

## Effects of Feeding *Lactobacillus* spp. on the Level of Cell Glutathione Sulphydryl and Immunoglobulin M in ICR Mice

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**ABSTRACT :** Effects of feeding seven strains of *Lactobacillus* spp. on the level of cell glutathione sulphydryl (GSH) in spleen, liver and erythrocyte of the ICR mice and on the level of immunoglobulin M in the spleen were determined. The level of cell glutathione sulphydryl in the spleen was dependent on the strain of *Lactobacilli*, it was significantly higher in the mice fed with *L. casei* CU 001, *L. rhamnosus* CU 02, *L. acidophilus* NCFM and *L. casei* YIT9018 ( $p > 0.05$ ). The level of cell glutathione sulphydryl in the liver increased in the mice fed with *L. casei* YIT9018, *L. acidophilus* NCFM, *L. casei* CU 001 ( $p > 0.05$ ), the level of glutathione sulphydryl of the erythrocyte showed significantly higher value than control mice when fed with *L. acidophilus* NCFM, *L. casei* YIT9018, *L. casei* CU 001 ( $p > 0.05$ ). The level of immunoglobulin M in the spleen of ICR mice expressed as the plaque count revealed significantly higher value than the control mice when fed with *L. casei* CU 001, *L. acidophilus* NCFM and *L. casei* YIT 9018. (*Asian-Aust. J. Anim. Sci.* 2004, Vol 17, No. 3 : 415-419)

**Key Words :** Immunoenhancing Activity, Glutathione Sulphydryl, Ig M Plaque Forming Cell

### INTRODUCTION

*Lactobacillus* spp. are one of the important component group of intestinal microflora which could afford the host animal not only with specific or nonspecific immunopotentiating capacity but with an antagonistic activities against pathogenic bacteria (Crawford, 1979; Perdigon et al., 1990; Yoon and Won 2002). Whey proteins consisting of alpha-lactalbumin and beta-lactoglobulin could be hydrolyzed and synthesize tripeptide glutathione, an important antioxidant defence mechanism in living cell, which can be used in the tissue as an antioxidant removing hydroxyl ions in the cell to form paired sulphydryl molecule (Bounous et al., 1983; Bounous and Gold 1991; Tammy and Taylor, 1994). They could stabilize DNA molecule during the course of cell multiplication (Anderson, 1985; Carol et al., 1993) and it has been known that sulfur containing amino acids cysteine, glutamic acid and glycine are important constituents synthesizing glutathione molecule which could play an important role in the immune system as a precursor of immunoglobulin (Rose, 1980; Bounous et al., 1989; Yihong et al., 1998).

This study was aimed for finding out the effects of lactobacilli on the synthesizing glutathione molecules in the laboratory animal ICR mouse system, based on the consideration that the proteolytic activities of *Lactobacilli* are variable depending on the species and strains of the lactobacilli (Salminen et al., 1996).

### MATERIALS AND METHODS

#### Bacterial strains and media

The strains and sources of bacteria used in this study are given in Table 1. *Lactobacillus* spp. were cultured in MRS broth (Difco, USA) at 37°C and maintained in 11% skim milk containing 0.75 M adonitol at -70°C.

#### Mice

Male specific pathogen free ICR mice were obtained from Daehan Biolink (Choongbook, Korea) and were acclimated for at least 1 wk before use. The mice were kept under the condition of 12 h light-dark cycle at a controlled temperature (22±2°C) and were supplied with water and feed *ad libitum* and experimental design is shown in Table 2.

