Screening and Characterization of *Lactobacillus casei* MCL Strain Exhibiting Immunomodulation Activity

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Abstract

As an appraisal for the application of a new starter culture, more than 200 lactic acid bacteria strains were isolated from raw milk and healthy human feces. The strains showing excellent growth and acid production ability in 10% skim milk media were selected and identified as *Lactobacillus casei* based on the results of their API carbohydrate fermentation patterns, as well as 16S rDNA sequence analysis. To assess the effect of *L. casei* strains on irritable bowel disease (IBD), the inhibitory effect of the selected strains against the nitric oxide (NO) production of lipopolysaccharide (LPS)-stimulated RAW 264.7 cells was measured. Among the tested *L. casei* strains, *L. casei* MCL was observed to have the greatest NO inhibitory activity. Additionally, *L. casei* MCL was found to inhibit mRNA expression of pro-inflammatory cytokines (interleukin-1 β , IL-6, TNF- α), as well as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) involved in pathophysiologic processes such as inflammation. The mRNA expression of anti-inflammatory cytokines, including IL-10 and transforming growth factor- β (TGF- β) of *L. casei* MCL, was confirmed using quantitative real-time PCR. In conclusion, *L. casei* MCL showed decreases in the expression of pro-inflammatory cytokines and up-regulated expression of the anti-inflammatory cytokine.

Key words: immunomodulating, *Lactobacillus casei*, probiotics, cytokine

Introduction

As it is a well-established fact that many gastrointestinal disorders are associated with changes in the intestinal mucosal function and the stability of the intestinal microflora by probiotics, it would be interesting to determine whether fermented milk products containing probiotics could serve as a new functional food to confer health care benefits. Probiotics can be defined as “live microbial food ingredients that, when administered in adequate amounts, confer a health benefit on a host” (FAO/WHO, 2001). Since lactic acid-producing bacteria confer various advantageous effects by improving the ecosystem in the gut, they are referred to as probiotics.

Lactic acid bacteria are widely used as probiotic microorganisms because of the health and nutritional benefits contributed to the host, such as maintaining the balance of the intestinal microflora, lowering of serum cholesterol, and enhancement of immunopotentiating activity and γ -

aminobutyric acid (GABA) production (Fuller, 1993; Gilliland and Walker, 1990; Sanders, 1993). In addition, a variety of convincing evidence regarding the anti-inflammatory properties of probiotics has been proposed by many studies (Lee *et al.*, 2008; Menard *et al.*, 2004). Among the probiotics, *L. casei* strains may be isolated from raw or fermented dairy products, gastrointestinal tracts, and the reproductive systems of humans and animals, as well as fermented plant products (Kandler and Weiss, 1986). The *L. casei* YIT 9029 strain is a typical common probiotic, the immune modulation activities of which have been studied extensively (de Waard *et al.*, 2001; Kato *et al.*, 1984; Matsumoto *et al.*, 2005; Matsubuchi *et al.*, 2003). This strain has yielded beneficial results in clinical trials with bladder cancer patients (Kakizoe, 2000) and has been shown to prevent atypia in colorectal tumors, although the exact mechanism of prevention has yet to be elucidated (Ishijawa *et al.*, 2005).

Inflammation occurs when the host's immune system attempts to counteract injurious agents such as invading pathogens (Henderson *et al.*, 1996; Ulevitch and Tobias, 1995). The immunoregulatory mechanisms that include regulatory cells, cytokines, and apoptosis, among others, contribute in controlling immune responses by preventing

