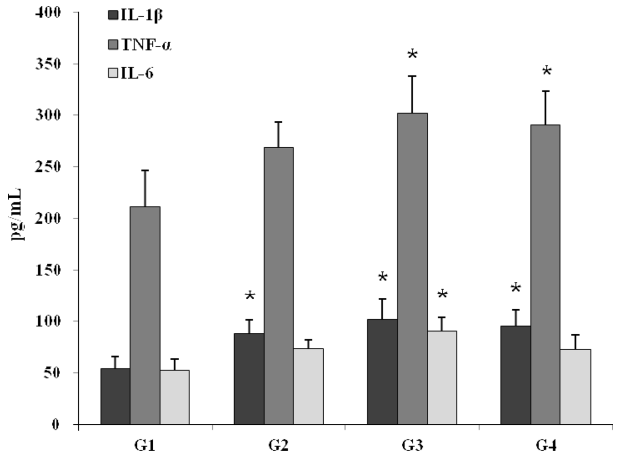

Fig. 3. IL-1β, TNF-α, IL-6 production by peritoneal macrophages of C57BL/6 mice fed experimental diets for 7 wk. *Significant difference from control (p<0.05); G1, Control group without cheese supplementation; G2, Diet group containing the cheese (1%) made with TCC-3 (Chr. Hansen A/S); G3, Diet group containing the cheese (1%) made with *S. macedonicus* LC743; G4, Diet group containing the cheese (1%) made with TCC-3 and *S. macedonicus* LC743

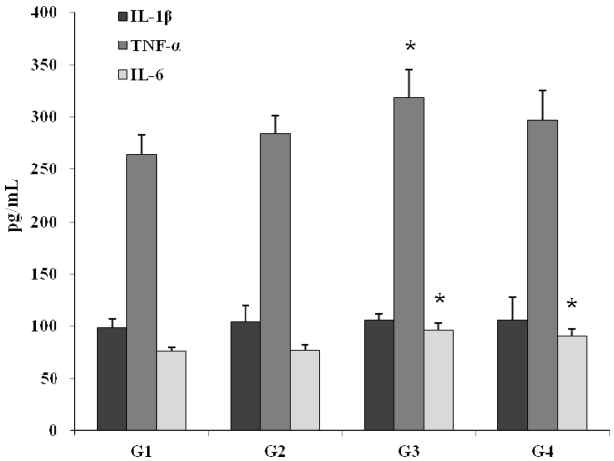

Fig. 4. IL-1β, TNF-α, IL-6 concentration in serum of C57BL/6 mice fed experimental diets for 7 wk. *Significant difference from control (p<0.05); G1, Control group without cheese supplementation; G2, Diet group containing the cheese (1%) made with TCC-3 (Chr. Hansen A/S); G3, Diet group containing the cheese (1%) made with *S. macedonicus* LC743; G4, Diet group containing the cheese (1%) made with TCC-3 and *S. macedonicus* LC743

cantly higher in G3 treated with cheeses containing *S. macedonicus* 743 than other groups. Fig. 4 showed that the serum IL-1β, IL-6 and TNF-α concentration in mice treated with cheese containing *S. macedonicus* LC743 and TCC-3. The IL-1β level was observed no significant differences in all group. G3 group treated with cheese containing *S. macedonicus* LC743 showed the highest production of TNF-α compared to the other groups. The IL-6 level was significantly the higher treated with *S. macedonicus* LC743 and *S. macedonicus* LC743 + TCC-3 (1:1) group than the control group. This result demonstrated that the ingestion of the cheeses containing *S. macedonicus* LC743 and *S. macedonicus* LC743 + TCC-3 (1:1) could be able to activate macrophages.

Differential white cell count and tissue examination

In differential white blood cell counts, the neutrophils percentages were significantly higher in G1 group than other groups. The lymphocytes percentages were significantly higher in G2, G3 and G4 treated with cheeses containing TCC-3, *S. macedonicus* 743 and *S. macedonicus* LC743 + TCC-3 (1:1) than control group. The monocytes, eosinophils and basophils percentages were observed no significant differences in all groups (Table 2). Fig. 5 showed result of photograph and light microscopic findings in the male C57BL/6 mice spleen of experimental diets and control group. The spleen of control group mice was appeared normal (A). The treated mice showed lightly lymphocyte depletion and enlargement of germinal center in spleen (B).

