***Lactococcus* strains treated with heat and hen-egg-white lysozyme induce abundant interleukin-12 production by J774.1 macrophages and murine spleen cells**

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ABSTRACT

The IL-12-inducing ability of lactic acid bacteria could be a critical index of immunomodulatory activity, especially in promoting T-helper-1 responses and in suppressing T-helper-2-mediated allergic responses. We aimed to develop a simple method for enhancing the IL-12-inducing ability of bacteria. We examined the in vitro effects of strains of lysozyme-modified *Lactococcus* (ML-LYS), prepared by heat treatment of the *Lactococcus* strain in the presence of lysozyme, on the ability of mouse macrophage-like J774.1 cells and spleen cells to produce IL-12. An IL-12-inducing ability greater than that of heat-killed bacteria was shown by 41 of 46 ML-LYS strains in J774.1 cells and by all 46 ML-LYS strains in mouse spleen cells. In contrast, bacteria modified by α -lactalbumin, β -lactoglobulin, or ovalbumin did not enhance IL-12 production in J774.1 cells. Microscopically, ML-LYS showed stronger resistance to lysozyme and macrophage digestion than did heat-killed bacteria or the other modified bacteria. Addition of chitotriose, a lysozyme inhibitor, enhanced IL-12 production by J774.1 cells stimulated with heat-killed bacteria. Therefore, enhancement of resistance to lysozyme may be a key factor in the strong IL-12-inducing ability of ML-LYS. These findings have important implications for the design of dairy products that have an immunomodulatory effect using the modified bacteria.

Key words: hen-egg-white lysozyme, *Lactococcus* strain, macrophage, interleukin-12

INTRODUCTION

Lactococcus strains are gram-positive bacteria of the lactic acid group. They have been widely used in fermented foods such as cheese, yogurt, and fermented vegetables. Lactococci have several health-promoting effects (i.e., probiotic properties), including improvement of lipid metabolism (Lee et al., 2005), immuno-

modulatory activity (Kimoto et al., 2004; Suzuki et al., 2008), and anti-aging effects (Kimoto-Nira et al., 2007).

Interleukin-12 produced by antigen-presenting cells, such as dendritic cells and macrophages, stimulates both natural killer cells and T-helper (Th) 1 cells to produce interferon- γ . Several studies have shown that specific lactic acid bacteria enhance the secretion of IL-12 by dendritic cells or macrophages and could promote the development of Th1 cells (Shida et al., 1998; Kato et al., 1999). The resulting stimulation of Th1 responses can suppress Th2 responses, which stimulate the production of IgE by B cells and increase the risk of allergic reactions (Winkler et al., 2007). Therefore, lactic acid bacteria strains with strong IL-12-inducing ability have been selected as immunomodulators to suppress IgE production in allergen-sensitized animal models (Fujiwara et al., 2004; Kimoto et al., 2004; Sashihara et al., 2006). Thus, the IL-12-inducing ability of lactic acid bacteria could be a critical index of immunomodulatory activity for the prevention and management of allergic diseases.

The IL-12-inducing ability of lactic acid bacteria varies with the strain (Fujiwara et al., 2004; Sashihara et al., 2006; Shida et al., 2006a; Suzuki et al., 2008). Shida et al. (2006a) reported that phagocytosis of lactobacilli was necessary for IL-12 induction, and that strains with strong IL-12 induction were relatively resistant to lysis in macrophages. This finding is important for selecting strains with strong IL-12-inducing ability. However, it takes large amounts of labor, time, and money to select a desirable strain. If it were possible to give a strain strong IL-12-inducing ability, it would not be necessary to choose it from among large numbers of strains; it would thus be possible to add a new function—namely immunomodulatory activity—to a strain that is already used in dairy food and has other useful functions.