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pathologic infection associated with excessive reactivity. The lack of these mechanisms results in many inflammatory diseases such as inflammatory bowel diseases (IBDs). The production of cytokines triggered by bacterial components such as lipopolysaccharide (LPS), lipoteichoic acid (LTA) and peptidoglycan can lead to the development of systemic inflammatory response syndrome. LPS activates the cells of the innate immune system to produce a variety of inflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-8, TNF- α , and NO (Wai *et al.*, 2004). Nitric oxide (NO) is highly reactive molecule generated endogenously from L-arginine by a family of NO synthase (NOS) isoenzymes (Coleman, 2001). The isoenzyme present in macrophage is an inducible form (iNOS), able to produce high concentrations to NO in many cells after stimulation with bacterial endotoxins, LPS or different proinflammatory cytokines including TNF- α , IFN- γ , IL-1, IL-6, etc (Nathan, 1994; Stuehr and Marlette, 1985; Stuehr and Marlette, 1987). Among several inflammatory diseases, IBDs are closely connected with lactic acid bacteria (Lee *et al.*, 2008). IBD patients may suffer from abdominal pain, diarrhea, intestinal obstruction, and/or bloody stool. To date, therapies such as anti-inflammatory drugs, steroids, or anti-tumor necrosis factor can effectively reduce the symptoms and signs associated with IBD, while there remains no known medical cure for IBD (Hanauer, 2002; Macdonald and Monteleone, 2005).

Therefore, the NO inhibitory ability and cytokine profile of *L. casei* strains were assessed in this study in order to determine the immunomodulating potential of *L. casei* strains.

Materials and methods

Identification of *L. casei* MCL

The bacterial 16S rRNA gene was amplified by PCR using the HotStarTaq[®] Plus Master Mix Kit (Qiagen, USA) containing DNA polymerase, dNTPs, Tris-HCl (pH 9.0), KCl, MgCl₂, and primers pBact27F (5'-AGAGTTTGA-TCMTGGCTCAG-3') and pUniv 1492R (5'-GGYTAC-CTTGTTACG ACTT-3') (Lane, 1991). The PCR product was purified using a PCR purification kit (Qiagen, USA). Gene cloning, DNA transformations using *E. coli*, DNA manipulations, and agarose gel electrophoresis were performed as reported by Sambrook *et al.* (1989). The 16S rRNA gene was sequenced at Solgent Corp. (Korea).

Carbohydrate fermentation patterns were measured using an API 50 CHL carbohydrate test kit (bioMerieux Co., France). Briefly, bacteria were cultured in de Man,

Rogosa and Sharpe (MRS) broth (Difco, USA) at 37°C for 12 h and centrifuged at 17,940 g for 10 min. After centrifugation, the cells were washed twice in 1X PBS buffer. Turbidity was assessed using the McFarland Standard 4, and 3 mL of the diluted sample was inoculated into API 50 CHL medium. Subsequently, 100 μ L of sample were inoculated into each API test well containing 49 different carbohydrates. The fermentation profiles were confirmed by the manufacturer's API web site (<https://apiweb.biomerieux.com>).

Culture characteristics in skim milk

L. casei MCL isolated from raw milk and *L. casei* YIT 9029 obtained from Korea Yakult Co. were tested for their viable cell count, pH, and acidimetry. To measure the viable cell count, cells were cultured in MRS broth twice and inoculated in 10% skim milk supplemented with 2% glucose. Cells were counted at an interval of 4 h for a total of 36 h. Cultured cells were diluted in anaerobic dilution solution and applied to MRS agar. The plates were cultured in an incubator for 48 h at 37°C. The pH was tested using a pH meter (M530P, Nova Analytics Corp., USA).

Preparation of bacterial components for nitric oxide assay

LAB were cultured in MRS broth at 37°C for 12 h and harvested by centrifugation at 10,000 g for 15 min. After bacterial cells were washed twice with 1×PBS buffer, samples were heated at 100°C for 10 min and centrifuged at 10,000 g for 30 min and the supernatant was used for the NO assay after filtration.

The strains grown in skim milk medium at 37°C for 24 h and were heated at 100°C for 10 min and centrifuged at 10,000 g for 30 min.