**Diets :** The detailed composition of protein free basal diet (Dyets # 111195 (Dyet Inc. USA)) provided in grams per kilogram diet: sucrose, 100; cornstarch, 550.5; dyetrose, 182; soybean oil, 70; cellulose, 50; mineral, 35; vitamin mix, 10; chlorine bitartrate, 2.5. The detailed composition of whey protein isolate (Alcacen 895, New Zealand) was as follows (w/w %): whey protein 94.3, milk fat 0.3, moisture 3.6, lactose 0.1, ash 1.5. The vitamin mixture (AIN-76A) contains grams per kilogram of diet: thiamine HCl, 0.6; riboflavin, 0.6; pyridoxine, 0.7; niacin, 3.0; calcium pantothenate, 1.6; folic acid, 0.6; biotin, 0.02; vitamin B<sub>12</sub>, 1.0; vitamin A palmitate (500,000 IU/g), 0.8; vitamin D<sub>3</sub> (400,000 IU/g), 0.25; vitamin E acetate (500 IU/g) 10.0; menadione sodium bisulfite, 0.08; sucrose, 981. The mineral mix (AIN-76) contains grams per kilogram of diet: calcium phosphate dibasic, 500; sodium chloride, 74; potassium citrate, 220; potassium sulfate, 52; magnesium oxide, 24; manganous carbonate, 3.5; ferric citrate, 6; zinc

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**Table 1.** Sources of strains of *Lactobacillus spp.* used in this investigation

Species	Strains	Source
<i>L. casei</i>	CU 001	Lab. of Dairy Microbiol. Chung-Ang University
<i>L. helveticus</i>	CU 631	"
<i>L. rhamnosus</i>	CU 02	"
<i>L. plantarum</i>	CU 03	"
<i>L. laifimolactis</i>	CU 04	"
<i>L. brevis</i>	CU 05	"
<i>L. brevis</i>	CU 06	"
<i>L. fermentum</i>	CU 07	"
<i>L. salivarius</i>	CU 041	"
<i>L. acidophilus</i>	ATCC 4356	Food Research Institute of Canada
<i>L. rhamnosus GG</i>	ATCC 5310	"
<i>L. acidophilus</i>	NCFM	Yakult R & D Center
<i>L. casei</i>	YIT 9018	"

**Table 2.** Experimental design of mouse trial

Group	No. of mouse	Treatment
Control	9	Basal diets <sup>1</sup> with WPI 20%
WPI 30%	9	Basal diets with WPI 30%
<i>L. casei</i> CU 001	9	Basal diets with WPI 20%, <i>Lactobacilli</i> <sup>2</sup>
<i>L. helveticus</i> CU 631	9	"
<i>L. rhamnosus</i> CU 02	9	"
<i>L. acidophilus</i> ATCC 4356	9	"
<i>L. rhamnosus</i> ATCC 5310	9	"
<i>L. acidophilus</i> NCFM	9	"
<i>L. casei</i> YIT 9018	9	"

<sup>1</sup> Protein free mouse diet. <sup>2</sup> Basal diets with WPI 20% and administrated with various *Lactobacilli* total viable cell of  $10^9$ /mouse/day.

carbonate, 1.6; cupric carbonate, 0.3; potassium iodate, 0.01; sodium selenite 0.01; chromium potassium sulfate, 0.55; sucrose 118.03.

**Immunization for the plaque assay :** The diet -fed mice were immunized by an intravenous injection of  $5 \times 10^6$  sheep red blood cells obtained weekly from Komed Ltd (Seoul, Korea).