Here, we examined the in vitro effects of lysozyme-modified strains of *Lactococcus* (ML-LYS), which were prepared by heat treatment of strains in the presence of lysozyme, on the ability of mouse macrophage-like J774.1 cells and spleen cells to elicit IL-12 production. To elucidate the mechanism by which IL-12-inducing ability was enhanced, we produced other *Lactococcus*

Received September 22, 2010.

Accepted March 13, 2011.

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Table 1. *Lactococcus* strains used in this study

Species and strains	Reference
<i>Lactococcus lactis</i> ssp. <i>cremoris</i> 341, C60, C68, J50, O21, O29, S19 H-17, Hc-1, HP	Nomura et al., 2006 Laboratory collection
<i>Lactococcus lactis</i> ssp. <i>lactis</i> 342, 1175, 1256, 1257, 1260, 1263, C13, G50, H45, H46, J44, J53, O06, O11, O19, O31, O52, O59, O62, P17, P79, Q14, Q20, S01 S63 ATCC19435	Nomura et al., 2006 Suzuki et al., 2008 ATCC ¹
<i>Lactococcus lactis</i> ssp. <i>lactis</i> biovar <i>diacetylactis</i> 772, C59, C61, C66, H59, O07, O09, O10, O17, O20	Nomura et al., 2006

¹American Type Culture Collection, Rockville, MD.

strains modified with common food proteins and examined their properties, including their IL-12-inducing ability, phagocytosis, bacterial adhesion to hydrocarbons (BAH), and resistance to cell-wall digestion.

MATERIALS AND METHODS

Mice

Female BALB/c mice were purchased from Charles River Japan (Kanagawa, Japan) and were used at 6 to 8 wk old. Mice were bred at the Animal Facility of the National Institute of Livestock and Grassland Science (Ibaraki, Japan) in accordance with guidelines for the care and use of experimental animals (Animal Care Committee, National Institute of Livestock and Grassland Science).

Reagents

Lysozyme from hen egg white was purchased from Sigma-Aldrich (St. Louis, MO). Ovalbumin was prepared from fresh egg white of White Leghorn hens in accordance with the method of Marshall and Neuberger (1972). Crude ovalbumin was further purified by ion-exchange chromatography. β -Lactoglobulin (genetic variant A) and α -lactalbumin were prepared from fresh raw milk and purified by ion-exchange chromatography as described previously (Aschaffenburg and Drewry, 1957). Their purity was assessed by using gel electrophoresis.

Cytochalasin D and lipopolysaccharide O55:B5 (LPS) were obtained from Sigma-Aldrich. Chitotriose was purchased from Seikagaku Biobusiness (Tokyo, Japan). Fluorescein isothiocyanate isomer-1 (FITC) was obtained from Dojindo Laboratories (Kumamoto, Japan), and 4',6-diamidino-2-phenylindole (DAPI) was obtained from Wako Chemicals (Osaka, Japan).

Bacterial Strains and Growth Conditions

The *Lactococcus* strains used are listed in Table 1 (Nomura et al., 2006; Suzuki et al., 2008). Bacterial

strains were grown for 48 h at 30°C in M17 broth (BD Difco, Detroit, MI) in which the lactose had been substituted with 0.5% glucose. Cultured bacteria were washed twice with 0.85% NaCl and resuspended to an optical density at a wavelength of 620 nm (OD_{620}) of 1.0 in 0.85% NaCl.

Modification of Bacterial Cells with Proteins

The lysozyme-modified *Lactococcus* strains were prepared by treating a solution of bacterial cells (OD_{620} of 1.0) and lysozyme (1 mg/mL) for 50 min at 100°C. The sample was then immediately placed on ice. Bacteria modified with α -LA, β -LG, or ovalbumin (ML-LA, ML-LG, and ML-OA, respectively) and heat-killed bacteria without added protein (HK) were prepared in the same manner. These modified bacteria were stored at 4°C until assay.