Cell culture

RAW 264.7 cells obtained from the Korean Cell Line Bank (Seoul, Korea) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% 100 U/mg penicillin/streptomycin. All cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂. The cell number and viability were assessed by Trypan blue dye exclusion on a Neubauer hemocytometer. Cells were cultured to confluence in sterile tissue culture dishes and gently detached by repeated pipetting. For the experiments, cells were cultured in triplicate at a density of 5×10^5 in 24-well tissue culture plates (Nunc, Denmark). Cell cultures were incubated for 24 h

and analyzed for NO concentration.

Nitric oxide assay

To assess the NO production-inhibitory activity of *L. casei* MCL, the RAW 264.7 cells ($1 \times 10^6/\text{mL}$) previously cultured in DMEM were stimulated for 24 h using LPS (50 ng/mL) and 10% of LAB fractions (the supernatant of bacterial cell suspension or skim milk culture). The concentration of NO was determined by measuring the amount of nitrite in the cell culture supernatant using Griess reagent (Sigma, USA) according to the manufacturer's protocol. The cultured RAW 264.7 cells were briefly centrifuged at 1,000 g for 10 min, with 150 μL of the cell culture supernatant mixed with 150 μL of Griess reagent and subsequently incubated for 10 min at room temperature. The absorbance was measured using a microplate reader at 540 nm and compared to a calibration curve generated using sodium nitrite as a standard.

Preparation of cDNA and real-time PCR

Total RNA was collected from stimulated RAW 264.7 cells using TRIzol reagent (Invitrogen, USA), as described by the manufacturer's protocol. RNA was further isolated using the RNeasy Mini Kit from Qiagen (USA), in accordance with the manufacturer's protocol. The remaining DNA in the extract solution was removed with the DNase I kit (Ambion, USA), and cDNA was synthesized using SuperScript III (Invitrogen, USA). The concentration of cDNA was measured using a NanoDrop spectrophotometer (Thermo Scientific, USA) and adjusted to 100 ng/ μL .

The expression of selected cytokines in RAW 264.7 cells exposed to *L. casei* MCL cellular components and skim milk cultures was determined by real-time quantitative PCR using SYBR Green technology with the ABI 7900HT (Applied Biosystems, USA). Primers used for the PCR included glyceraldehydes-3-phosphate dehydrogenase (GAPDH), COX-2, iNOS, TNF- α , IL-1 β , IL-6, IL-10, and TGF- β , and the cDNA sequences were obtained from GenBank with accession numbers XM_124295.1, NM_000963, NM_010927, XM_123058.1, NM_008361.3, NM_031168.1, NM_010548.2, NM_011577.1, respectively. The nucleotide sequences of the primers and probes are shown in Table 1.

Statistical analysis

All samples were measured in triplicate. Statistical analyses were performed using SPSS 18.0 (SPSS for windows, USA). The results of cytokine expression are analyzed by one way ANOVA followed by Turkey's multiple comparisons test. All samples were considered statistically significant at $p < 0.05$.

Result and Discussion

Identification and DNA sequencing of *L. casei* MCL

The strain MCL isolated from raw milk was a catalase negative, non-motile, gram-positive, rod-shaped bacterium. Most of these features are characteristic of the genus *Lactobacillus*. Biochemical and molecular biological identification were attempted. Carbohydrate fermenta-

Table 1. Primer sequences used for real-time PCR analysis

Gene		Primer sequences	Size (bp)
GAPDH	F	5'-CATGGCCTTCCGTGTTCTCTAC-3'	122
	R	5'-TCAGTGGGCCCTCAGATGC-3'	
COX-2	F	5'-CTCAGCCATACAGCAAATCCTT-3'	101
	R	5'-GTCCGGGTACAATCGCACTTAT-3'	
iNOS	F	5'-CCAGCCTGCCCTTCAAT-3'	104
	R	5'-ATCCTTCGGCCCACTTCCT-3'	
IL-1 β	F	5'-TGACGGACCCCAAAGAT-3'	122
	R	5'-GTGATACTGCCTGCCTGAAG-3'	
IL-6	F	5'-CCGAGAGGAGACTTCACAGAG-3'	107
	R	5'-TCATTCCACGATTCCCAGAG-3'	
TNF α	F	5'-AGGCACTCCCCAAAAGATG-3'	122
	R	5'-CACCCCGAAGTTCAGTAGACAGA-3'	
IL-10	F	5'-GCTGGACAACATACTGCTAACCGACTC-3'	141
	R	5'-TCCTTGATTCTGGGCCATGCTTCTCT-3'	
TGF- β	F	5'-GCTACCATGCCAACTTCTGTCTG-3'	106
	R	5'-GAAGCGCCCGGGTTGTGTTGGTTGTAG-3'	