#### Plaque forming cell assay

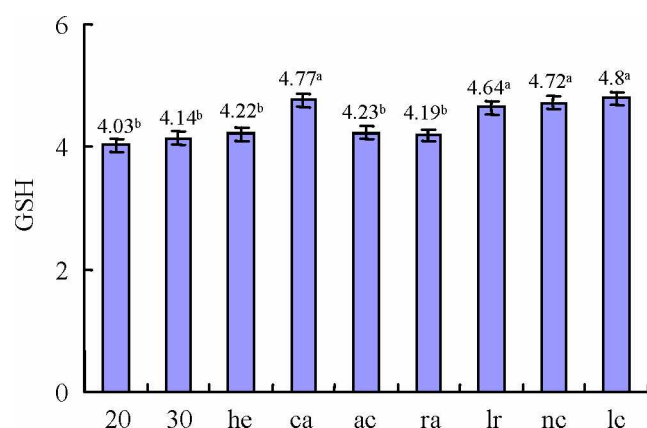
Ig M plaque forming cell were determined by the procedures described by Cunningham and Szenberg (1968). On the fifth day after immunization, spleen cell suspensions were prepared by gently tamping the spleen through a 50 mesh stainless steel screen, and collecting the cells in Earle's balanced salt solution (BSS, Sigma E3261, USA) supplemented with 10% heat inactivated calf serum. The spleen cells were washed with BSS and made up to 15ml with BSS. Sheep red blood cells were washed twice with BSS and made up to a 20% concentration. Guinea pig serum (Komed Ltd, Seoul, Korea) as a source of complement was diluted 1/15 with BSS. All stock solutions were kept on ice water until used. The test consisted of mixing 0.05 ml of spleen cells, 0.15 ml of sheep red blood cells complement solution in a test tube at 37°C. The whole mixture was immediately withdrawn and put into slide

chambers, sealed with warm paraffin wax, and incubated at 37°C for 60 min. The number of plaque forming cells was counted and the total number of plaque forming cells per spleen was estimated by multiplying in each sample (0.05 ml spleen cells) by 300. Plaque forming cells have been expressed per total organ, since this appears to reflect more accurately the functional status of the spleen per se (Cunningham, 1965).

#### Determination of cell glutathione of spleen, liver and erythrocyte

Spleen and liver was washed in ice-cold 0.9% NaCl. Ninety mg of tissue was homogenized by homogenizer, centrifuged at  $3,000 \times g$  for 10 min at 4°C. took 50-100  $\mu$ l of supernatant as a sample and determined the content using assay kit (Calbiochem, USA) following the manufacturers directions. The total volume was adjusted to 900  $\mu$ l with buffer (Solution 3), 50  $\mu$ l of solution R1 was added and mixed thoroughly. After adding 50  $\mu$ l of solution (R2) and mixing thoroughly, the reaction mixture was incubated at 25°C in the dark and  $A_{420}$  was measured (Anderson, 1989).

500  $\mu$ l of whole blood was taken from the heart into the heparin treated tube, centrifuged at  $2,500 \times g$  for 10 min at 4°C and erythrocyte pellet was collected, glutathione in the erythrocyte was determined by the assay kit (Calbiochem,



**Figure 1.** Spleen glutathione content ( $\mu\text{mol/g/wet tissue}$ ) on 5th day following immunization with  $5 \times 10^6$  SRBC. 20; control diet containing 20% WPI, 80% basal diet. 30; WPI fortified diet (30/70) 30% WPI and 70% basal diet. he; control diet with *L. helveticus* CU 631 ( $2 \times 10^9$  cfu/ml), ca; control diet with *L. casei* CU 001 ( $2 \times 10^9$  cfu/ml). ac; control diet with *L. acidophilus* ATCC 4356 ( $2 \times 10^9$  cfu/ml). ra; control diet with *L. rhamnosus* ATCC 5310 ( $2 \times 10^9$  cfu/ml). lr; control diet *L. rhamnosus* CU002 ( $2 \times 10^9$  cfu/ml). nc; control diet with *L. acidophilus* NCFM ( $2 \times 10^9$  cfu/ml). lc; control diet with *L. casei* YIT9018 ( $2 \times 10^9$  cfu/ml).

USA). The results were expressed as  $\mu\text{mol/g}$ .

#### Statistical analysis

Within the same treatment group, for the comparison of the glutathione and IgM values between the treatments, values were compared using SAS Duncan's multiple-range test.

## RESULTS AND DISCUSSION

#### Effects of feeding *Lactobacillus* spp. on the level of spleen cell glutathione sulphydryl (GSH)

The spleen glutathione levels were shown in Figure 1. Mice fed with control diet containing whey protein concentrate and basal diet (20/80) was determined as containing  $4.03 \mu\text{mol/g}$  of reduced glutathione per spleen, whereas mice fed with the diet fortified whey protein isolate (30/70) contained  $4.14 \mu\text{mol/g}$  of glutathione were higher than that of Bounous et al. (1989). The difference between the values of mice fed on control diet and mice fed the fortified diet with whey protein was not statistically significant.

