**Note**

**Cytokine Production by the Murine Macrophage Cell Line J774.1 after Exposure to Lactobacilli**

Hirotsugu Morita,¹ Fang He,¹* Tetsuo Fuse,¹ Arthur C. Ouwehand,² Hideo Hashimoto,¹ Masataka Hosoda,¹ Koko Mizumachi,³ and Jun-Ichi Kurisaki⁴

¹Technical Research Laboratory, Takanashi Milk Products Co., Ltd., Yokohama, Kanagawa 241-0023, Japan
²Departments of Biochemistry and Food Chemistry, University of Turku, Fin-20014 Turku, Finland
³National Institutes of Livestock and Grassland Science, Ikenodai, Kukizaki, Inashiki, Ibaraki 305-0901, Japan
⁴National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan

Received February 18, 2002; Accepted May 2, 2002

Eleven strains of lactobacilli were tested for their ability to induce the murine macrophage-like cell line J774.1 to secrete cytokines. Some of the bacteria tested induced the production of interleukin(IL) 6, IL-12, and tumor necrosis factor α (TNF-α) by J774.1 cells. Seven strains also induced the production of IL-10. However, no IL-1β was produced. *Lactobacillus acidophilus* TMC 0356 significantly induced the production of more IL-6, IL-10, IL-12, and TNF-α than the other bacteria tested (p < 0.0001; ANOVA). These results suggest that lactobacilli can activate macrophages to secrete both proinflammatory and anti-inflammatory cytokines. Selected strains might be used to bring about pro or anti-inflammatory immune reactions.

**Key words:** cytokine; macrophage; *Lactobacillus*; inflammation; probiotics

The genus Lactobacillus is a heterogeneous group of microorganisms comprising about 70 recognized species and subspecies with a broad ecological distribution.¹ Lactobacilli are one of the main constituents of the normal indigenous flora of humans, especially in the oral cavity, the female genital tract, and the small and large intestines, where they contribute to health.²,³ Lactobacilli have long been used in the preparation of fermented foods, in which form also they contribute to well being;⁴ they have received attention as a live-microbial food ingredient beneficial to their host.⁴ Evidence from clinical and animal studies has supported the idea that lactobacilli, especially some selected strains, can modify immune responses of the host.⁵ However, the mechanisms by which lactobacilli alter the immunological responses are unclear.

Macrophages are tissue-based phagocytes derived from monocytes. They participate in both innate and adaptive immune responses.⁶,⁷ Macrophages are activated by microbial metabolites, such as endotoxin, by molecules such as CD40 ligand, and by T cell cytokines such as interferon-γ (IFN-γ). Activated macrophages phagocytose microorganisms, secrete proinflammatory cytokines, and present antigens to helper T cells. Many of these activities are mediated through the release of different cytokines. Therefore, a change in profile of macrophage-derived cytokines is one way human immunity is influenced. Recently, we found that bifidobacteria stimulate the production of macrophage-derived cytokines in a strain-dependent manner,⁸ although the same bacteria did not stimulate proinflammatory cytokine secretion from human intestinal epithelial cells.⁹ Here, we examined the ability of lactobacilli to induce cytokine secretion by macrophages using the murine macrophage-like cell line J774.1.

The cell line was purchased from Riken Gene Bank (Tsukuba, Japan) and maintained with RPMI 1640 medium (Sigma, St. Louis, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Rockville, USA) at 37°C in an atmosphere of air and 5% CO₂. Nine *Lactobacillus* strains stored in the Microbiological Laboratory of Takanashi Milk Products Co., Ltd. (Yokohama, Japan) were used as

---

**Table 1. Strains of Lactobacilli Tested**

<table>
<thead>
<tr>
<th>TMC Strain No.</th>
<th>Bacteria</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0313</td>
<td><em>L. acidophilus</em></td>
<td>Dairy food</td>
</tr>
<tr>
<td>0356</td>
<td><em>L. acidophilus</em></td>
<td>Human intestine</td>
</tr>
<tr>
<td>0402</td>
<td><em>L. casei</em></td>
<td>Dairy food</td>
</tr>
<tr>
<td>0413</td>
<td><em>L. casei</em> Shirotake</td>
<td>Dairy food</td>
</tr>
<tr>
<td>0409</td>
<td><em>L. casei</em></td>
<td>Dairy food</td>
</tr>
<tr>
<td>1001</td>
<td><em>L. casei</em></td>
<td>Human intestine</td>
</tr>
<tr>
<td>1002</td>
<td><em>L. casei</em></td>
<td>Human intestine</td>
</tr>
<tr>
<td>1003</td>
<td><em>L. casei</em></td>
<td>Human intestine</td>
</tr>
<tr>
<td>0503</td>
<td><em>L. rhamnosus</em></td>
<td>Dairy food</td>
</tr>
<tr>
<td>0510</td>
<td><em>L. rhamnosus</em></td>
<td>Dairy food</td>
</tr>
<tr>
<td>0514</td>
<td><em>L. rhamnosus</em></td>
<td>Human intestine</td>
</tr>
</tbody>
</table>

¹ To whom correspondence should be addressed. Fax: +81-45-364-2160; E-mail: he-fang@takanashi-milk.co.jp
the test bacteria (Table 1). These bacteria withstand gastric and bile acids better than 80 other strains of lactobacilli in the same microbiological collection (unpublished results). In addition, two probiotic lactobacilli were included in the study. L. rhamnosus GG (ATCC 53103) was obtained from Valio Ltd. (Helsinki, Finland) and L. casei Shirota, isolated from a Yakult product of Yakult Honsha Co., Ltd. (Tokyo, Japan). Lactobacilli were cultured first in de Man, Rogosa and Sharp broth (MRS broth; Difco Laboratories, Detroit, Michigan, USA) for 18 h. at 37°C two or three times. After incubation, the bacteria were collected by centrifugation and washed three times with phosphate-buffered saline (pH 7.1). The bacteria were then suspended in RPMI medium at 10^8 CFU/ml and heat-inactivated at 65°C for 30 min. This amount was chosen because it was the smallest to induce cytokine secretion by the two commercial probiotic strains we tested (results not shown). The bacterial preparations were stored at −70°C until use.