tion patterns showed that this strain was identified as *L. paracasei* (99.9%) according to the API database. In addition, the 16S rDNA gene sequence of *L. casei* MCL was deposited into GenBank under the accession number of JN974882. Identification of *L. casei* at the strain level is crucial for a variety of industrial applications, as it facilitates the tracking of specific strains with industrially-relevant properties.

The *L. casei* MCL isolated from raw milk and *L. casei* YIT 9029 were tested for their carbohydrate fermentation patterns using the API 50 CHL kit. The results showed that the two strains used carbohydrates such as ribose, galactose, D-glucose, D-fructose, D-mannose, mannitol, sorbitol, N-acetyl glucosamine, esculin, salicin, D-cellobiose, maltose, lactose, saccharose, trehalose, D-turanose, gentiobiose, D-tagatose and gluconate (Table 2). D-adonitol, L-sorbose, amygdalin, and arbutin were only fermented by *L. casei* YIT 9029, and inulin and D-melezitose were only fermented by *L. casei* MCL.

Culture characteristics of *L. casei* MCL in skim milk

Recently, *L. casei* strains have been widely used as a starter culture for yogurt and other fermented milk prod-

ucts in the dairy industry. To use LAB as a starter culture, the viable count should be above 6 Log CFU/g in order to supply a sufficient dairy dose (Samona and Robinson, 1991). Additionally, the pH should be between 4.2 and 4.4 in order to produce the organoleptic qualities of sourness and for the firm coagulum to be satisfactory (Narvhus, 1996). Among the 204 strains isolated from healthy adult feces and raw milk, only six strains were selected based on their consecutive culturability in 10% skim milk. Finally, *L. casei* MCL isolated from raw milk was assessed for viable cell count, pH, and titratable acidity. As shown in Fig. 1, the pH change of *L. casei* MCL was slightly higher than *L. casei* YIT 9029. After 16 h, the pH of *L. casei* YIT 9029 rapidly decreased, while the final pH for *L. casei* MCL was found to be similar to that of *L. casei* YIT 9029 (Fig. 1). The viable cell count was 9 Log CFU/g for both *L. casei* YIT 9029 and *L. casei* MCL. Based on these results, *L. casei* MCL was determined to have potential as a starter culture.

Effect of *L. casei* MCL on nitric oxide production

NO is a suspected mediator in the development of diseases that are associated with chronic inflammation, including cancer. Practically every cell and many immu-

Table 2. Carbohydrate fermentation profiles of *L. casei* YIT 9029 and *L. casei* MCL*

Carbohydrate	9029	MCL	Carbohydrate	9029	MCL
Control	-	-	Esculin ferric citrate	+	+
Glycerol	-	-	Salicin	+	+
Erythritol	-	-	D-cellobiose	+	+
D-arabinose	-	-	D-maltose	+	+
L-arabinose	-	-	D-lactose	+	+
D-ribose	+	+	D-melibiose	-	-
D-xylose	-	-	D-saccharose	+	+
L-xylose	-	-	D-trehalose	+	+
D-adonitol	+	-	Inulin	-	+
Methyl-β-D-xylopyranoside	-	-	D-melezitose	-	+
D-galactose	+	+	D-raffinose	-	-
D-glucose	+	+	Amidon	-	-
D-fructose	+	+	Glycogen	-	-
D-mannose	+	+	Xylitol	-	-
L-sorbose	+	-	Gentiobiose	+	+
L-rhamnose	-	-	D-turanose	+	+
Dulcitol	-	-	D-lyxose	-	-
Inositol	-	-	D-tagatose	+	+
D-mannitol	+	+	D-fucose	-	-
D-sorbitol	+	+	L-fucose	-	-
Methyl-α-D-mannopyranoside	-	-	D-arabitol	-	-
Methyl-α-D-glucopyranoside	-	-	L-arabitol	-	-
N-acetylglucosamine	+	+	Potassium gluconate	+	+
Amygdalin	+	-	Potassium 2-ketogluconate	-	-
Arbutin	+	-	Potassium 5-ketogluconate	-	-