The glutathione contents in spleen of mice fed on the diet fortified with *Lactobacilli* ( $2 \times 10^9$  cfu/ml) were higher than that of control mice fed on control diet. But all treatments do not show statistically significant difference, only mice fed on *L. casei* CU 001, *L. rhamnosus* CU 02, *L. acidophilus* NCFM and *L. casei* YIT 9018 had significantly higher in spleen glutathione than mice fed on

**Table 3.** Liver glutathione (GSH) contents of mice fed with whey protein isolates (WPI) and administrated with *Lactobacillus* spp.

Group	Treatment	GSH content ( $\mu\text{mol/g}$ ) <sup>1</sup>
20	Basal diets <sup>2</sup> with WPI 20%	9.31 <sup>c</sup>
30	Basal diets with WPI 30%	10.12 <sup>bc</sup>
ca	Administrated with LAB <sup>3</sup>	11.22 <sup>a</sup>
he	"	10.59 <sup>ab</sup>
lr	"	11.01 <sup>ab</sup>
ac	"	10.17 <sup>bc</sup>
ra	"	10.18 <sup>bc</sup>
nc	"	11.19 <sup>a</sup>
lc	"	11.31 <sup>a</sup>

<sup>1</sup> Liver glutathione content following immunization with  $5 \times 10^6$  sheep red blood cell.

<sup>2</sup> Protein free mouse diet.

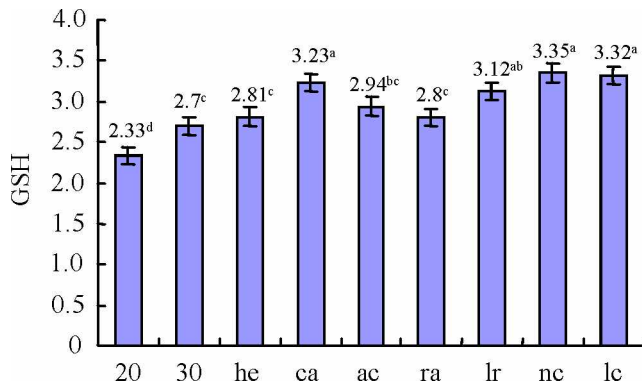
<sup>3</sup> Basal diets with WPI 20% and administrated various lactic acid bacteria. ca; *L. casei* CU 001 ( $2 \times 10^9$  cfu/ml), he; *L. helveticus* CU 631 ( $2 \times 10^9$  cfu/ml), lr; *L. rhamnosus* CU 02 ( $2 \times 10^9$  cfu/ml), ac; *L. acidophilus* ATCC 4356 ( $2 \times 10^9$  cfu/ml), ra; *L. rhamnosus* GG ATCC 5310 ( $2 \times 10^9$  cfu/ml), nc; *L. acidophilus* NCFM ( $2 \times 10^9$  cfu/ml), lc; *L. casei* YIT9018 ( $2 \times 10^9$  cfu/ml). <sup>a-c</sup> Values with different superscripts in the same with are different ( $p < 0.05$ ).

control diet ( $p < 0.05$ ). Beneficial activities of lactic acid bacteria could be explained on the basis of the boosting effect of cell glutathione, an antioxidant in cell, which neutralize free radicals by handing off an electron then pairing off to remain neutral themselves. The destructive hydroxyl radicals is changed to  $\text{H}_2\text{O}$  by getting electron from glutathione sulphydryl molecule (Anderson, 1985). Many kinds of reactive oxygen species can be formed in the human body (oxidative stress), free radicals can damage or destroy cell walls causing apoptosis and disrupt DNA, then pathogenesis could be proceeded. Existence of enough antioxidants is effective for the reduction of reactive radicals by scavenging reactive oxygen species, otherwise glutathione depletion and the subsequent low stores of protein thiol result in both calcium release from intracellular calcium and cytotoxicity (Anderson, 1985; Bounous and Gold, 1991) Elevated scavenging of reactive oxygen by glutathione would be a good trait of *Lactobacilli*.