Modification of the bacteria by lysozyme was confirmed by immunofluorescence microscopy. Heat-killed cells or ML-LYS was incubated with rabbit anti-lysozyme IgG antibody (Nordic Immunology, Tilburg, the Netherlands) for 60 min at 20°C. After 3 washes with PBS, samples were stained with Alexa 594-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA). The bacteria were stained with DAPI (1 μ g/mL) for 60 min at room temperature. After a further 3 washes with PBS, bacterial cells were observed by fluorescence microscopy (Axioplan2, Carl Zeiss, Oberkochen, Germany).

Cell Culture

The J774.1 cells were obtained from the American Type Culture Collection (Rockville, MD). Spleen cells were harvested from BALB/c mice, as described previously (Kimoto et al., 2004). Briefly, the spleen was taken out aseptically, teased apart, and passed through a cell strainer (BD Falcon, Franklin Lakes, NJ). Then, the erythrocytes were lysed with ACK buffer (0.15 M NH_4Cl , 10 mM $KHCO_3$, and 0.1 mM EDTA, pH

7.2), and the cells were washed twice with RPMI-1640 (Sigma-Aldrich). The J774.1 cells and spleen cells were cultured in a 5% CO₂ atmosphere at 37°C in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL of penicillin, 100 µg/mL of streptomycin, 10 mM HEPES, and 50 µM 2-mercaptoethanol.

In Vitro IL-12 Production Assay

The J774.1 cells (1×10^5) were cultured with HK or modified bacteria (final OD₆₂₀ of 0.01) in 200 µL of culture medium (see above) in a 96-well culture plate (BD Falcon). After 24 h, the level of IL-12p40 in the culture supernatants was analyzed by using commercially available ELISA kits (BD PharMingen, San Diego, CA) in accordance with the manufacturer's instructions. In some experiments, J774.1 cells were pretreated with cytochalasin D (5 µg/mL) or chitotriose (1 mM) for 30 min before the addition of HK or modified bacteria. Spleen cells (8×10^5) were cultured with HK or modified bacteria (final OD₆₂₀ of 0.001) for 96 h in a 96-well culture plate. The level of IL-12p70 in the culture supernatants was determined by ELISA kits (BD PharMingen).

Phagocytosis Assay

Bacteria (OD₆₂₀ of 1.0) were incubated overnight at 4°C with FITC (0.1 mg/mL) in 0.1 M sodium carbonate buffer (pH 9.0) and then were washed 3 times with 0.85% NaCl. To prepare FITC-labeled ML-LYS, ML-LA, ML-LG, ML-OA, or HK, FITC-labeled bacteria were suspended in one of the protein solutions or in 0.85% NaCl alone and the solution was then heat-treated at 100°C for 50 min. The J774.1 cells (1×10^5) were cultured with FITC-labeled HK or modified bacteria (final OD₆₂₀ of 0.01) in 200 µL of culture medium in a 96-well plate for 1 h at 37°C in a 5% CO₂ atmosphere. The cells were washed 3 times with PBS and then fixed with 4.0% (wt/vol) paraformaldehyde in PBS for 15 min on ice. After being washed, the cells were resuspended in PBS. The mean fluorescence intensity (MFI) of the bacteria within the cells and the rate of phagocytosis were analyzed by flow cytometer (FACSsort, BD Biosciences).

BAH

The BAH test was performed by the method of Rosenberg (1984), with some modifications. Heat-killed or modified bacteria (1.2 mL) were washed with distilled water and resuspended in an equal volume of distilled water. The OD₆₂₀ was then determined (OD_{620before}). The bacterial suspension was poured into a glass tube,

and then 1 mL of n-hexadecane (Nakalai Tesque, Kyoto, Japan) was added to the tube. After 10 min of preincubation at 30°C, the mixture was vortexed for 120 s. After the hydrocarbon phase had been allowed to rise for 15 min, the aqueous phase was transferred to a new tube and the OD₆₂₀ was then determined again (OD_{620after}). The BAH was calculated by using the following formula:

$$\text{BAH (\%)} = (\text{OD}_{620\text{before}} - \text{OD}_{620\text{after}}) / \text{OD}_{620\text{before}} \times 100.$$