*The values indicate positive (+) and negative (-) fermentation for each strain.

Fig. 1. Culture characteristics of *L. casei* MCL and *L. casei* YIT 9029 in 10% skim milk supplemented with 2% glucose. (a) pH and titratable acidity, (b) viable cell count of the bacteria.

nological parameters are modulated by NO (Cirino *et al.*, 2006). NO also has several biological functions in many types of immune cells including the bactericidal effects of macrophages and signal transduction during inflammation (Jeong *et al.*, 2010). The NO inhibiting rate of *L. casei* strains expressed relative rate (%) compared with NO concentration of positive control. The positive control is only treated to LPS and the negative control is only treated to 1X PBS buffer in RAW264.7. As shown in Fig. 2, the rate of NO inhibition by *L. casei* MCL was significantly higher than that by the *L. casei* YIT 9029 strain. Moreover, *L. casei* MCL exhibited the highest inhibitory rate (%) among the other *L. casei* strains (Fig. 2b). The positive control (LPS-treatment sample) produced 21.91 μ M NO and *L. casei* MCL produced 13.02 μ M NO (Fig. 2a). Therefore, we infer that *L. casei* MCL may exert an anti-inflammatory effect by inhibiting NO production.

Anti-inflammatory effect of cell components obtained from *L. casei* MCL

The mRNA expression of pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6) and enzymes involved in NO

Fig. 2. (a) NO concentration (μ M) and (b) NO inhibitory rate (%) in RAW 264.7 cells treated with the cellular components of seven *L. casei* strains.

production (iNOS and COX-2) were determined using quantitative real-time PCR. iNOS is a key factor in the production of NO in macrophages stimulated by bacterial endotoxin (LPS), which in turn participates in the inflammatory response with pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 (Nathan and Xie, 1994). IL-6 is a potent mitogenic polypeptide and stimulates cell proliferation in a various types of cells (Stein and Sutherland, 1998). Therefore, pro-inflammatory cytokines are important indicators of inflammation. According to Morotomi *et al.* (1996), the *L. casei* YIT 9029 strain has been demonstrated potential, when administered dietarily, to prevent cancer through its immunostimulating activity on experimentally-implanted tumors. Similarly, our results have also shown that *L. casei* YIT 9029 increases the expression of COX-2, iNOS, and IL-1 β (data not shown). On the other hand, *L. casei* MCL was found to suppress all pro-inflammatory genes (Fig. 3). This indicates that *L. casei* MCL has the potential to enhance anti-inflammatory responses. All experiments were conducted in triplicate. Following stimulation with LPS for 24 h, lower levels of pro-inflammatory cytokines were observed in RAW 264.7 cells from the *L. casei* MCL and LPS-treated sample compared with the sample treated with LPS only.