#### Effects of feeding *Lactobacillus* spp. on the level of liver cell glutathione sulphydryl (GSH)

The liver glutathione levels were shown in Table 3. In the liver cell of mice fed on control diet, the concentration of reduced glutathione was  $9.31 \mu\text{mol/g}$ , whereas that in the liver cell of mice fed on the diet fortified with whey protein isolate (30/70) was  $10.12 \mu\text{mol/g}$ . The difference between whey protein fortified diet and control diet showed no statistical significance, a similar degree of glutathione increasing effects were shown in the group fed on *L. acidophilus* 4356 and *L. rhamnosus* GG ATCC 5310.

All the *Lactobacilli* treated groups except those above two groups significantly higher liver glutathione level than control ( $p < 0.05$ ), and they could be divided into two groups



**Figure 2.** Erythrocytes glutathione content ( $\mu\text{mol/g/wet tissue}$ ) on day 5 following immunization with  $5 \times 10^6$  SRBC. 20; control diet which contains 20% WPI, 80% basal diet, 30; WPI fortified diet (30/70) WPI and 70 basal diet, he; control diet with *L. helveticus* CU 631 ( $2 \times 10^9$  cfu/ml), ca; control diet with *L. casei* CU 001 ( $2 \times 10^9$  cfu/ml), ac; control diet with *L. acidophilus* ATCC 4356 ( $2 \times 10^9$  cfu/ml), lr; control diet with *L. rhamnosus* CU02 ( $2 \times 10^9$  cfu/ml), ra; control diet *L. rhamnosus* GG ATCC 5310 ( $2 \times 10^9$  cfu/ml), nc; control diet with *L. acidophilus* NCFM ( $2 \times 10^9$  cfu/ml), lc; control diet with *L. casei* YIT9018 ( $2 \times 10^9$  cfu/ml).

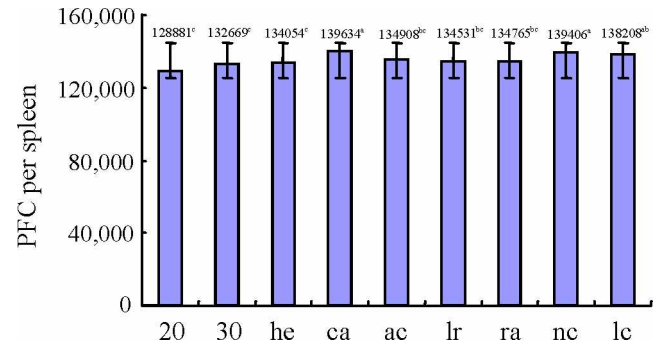
on the basis of glutathione level. The moderately higher group includes *L. helveticus* CU 631 and *L. rhamnosus* CU 02, and intensively higher group is *L. casei* CU 001, *L. acidophilus* NCFM, and *L. casei* YIT9018. Those effects by the *Lactobacilli* were dependent on the species or strains of lactic acid bacteria. average level of glutathione in the liver cells were different. approximately twice of that in spleen. the glutathione level increasing effects of *Lactobacilli* were similar with those in spleen showing the different glutathione synthesis capability in metabolism of each lactobacilli (Fahey et al., 1978).

#### Effects of feeding *Lactobacillus spp.* on the level of erythrocyte cell glutathione sulfhydryl (GSH)

The erythrocyte glutathione levels are shown in Figure 2. Mice fed on control diet showed containing whey 2.33  $\mu\text{mol/g}$  of reduced glutathione. whereas erythrocyte of mice fed on the diet fortified with whey protein isolate (30/70) contained 2.70  $\mu\text{mol/g}$ . significantly higher than that of control diet.