Microscopic Analysis of Degradation of Bacterial Cells

To examine the cell-wall sensitivity to lysozyme, the HK and modified bacteria were digested with 100 µg/mL lysozyme in sodium phosphate buffer (pH 6.2) for 24 h at 37°C. After 3 washes with PBS, the bacteria were fixed with methanol for 10 min and then stained with Giemsa (Nakalai Tesque) and observed by light microscopy.

To analyze macrophage digestion, J774.1 cells (5×10^5) were cultured with HK or the modified bacteria (final OD₆₂₀ of 0.001) on round 13-mm coverslips in a 24-well culture plate for 24 h at 37°C. Then, J774.1 cells were harvested by centrifugation at $200 \times g$ for 10 min. The cells were washed 3 times with PBS, fixed with methanol for 10 min, and stained with Giemsa. The bacteria in the macrophage were analyzed morphologically by light microscopy.

Statistical Analysis

Correlations between BAH and phagocytosis or between IL-12 production and phagocytosis were evaluated by Pearson correlation coefficient (r); 2-tailed *P*-values <0.05 were considered to indicate significance.

RESULTS

Microscopic Analysis of ML-LYS

Lysozyme modification of bacterial cells was confirmed by observation by immunofluorescence microscopy. The ML-LYS were individually covered by lysozyme, which seemed tightly bound to the bacterial cell walls (Figure 1). We considered that lysozyme on the surfaces of the modified bacterial cells would have been inactivated because the ML-LYS were not digested and their ability to induce IL-12p40 production by J774.1 cells remained stable for a few weeks (data not shown).

Effect of ML-LYS on IL-12 Production by J774.1 Cells and Spleen Cells

We examined IL-12p40 production by J774.1 cells stimulated with HK or ML-LYS from 46 *Lactococcus*

Figure 1. Immunofluorescence microscopic analysis of heat-killed bacteria without added protein (HK) and lysozyme-modified *Lactococcus* strain (ML-LYS; represented here by C59 strain); 4',6-diamidino-2-phenylindole (DAPI) staining (upper panel) and anti-lysozyme staining (lower panel).

strains. All of the ML-LYS, except for strains H-17, Hc-1, J50, O21, and O17, induced higher levels of IL-12p40 than were induced by HK (Figure 2A). All of the ML-LYS showed stronger IL-12-inducing ability than HK in spleen cells (Figure 2B). Addition of heat-treated lysozyme did not enhance IL-12p40 production by HK-stimulated J774.1 cells (data not shown).

Phagocytosis of *Lactococcus* Strains by J774.1

The J774.1 cells were cultured with FITC-labeled HK or ML-LYS of 46 *Lactococcus* strains for 1 h, and then the cells were analyzed by flow cytometer. In all 46 strains, the MFI of the J774.1 cells that engulfed the ML-LYS was higher than that of the J774.1 cells that engulfed the HK (Figure 3). It is known that the MFI is correlated with the number of bacteria taken up per cell. Therefore, the results showed that the ML-LYS were engulfed by the cells to a greater degree than were the HK. Addition of heat-treated lysozyme had no effect on the phagocytosis of HK by J774.1 cells (data not shown).

Specificity of Lysozyme as a Modulator for Enhancement of IL-12-Inducing Ability

To assess whether enhancement of IL-12 production was observed specifically in ML-LYS, we randomly selected 5 *Lactococcus* strains (HP, 1263, H45, C59, and O07). They were modified by α -LA, β -LG, and ovalbumin (ML-LA, ML-LG, and ML-OA, respectively), and then their IL-12-inducing ability and phagocytosis were examined. In all 5 strains, ML-LYS induced the greatest production of IL-12p40 by J774.1 cells (Figure 4A). In the phagocytosis study, the rates of engulfment of ML-LG, ML-OA, and ML-LYS by J774.1 cells were higher than those of HK, but the engulfment rates of ML-LA were similar to or less than those of HK (Figure 4B).