Stimulation with lipopolysaccharides causes an increase in the secretion of anti-inflammatory IL-10, which may respond to the local effects of inflammation (Yeganegi *et*

Fig. 3. mRNA expression levels of pro-inflammatory cytokines (a) COX-2, (b) iNOS, (c) TNF- α , (d) IL-1 β , (e) IL-6 in RAW 264.7 cells treated with the cellular components of *L. casei* MCL. ^{a-c}Means with different letters are significantly different ($p < 0.05$).

al., 2010). IL-10 has also been shown to inhibit IL-1 β , prostaglandin and TNF- α production in rhesus monkeys (Sadovsky *et al.*, 2003). In addition, it was reported to act

Fig. 4. mRNA expression levels of anti-inflammatory cytokines (a) IL-10, (b) TGF- β in RAW 264.7 cells treated with the cellular components of *L. casei* MCL. ^{a-c}Means with different letters are significantly different ($p < 0.05$).

as a potent macrophage deactivator by blocking the induced synthesis of IL-6, IL-8, and COX-2 in human monocytes (Lee *et al.*, 2009; Pena *et al.*, 2003; Trushin *et al.*, 2003).

Lactobacillus paracasei induces populations of regulatory CD4⁺ cells, which produce high levels of modulatory cytokines including IL-10 and TGF- β . As shown in Fig. 4, *L. casei* MCL up-regulated the level of IL-10, but did not have an effect on the expression of TGF- β . An increase in the secretion of the IL-10 anti-inflammatory cytokine by *L. casei* MCL may serve as a potential mechanism for preventing gastrointestinal infection and inflammation. Conclusively, *L. casei* MCL significantly decreased the secretion of pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α and increased the secretion of anti-inflammatory cytokines such as IL-10.

Anti-inflammatory effect of fermented milk from *L. casei* MCL

To confirm the immune effects of milk fermented with the *L. casei* MCL strain, the strain was cultured in 10% skim milk supplemented with 2% glucose for 24 h. The fermented milk containing *L. casei* MCL tended to reduce the secretion of particular pro-inflammatory cytokines such as COX-2, IL-6, and TNF- α in RAW 264.7 cells ($p < 0.05$) (Fig. 5) and TGF- β to levels similar to those observed in the control sample ($p < 0.05$) (Fig. 6). How-

Fig. 5. mRNA expression levels of pro-inflammatory cytokines (a) COX-2, (b) iNOS, (c) TNF- α , (d) IL-1 β , (e) IL-6 in RAW 264.7 cells treated with milk fermented by *L. casei* MCL. ^{a-c}Means with different letters are significantly different ($p < 0.05$).

Fig. 6. mRNA expression levels of anti-inflammatory cytokines (a) IL-10, (b) TGF- β in RAW 264.7 cells treated with milk fermented by *L. casei* MCL. ^{a,b}Means with different letters are significantly different ($p < 0.05$).

ever, contrary to the bacterial component, milk fermented with the *L. casei* MCL was only able to repress the expression of COX-2, TNF- α , IL-6 ($p < 0.05$), and TGF- β to levels similar to those observed in the control sample ($p < 0.05$). These results suggest that milk fermented by *L. casei* MCL may influence both specific and nonspecific immune responses. *Lactobacillus rhamnosus* GG down-regulates the inflammatory response after milk consumption in milk-hypersensitive adults (Majamaa and Isolauri, 1997). Thus, the fermented milk from *L. casei* MCL may reduce the inflammatory response by decreasing the secretion of pro-inflammatory cytokines. It may also have the potential for application in immunomodulating fermented milk. Future studies are required to confirm the immunomodulating activity of *L. casei* MCL *in vivo*.

Conclusions

We investigated the effect of immunomodulating of *L. casei* strains on NO and cytokine expression in several experiments. This study confirms that when the macrophages were infected with LPS, *L. casei* MCL inhibited production of NO and proinflammatory cytokines. It is probably assume that *L. casei* MCL had immune regulation activity in macrophage. Inhibiting the substances related with inflammation reaction might show beneficial effect

about gastrointestinal tract.

To get more insight into immunomodulating effect by probiotics, future studies combining cytokine gene expression and protein assay in disease models would be useful.