The difference between the increased value by feeding whey protein fortified diet and that of control diet showed statistical significance.

All the lactobacilli treated group showed significantly higher erythrocyte glutathione level than control ( $p < 0.05$ ). The treated group could be divided into three groups on the basis of glutathione level, a moderately higher group including *L. helveticus* CU 631 and *L. rhamnosus* ATCC 5310, a higher group of *L. acidophilus* ATCC 4356 and *L. rhamnosus* CU02, and an intensively higher group consisting *L. casei* CU 001, *L. acidophilus* NCFM, and *L.*



**Figure 3.** Plaque forming cells/spleen (PFC) on 5th day following immunization with  $5 \times 10^6$  SRBC in ICR mouse. 20; control diet which contains 20% WPI, 80% basal diet, 30; WPI fortified diet (30/70) WPI and 70 basal diet, he; control diet with *L. helveticus* CU 631 ( $2 \times 10^9$  cfu/ml), ca; control diet with *L. casei* CU 001 ( $2 \times 10^9$  cfu/ml), ac; control diet with *L. acidophilus* ATCC 4356 ( $2 \times 10^9$  cfu/ml), lr; control diet with *L. rhamnosus* CU02 ( $2 \times 10^9$  cfu/ml), ra; control diet *L. rhamnosus* GG ATCC 5310 ( $2 \times 10^9$  cfu/ml), nc; control diet with *L. acidophilus* NCFM ( $2 \times 10^9$  cfu/ml), lc; control diet with *L. casei* YIT9018 ( $2 \times 10^9$  cfu/ml).

*casei* YIT9018. Those effect of *Lactobacilli* on glutathione content in erythrocyte were similar in spleen and liver showing that the value of erythrocyte glutathione level seemed to be the indicative to those of spleen and liver, and could be used as an indicative values.

The level of cellular glutathione depends upon the supply of its constituent amino acids. of which cysteine is usually limiting, and the upper level of cellular glutathione is controlled by feedback inhibition of gamma-glutamylcysteine synthetase by glutathione. Thus enhancement of glutathione levels by the lactic acid bacteria could be achieved by the supply of cysteine by the variable proteolytic activity of lactobacilli (Fahey et al., 1978; Johnston et al., 1993).

#### Enhancement of Ig M level by feeding the *Lactobacilli*

Immunoglobulin M levels in the spleen of mice was determined by the IgM plaque forming cell assay methods (Cunningham and Szenberg, 1968) and the results are shown in Figure 3. Mice fed with control diet containing whey protein isolate and basal diet (20/80) were determined as containing  $128 \times 10^3$  plaque forming cells per spleen. whereas mice fed with the diet fortified whey protein isolate (30/70) contained  $132 \times 10^3$  plaque forming cells per spleen. The difference between the increased value by feeding whey protein fortified diet and that of control diet showed no statistical significance. The results of control diet and control diet and diet with *Lactobacilli* ( $2 \times 10^9$  cfu/ml) revealed significantly different. All the lactobacilli treated group showed significantly higher plaque forming cells than control ( $p < 0.05$ ). *L. casei* CU 001, *L. acidophilus* NCFM

exerted a most intensive immunomodulating effect. Those effects by the *Lactobacilli* were dependent on the species or strains of lactic bacteria. As dietary cysteine content is a rate limiting substrate for the synthesis of glutathione, which is necessary for the proliferation of lymphocyte, modulation of intracellular glutathione by feeding lactic acid bacteria may have affected immune responsiveness enhancing immunomodulatory activity. *In vivo* administration of GSH has been demonstrated to enhance the activation of cytotoxic T-cells, but depletion of GSH intracellularly inhibited the activation of lymphocytes, increased susceptibilities of human lymphoid cells to radiation, suggesting that intracellular GSH can modulate the function of immune cells, and a deficiency in GSH may contribute to the immunodeficiency (Roederer et al., 1992). There seemed a tendency that a higher GSH level paralleled a higher level of IgM forming plaque cells in this experiment.

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