Based on the result of this study, it could be concluded that the *S. macedonicus* LC743 could stimulate the immune functions of mice.

Table 2. Differential white cell counts of C57BL/6 mice fed experimental diets for 7 wk (%)

Group	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
G1	13.14 (±5.84)[a]	84.16 (±7.65)[b]	1.46 (±0.62)	0.68 (±0.36)	0.56 (±0.48)
G2	11.78 (±3.26)[b]	86.12 (±4.04)[a]	1.14 (±0.25)	0.52 (±0.45)	0.44 (±0.55)
G3	11.70 (±6.52)[b]	85.82 (±7.64)[a]	1.46 (±0.82)	0.58 (±0.24)	0.44 (±0.55)
G4	11.75 (±3.33)[b]	85.89 (±3.06)[a]	1.46 (±0.54)	0.41 (±0.63)	0.49 (±0.68)

Values are mean±S.D.

[a-b]Means within a same column with different superscripts are significantly different (p<0.05).

G1, Control group without cheese supplementation; G2, Diet group containing the cheese (1%) made with TCC-3 (Chr. Hansen A/S); G3, Diet group containing the cheese (1%) made with *S. macedonicus* LC743; G4, Diet group containing the cheese (1%) made with TCC-3 and *S. macedonicus* LC743

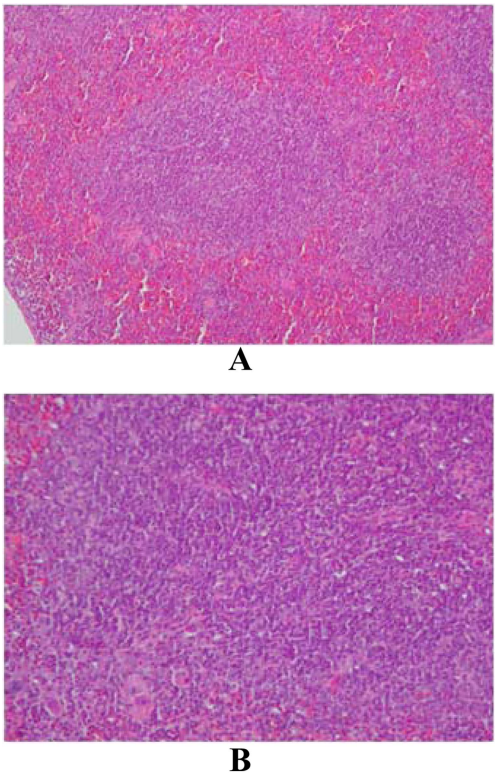

Fig. 5. Photograph and light microscopic findings in the male C57BL/6 mice spleen from control groups (A) and experimental diets (B) (Hematoxylin & Eosin; Magnification: ×200).

Acknowledgement

The study was financially supported by Technology Development Program for Agriculture and Forestry Ministry for Food, Agriculture, Forestry and Fisheries, 2010.