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References

1. Cirino, G., Distrutti, E., and Wallace, J. L. (2006) Nitric oxide and inflammation. *Inflamm. Allergy Drug Target* **5**, 115-119.
2. Coleman, J. W. (2001) Nitric oxide in immunity and inflammation. *Int. Immunopharmacol.* **1**, 1397-1406.
3. de Waard, R., Garssen, J., Snel, J., Bokken G. C., Sako, T., Veld, J. H., and Vos, J. G. (2001) Enhanced antigen-specific delayed-type hypersensitivity and immunoglobulin G2b responses after oral administration of viable *Lactobacillus casei* YIT 9029 in Wistar and Brown Norway rats. *Clin. Diagn. Lab. Immunol.* **8**, 762-767.
4. Food and Agriculture Organization of the United Nations. (2001) Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. (http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf)
5. Fuller, R. (1993) Probiotic foods, current use and future developments. *Int. Food Inged.* **3**, 23-26.
6. Gilliland, S. E. and Walker, D. K. (1990) Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans. *J. Dairy Sci.* **73**, 905-911.
7. Hanauer, S. B. (2002) New steroids for IBD: progress report. *Gut* **51**, 182-183.
8. Henderson, B., Poole, S., and Wilson, M. (1996) Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. *Microbiol. Rev.* **60**, 316-341.
9. Ishikawa, H., Akedo, I., Otani, T., Suzuki, T., Nakamura, T., Takeyama, I., Ishiquro, S., Miyaoka, E., Sobue, T., and Kakizoe, T. (2005) Randomized trial of dietary fiber and *Lactobacillus casei* administration for prevention of colorectal tumors. *Int. J. Cancer* **116**, 762-767.
10. Jeong, I. Y., Lee, H. J., Jin, C. H., Park, Y. D., Choi, D. S., and Kang, M. A. (2010) Anti-inflammatory activity of *Stevia rebaudiana* in LPS-induced RAW 264.7 cells. *J. Food Sci. Nutr.* **15**, 14-18.
11. Kakizoe, T. (2000) Asian studies of cancer chemoprevention: latest clinical results. *Eur. J. Cancer* **36**, 1303-1309
12. Kandler, O. and Weiss, N. (1986) Genus *Lactobacillus* Beijerinck 1901. In: Bergey's manual of systematic bacteriology, Vol. 2. Sneath, P. H. A., Mair, N. S., Sharpe, M. E., and Holt, J. G. (eds) Williams and Wilkins, Baltimore, pp. 1209-1234.
13. Kato, I., Yokokura, T., and Mutai, M. (1984) Augmentation of mouse natural killer cell activity by *Lactobacillus casei* and its surface antigens. *Microbiol. Immunol.* **27**, 209-217.
14. Lane, D. J. (1991) 16S/23S rRNA sequencing. In: Nucleic acid techniques in bacterial systematics. Stackebrandt, E. & Goodfellow, M. (eds) John Wiley, Chichester, pp. 115-175.
15. Lee, J. H., Lee, B., Lee, H. S., Bae, E. A., Lee, H., Ahn, Y. T., Lim, K. S., Huh, C. S., and Kim, D. H. (2009) *Lactobacillus suntoryeus* inhibits pro-inflammatory cytokine expression and TLR-4-linked NF- κ B activation in experimental colitis. *Int. J. Colorectal Dis.* **24**, 231-237.
16. Lee, H. S., Han, S. Y., Bae, E. A., Huh, C. S., Ahn, Y. T., Lee, J. H., and Kim, D. H. (2008) Lactic acid bacteria inhibit proinflammatory cytokine expression and bacterial glycosaminoglycan degradation activity in dextran sulfate sodium-induced colitic mice. *Int. Immunopharmacol.* **8**, 574-580.
17. Macdonald, T. T. and Monteleone, G. (2005) Immunity, inflammation, and allergy in the gut. *Science* **307**, 1920-1925.
18. Majamaa, H. and Isolauri, E. (1997) Probiotics: a novel approach in the management of food allergy. *J. Allergy Clin. Immunol.* **99**, 175-185.
19. Matsubuchi, T., Takagi, A., Matsuzaki, T., Nagaoka, M., Ishikawa, K., Yokokura, T., and Yoshikai, Y. (2003) Lipoteichoic acids from *Lactobacillus* strains elicit strong tumor necrosis factor α -inducing activities in macrophages through Toll-like receptor 2. *Clin. Diagn. Lab. Immunol.* **10**, 259-266.
20. Matsumoto, S., Hara, T., Hori, T., Mitsuyama, K., Nagaoka, M., Tomiyasu, N., Suzuki, A., and Sata, M. (2005) Probiotic *Lactobacillus*-induced improvement in murine chronic inflammatory bowel disease is associated with the down-regulation of pro-inflammatory cytokines in lamina propria mononuclear cells. *Clin. Exp. Immunol.* **140**, 417-426.
21. Menard, S., Candali, C., Bambou, J. C., Terpend, K., Cerf-Bensussan, N., and Heyman, M. (2004) Lactic acid bacteria secrete metabolites retaining anti-inflammatory properties after intestinal transport. *Gut* **53**, 821-828.
22. Morotomi, M. (1996) Properties of *Lactobacillus casei* Shirota strain as probiotics. *Asia Pacific J. Clin. Nutr.* **5**, 29-30.
23. Narvhus, J. (1996) Probiotiske bakterier-metabolisme in melk. *Meieriposten* **12**, 341-343.
24. Nathan, C. and Xie, Q. W. (1994) Nitric oxide synthases: roles, tolls and controls. *Cell* **78**, 915-918.
25. Pena, J. A. and Versalovic, J. (2003) *Lactobacillus rhamnosus* GG decreases TNF- α production in lipopolysaccharide-activated murine macrophages by a contact-independent mechanism. *Cell Microbiol.* **5**, 277-285.
26. Sadowsky, D. W., Novy, M. J., Witkin, S. S., and Gravett, M. G. (2003) Dexamethasone or interleukin-10 blocks interleukin-1 β -induced uterine contractions in pregnant rhesus monkeys. *Am. J. Obstet. Gynecol.* **188**, 252-236.
27. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