Figure 2. Effect of lysozyme-modified *Lactococcus* strain (ML-LYS) and heat-killed bacteria without added protein (HK) on IL-12 production by J774.1 cells (A) and spleen cells (B). Data are the means of triplicates with SD and are representative of 2 independent experiments.

Figure 3. Phagocytosis of lysozyme-modified *Lactococcus* strain (ML-LYS) and heat-killed bacteria without added protein (HK) by J774.1 cells. The mean fluorescence intensity (MFI) of macrophages was analyzed by flow cytometry, and its ratio (ML-LYS:HK) in 46 *Lactococcus* strains was calculated for 1 h. Data are representative of 2 independent experiments.

Relationship Between BAH and Phagocytosis

To understand the mechanism of enhancement of phagocytosis by the modification, the surface hydrophobicity of the modified bacteria was investigated by the BAH method. Bacterial surface hydrophobicities were enhanced in all modified bacteria (ML-LYS, ML-LA, ML-LG, and ML-OA) prepared from the 5 strains described above. The relationship between the surface hydrophobicity of the modified bacteria and the rate of phagocytosis of the modified bacteria was analyzed. Strong positive correlations ($r > 0.7$) were observed in 4 of the 5 strains (Figure 5A). Overall, the rate of phagocytosis was well correlated with BAH ($r = 0.744$, $P < 0.05$; Figure 5B).

Relationship Between Phagocytosis and IL-12 Production

We examined the ability of HK, ML-LYS, or ML-LG to induce IL-12 production by J774.1 cells preincubated with or without cytochalasin D, a phagocytosis inhibitor. With all modified strains, cytochalasin D treatment of the macrophages dramatically inhibited IL-12 production (Figure 6A). However, the correlation between IL-12-inducing ability and the rate of phagocytosis of modified bacteria, except in the case of strain 1263, was only weakly positive (Figure 6B). Overall, the rate of phagocytosis was modestly positive correlated with IL-12-inducing ability ($r = 0.505$, $P < 0.05$; Figure 6C).

Resistance to Lysozyme and Digestion by Macrophages

To elucidate the factor enhancing the IL-12-inducing ability of ML-LYS, we investigated the lysozyme sensitivity of the modified bacteria. Giemsa staining showed that only ML-LYS maintained their reactivity to Giemsa stain upon lysozyme digestion for 24 h; HK and the other modified bacteria lost their Giemsa stain reactivity (Figure 7A). Macrophage digestion of the modified bacteria was also investigated. After co-incubation of macrophages and bacteria for 24 h, ML-LYS were still observed in the macrophages, but HK or the other modified bacteria were not observed (Figure 7B).

We next examined the relationship between resistance to lysozyme or macrophage digestion and the IL-12-inducing ability of the bacteria; that is, we examined the additive effect of chitotriose, a lysozyme inhibitor, on IL-12 production by J774.1 cells stimulated with HK. Addition of chitotriose enhanced IL-12 induction by HK of all 5 strains but did not enhance IL-12 induction by LPS (Figure 7C).

DISCUSSION

The IL-12-inducing ability of lactic acid bacteria is an important factor in the selection of appropriate strains with the potential to prevent and treat allergic diseases (Fujiwara et al., 2004; Kimoto et al., 2004; Sashihara et al., 2006). We demonstrated here that modification with lysozyme could enhance the IL-12-inducing abil-

Figure 4. Comparisons of IL-12-inducing abilities (A) and phagocytosis (B) of *Lactococcus* modified by lysozyme (ML-LYS), α-LA (ML-LA), β-LG (ML-LG), and ovalbumin (ML-OA) from 5 *Lactococcus* strains. Phagocytosis was analyzed after incubation for 1 h. Data are means of triplicates with SD. HK = heat-killed bacteria without added protein.

ity of *Lactococcus* strains. Moreover, we found that this modification altered bacterial-cell surface properties such as hydrophobicity and resistance to lysozyme or macrophage digestion, and that these altered properties were important for enhancing the IL-12-inducing ability of lactococci.