References

1. Blanchette, L., Roy, D., Bélanger, G., and Gauthier, S. F. (1996) Production of cottage cheese using dressing fermented by bifidobacteria. *J. Dairy Sci.* **79**, 8-15.
2. Corbo, M., Albenzio, M., De Angelis, M., Sevi, A., and Gobbetti, M. (2001) Microbiological and biochemical properties of Canestrato Pugliese hard cheese supplemented with bifidobacteria. *J. Dairy Sci.* **84**, 551-561.
3. Czarniecki, C. A. (1993) The role of tumor necrosis factor in viral disease. *Antiviral Res*. **22**, 223-258.
4. Dinakar, P. and Mistry, V. V. (1994) Growth and viability of *Bifidobacterium bifidum* in Cheddar cheese. *J. Dairy Sci.* **77**, 2854-2864.
5. Dinarello, C. A. (1987) The biology of interleukin 1 and comparison to tumor necrosis factor. *Immuno. Letters*. **16**, 227-232.
6. Furtado, M. M., Partridge, J. A., and Ustunol, Z. (1993) Ripening characteristics of reduced fat cheddar cheese using heat-shocked and live *Lactobacillus casei* as adjunt culture. Abstract D28. *J. Dairy Sci.* **76**, 101.
7. Gardiner, G., Ross, R., Collins, J., Fitzgerald, G., and Stanton, C. (1998) Development of a probiotic Cheddar cheese containing human-derived *Lactobacillus paracasei* strains. *Appl. Environ. Microbiol.* **64**, 2192-2199.
8. Gardiner, G., Stanton, C., Lynch, P., Collins, J., Fitzgerald, G., and Ross, R. (1999) Evaluation of Cheddar cheese as a food carrier for delivery a probiotic strain to the gastrointestinal tract. *J. Dairy Sci.* **82**, 1379-1387.
9. Gill, H. S. (1998) Stimulation of the immune system by lactic cultures. *Int. Dairy. J.* **8**, 535-544.
10. Gobbetti, M., Corsetti, A., Smacchi, E., Zocchetti, A., and De Angelis, M. (1998) Production of Crescenza cheese by incorporation of bifidobacteria. *J. Dairy Sci.* **81**, 37-47.
11. Gomes, A. M. P. and Malcata, F. X. (1998) Development of probiotic cheese manufactured from goat milk. *J. Dairy Sci.* **81**, 1492-1507.
12. Kang, H. S., Kim, Y. H., Lee, C. S., Lee, J. J., Choi, I. P., and Pyun, K. H. (1998) Suppression of interleukin-1 and tumor necrosis factor-α production by acanthoic acid, (-)-pimara-9(11), 15-dien-19-oic acid, and its antifibrotic effects *in vivo*. *Cell. Immunol.* **170**, 212-221.
13. Mantovani, A., Bottazzi, B., Colotta, F., Sozzani, S., and Ruco, L. (1992) The origin and function of tumor-associated macrophages. *Immunol. Today* **13**, 265-270.
14. Medici, M., Vinderola, C. G., and Perdigón, G. (2004) Gut mucosal immunomodulation by probiotic fresh cheese. *Int. Dairy J.* **14**, 611-618.
15. Morrissette, N., Gold, E., and Aderem, A. (1999) The macrophage-a cell for all seasons. *Trends Cell Biol*. **9**, 199-201.
16. Murad, H. A., Sadek, Z. I., and Fathy, F. A. (1998) Production of bifidus Kariesh cheese. *Deutsche Lebensmittel-Rundschau*, **94**, 409-412.
17. Oberg, C. J., Wang, A., Moyes, L. V., Brown, R. J., and Richardson, G. H. (1991) Effects of proteolytic activity of thermolactic cultures on physical properties of Mozzarella cheese. *J. Dairy Sci.* **74**, 389-397.
18. Riordan, K. O. and Fitzgerald, G. F. (1998) Evaluation of bifidobacteria for the production of antimicrobial compounds and assessment of performance in cottage cheese at refrigeration temperature. *J. Appl. Microbiol.* **85**, 103-114.
19. Shaw, S. K. and White, C. H. (1994) Survival of bifidobacteria in reduced fat Cheddar cheese. *J. Dairy Sci.* **77**, 4.
20. Stanton, C., Gardiner, G., Lynch, P. B., Collins, J. K., Fitzgerald, G., and Ross, R. P. (1998) Probiotic cheese. *Int. Dairy J.* **8**, 491-496.
21. van der Poll, T., Keogh, C. V., Guirao, X., Buurman, W. A., Kopf, M., and Lowry, S. F. (1997) Interleukin-6 gene-deficient mice show impaired defense against pneumococcal pneumonia. *J. Infect. Dis.* **176**, 439-444.
22. Vinderola, C. G., Prosello, W., Ghiberto, D., and Reinheimer, J. (2000) Viability of probiotic (*Bifidobacterium*, *Lactobacillus acidophilus* and *Lactobacillus casei*) and nonprobiotic microflora in argentinian fresh cheese. *J. Dairy Sci.* **83**, 1905-1911.

(Received 2011.11.25/Revised 2012.1.5/Accepted 2012.1.10)