28. Samona, A. and Robinson, R. K. (1991) Enumeration of bifidobacteria in dairy products. *J. Soc. Dairy Technol.* **44**, 64-66.
29. Sanders, M. E. (1993) Effect of consumption of lactic cultures on human health. In: *Advance in Food and Nutrition Research*. Kinsella, J. (ed) Academic Press, San Diego, pp. 67-130.
30. Stein, B. and Sutherland, M. S. K. (1998) IL-6 as a drug discovery target. *Drug Discov. Today* **3**, 202-213.
31. Stuehr, D. J. and Marlette, M. A. (1985) Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. *Proc. Nat'l. Acad. Sci. USA* **82**, 7738-7742.
32. Stuehr, D. J. and Marlette, M. A. (1987) Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines or interferon- γ . *J. Immunol.* **39**, 518-525.
33. Trushin, S. A., Pennington, K. N., Carmona, E. M., Asin, S., Savoy, D. N., Billadeau, D. D., and Paya, C. V. (2003) Protein kinase C α (PKC α) acts upstream of PK theta to activate I κ B kinase and NF- κ B in T lymphocytes. *Mol. Cell Biol.* **23**, 7068-7081.
34. Ulevitch, R. J. and Tobias, P. S. (1995) Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Ann. Rev. Immunol.* **13**, 437-457.
35. Wai, P. Y. and Kuo, P. C. (2004) Nitric oxide and endotoxin-mediated sepsis: the role of osteopontin. *Gene Ther. Mol. Biol.* **8**, 501-508.
36. Yeganegi, M., Leung, C. G., Martins, A., Kim, S. O., Reid, G., Challis, J. R. G., and Bocking, A. D. (2010) *Lactobacillus rhamnosus* GR-1-induced IL-10 production in human placental trophoblast cells involves activation of JAK/STAT and MAPK pathways. *Reprod. Sci.* **17**, 1043-1051.

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