The bacteria modified by lysozyme or other proteins were more hydrophobic than HK. The hydrophobicity of lysozyme is increased by heat treatment in urea solution because of the structural alteration induced by heat denaturation (Sugahara et al., 2002). On the surfaces of the modified bacteria that we used, lysozyme or other proteins denatured by heat treatment may have altered the hydrophobicity and thus enhanced the BAH. Microspheres with hydrophobic surfaces are more easily engulfed by macrophages than those with hydrophilic surfaces (Tabata and Ikada, 1988). We observed strong positive correlations between the BAH of modified bacteria (HK, ML-LYS, ML-LA, ML-LG, and ML-OA) and the rate of engulfment by macrophages. These findings suggest that modification of the bacteria by heat treatment in the presence of protein enhanced the hydrophobicity of the bacterial cell surface and thus facilitated engulfment of the bacteria by macrophages.

Figure 5. Relationship between bacterial adhesion to hydrocarbons (BAH) and phagocytosis of *Lactococcus* cells by J774.1 cells. The BAH of heat-killed bacteria without added protein (HK), and *Lactococcus* modified by lysozyme (ML-LYS), α-LA (ML-LA), β-LG (ML-LG), and ovalbumin (ML-OA) of 5 strains and the percentages of macrophages that had engulfed them within 1 h (Figure 4B) were plotted. Each strain (A) and overall (B) are shown. Correlation coefficients are shown.

Production of IL-12 by macrophages stimulated with modified *Lactococcus* strains was dramatically suppressed by the blocking of phagocytosis. This result suggests that phagocytosis is essential for strong IL-12 induction by lactococci. This is reminiscent of a recent report showing that phagocytosis of lactobacilli was necessary for IL-12 induction (Shida et al., 2006b). However, we found weak correlations between IL-12 production and the rate of engulfment of bacteria by macrophages; this indicates that enhancement of IL-12-inducing ability by ML-LYS does not result merely from an increase in the rate of phagocytosis. However, in the case of strains such as 1263, which could not be easily engulfed by the cells, increasing hydrophobicity and phagocytosis by modification may be important for enhancing the strains' IL-12-inducing ability.

The lysozyme resistance of bacterial cells was enhanced only by the modification of bacteria with lysozyme. Our results indicated that lysozyme could successfully cover the bacterial cell individually. However,

Figure 6. Relationship between IL-12 production by J774.1 cells stimulated with *Lactococcus* (5 strains) and phagocytosis of the bacteria by the cells. (A) IL-12p40 production upon stimulation with modified bacteria with or without cytochalasin D. Data are means of triplicates with SD. (B and C) IL-12-inducing abilities of heat-killed bacteria without added protein (HK), and *Lactococcus* modified by lysozyme (ML-LYS), α -LA (ML-LA), β -LG (ML-LG), and ovalbumin (ML-OA) (5 strains, see Figure 4A), and the percentages of macrophages that had engulfed the bacteria within 1 h (see Figure 4B) were plotted. Each strain (B) and overall (C) are shown. Correlation coefficients are shown.

Figure 7. Lysozyme resistance and IL-12-inducing ability. (A) Microscopic images of lysozyme digestion of heat-killed bacteria without added protein (HK), and *Lactococcus* modified by lysozyme (ML-LYS), α -LA (ML-LA), β -LG (ML-LG), and ovalbumin (ML-OA) (represented by strain C59). (B) Microscopic images of macrophage digestion of HK, ML-LYS, ML-LA, ML-LG, and ML-OA (strain C59). Arrows show undigested bacterial cells. (C) IL-12p40 production by J774.1 cells upon stimulation by HK of the 5 strains with or without chitotriose. Data are means of triplicates with SD. Color version available in the online PDF.

we observed that α -LA was aggregated partially on the bacterial cell and that β -LG and ovalbumin were aggregated themselves including the bacterial cell (data not shown). Only ML-LYS consist of bacteria surrounded by inactivated lysozyme. Therefore, the ML-LYS are probably protected from attack by the endogenous lysozyme *N*-acetylmuramidase. It is well known that *N*-acetylmuramidase in macrophages plays an important role in digesting bacterial cells (Ganz et al., 2003). The ML-LYS also had strong resistance to macrophage digestion, probably because of the enhancement of resistance to endogenous lysozyme. In addition, inhibition of lysozyme activity enhanced IL-12 production when J774.1 cells were stimulated with HK in the presence of chitotriose. Therefore, it seems that modification of

Lactococcus strains by heat treatment in the presence of lysozyme endows the bacteria with resistance to lysozyme and macrophage digestion and enhances IL-12 production by J774.1 cells. *Lactobacillus* strains with a rigid cell wall resistant to macrophage digestion effectively stimulate macrophages to produce IL-12 (Shida et al., 2006a); the findings of these authors strongly support this hypothesis.

Activation of macrophages by microorganisms is mediated by pattern recognition systems, such as toll-like receptors, which recognize specific bacterial components. Several components of bacteria, such as lipoteichoic acid, peptidoglycan, and CpG DNA, are candidates for activators (Michelsen et al., 2001; Fujiwara et al., 2004; Bafica et al., 2005). Here, we compared the IL-12-inducing ability of HK and ML-LYS prepared from the same strains and presumably with the same bacterial components. Therefore, the bacterial components per se were unlikely to have been the factors responsible for the enhancement of IL-12-inducing ability by modification with lysozyme.

Lactic acid bacteria and bifidobacteria are widely used as starter bacteria in the manufacture of cheese and other fermented dairy products. Several studies showed that some of them had probiotic activity but their effects were strain-dependent. We confirmed that this lysozyme modification could enhance IL-12-inducing ability of some *Lactobacillus* strains and *Bifidobacterium* strains (data not shown). Therefore, the modification method can be widely applicable to other probiotic bacteria. In addition, the method established in this study is expected as a time- and cost-saving tool for adding immunomodulatory activity such as anti-allergic function to lactic acid bacteria. The production of functional probiotic foods presupposes stability of strain features in the final product. However, a recent study showed that the original properties used in the selection of specific probiotic strains may indeed be influenced by industrial production processes (Grześkowiak et al., 2011). To achieve a beneficial effect in the dairy food, this modification method may be applicable in stabilizing probiotic properties, and the modified strains could be suitable for use as novel supplements for functional dairy foods.

In conclusion, our results show that 2 alterations are related to why the lysozyme modification enhanced IL-12-inducing ability of lactococci. One is the alteration in hydrophobicity of *Lactococcus* strains. Modification of the bacteria with lysozyme and other proteins increased their hydrophobicity, which would result in enhancement of phagocytosis. Thus, the modification seems a good method to enhance IL-12-inducing ability of the bacteria that are poorly engulfed. Another alteration is the enhancement of resistance of lysozyme

and macrophage digestion and it is specific to the bacteria modified with lysozyme. The bacteria modified with other proteins had no alteration in resistance. Therefore, lysozyme modification would be widely applicable for enhancement of the IL-12-inducing ability of the bacteria. The present study provides insights into mechanisms of IL-12-inducing ability and should facilitate the manufacture of new dairy products with immunomodulatory effects.

ACKNOWLEDGMENTS

This study was supported in part by a grant for “Development of evaluation and management methods for the supply of safe, reliable, and functional food and farm produce” from the Ministry of Agriculture, Forestry and Fisheries of Japan.

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