Cultured J744.1 cells were spread onto a 24-well flat-bottomed plate (Nalge Nunc International, Rochester, N.Y., USA) with 5 × 10^5 cells/2 ml in each well. To the wells, 100 μl of heat-inactivated bacteria were added. After 24 h. incubation at 37°C in an air-5% CO₂ atmosphere, the supernatant was collected and stored at −70°C until analysis. The concentrations of interleukin (IL) 6, IL-10, IL-12 and tumor necrosis factor α(TNF-α) were assayed with commercial ELISA kits (Endogen, Inc., Woburn, MA, USA). The results are expressed as means and standard deviation of three independent experiments (mean ± SD). Each experiment was done with three for evaluation and of correction for intra-assay variation. The statistical significance of differences in the concentration of cytokine secretion was evaluated by analysis of variance (ANOVA) with Fisher’s protected least significant difference (PLSD) post-hoc test.

The lactobacilli induced secretion of cytokines by some strains (Fig. 1). All bacteria tested stimulated the secretion of IL-6, IL-12, and TNF-α. Seven of the

---

**Fig. 1.** Cytokine Production by J744.1 Cells after Exposure to Lactobacilli.

The murine macrophage cell line J744.1 was cultured in the presence of heat-inactivated lactobacilli for 24 hours. The concentration of cytokine in supernatant were analyzed with commercial cytokine ELISA kits. The results were expressed as average of three independent experiments and SD(error bar). Each experiment was performed with three parallels. Statistical significance of the difference in the level of cytokine secretion induced by the lactobacilli was determined by analysis of variance (ANOVA) using Fisher’s PLSD post-test (**p<0.01).
tested strains induced the secretion of IL-10. No IL-1β was detected in the medium after incubation of any of the lactobacilli tested. L. acidophilus TMC 0356 produced the highest concentrations of IL-6 (17.6±2.0 ng/ml), IL-10 (422.4±208.7 pg/ml), IL-12 (54.1±16.8 ng/ml) (p<0.0001; ANOVA).

The probiotic strain L. casei Shirota induced IL-12 production, which is as reported previously.\textsuperscript{10,11} Increases in IL-12 macrophage-derived increases the cytotoxicity mediated by natural killer cells against tumors, which may explain how this bacterium has antitumor effects in mice and humans.\textsuperscript{10,11} Another well-investigated probiotic bacterium, L. rhamnosus GG, help to prevent atopic dermatitis in infants\textsuperscript{10} and to alleviate food allergy.\textsuperscript{10} We found here that this strain induced as much IL-12 to be produced, as L. casei Shirota. The concentrations of IL-6 and TNF-α produced in the presence of L. rhamnosus GG were greater than L. casei Shirota. L. rhamnosus GG induced the production of IL-6, IL-12, and IFN-γ but not IL-10 from human peripheral blood mononuclear cells and activated nuclear factor κB and state signaling pathways in human monocytes.\textsuperscript{14-17} These results suggest that L. rhamnosus GG is similar to L. casei Shirota in their immunomodulatory effects, because IL-12 favors a type 1 helper T cell (Th1) response. However, more detailed studies need to be conducted in order to obtain more conclusive evidence for this hypothesis.

Proinflammatory cytokines IL-1β, IL-6, IL-12, and TNF-α are among the first cytokines produced by phagocytic cells in response to encounters with pathogenic bacteria and agents. Macrophage-derived IL-12 stimulates IFN-γ production in T cells and natural killer cells, which accelerates the development of native CD4+ T cells into Th1-type cells.\textsuperscript{6} Therefore, IL-12 is a key immunoregulator favoring Th1-type responses. On the other hand, IFN-γ, which induces IL-12 production, can act in positive feedback in the inflammatory responses by increasing IL-12 production. Such amplification of IL-12 production mediated by IFN-γ is risky, because it can lead to uncontrolled cytokine production and possible shock. The over expression of IL-12 impairs the organ specific auto-immunity balance. In contrast, IL-10 inhibits IL-12 production by phagocytic cells by inhibiting of IL-12 production by antigen-presenting cells including macrophages and by inhibiting the expression of other co-stimulatory surface molecules and soluble cytokines.\textsuperscript{18-20} The balance between the proinflammatory cytokine IL-12 and anti-inflammatory cytokine IL-10 must be important for host immunity. Gram-positive bacteria seem to stimulate IL-12 production and gram-negative bacteria preferentially stimulate IL-10 production.\textsuperscript{21-23} The mechanisms by which some bacteria induce the production of IL-10 is unclear, lipopolysaccharide of gram-negative bacteria may stimulate this anti-inflammatory response.\textsuperscript{18,19} Our finding here that most of the tested bacteria, and especially L. acidophilus TMC 0356, induced IL-10 secretion was in line with earlier studies on bifidobacteria. Bifidobacteria, especially those from healthy adults and infants, stimulate macrophage-derived IL-10 production but those from allergic infants induced little or no IL-10 production from the macrophages.\textsuperscript{9} Compounds other than lipopolysaccharides in bifidobacteria and lactobacilli probably contribute to the ability of these bacteria to stimulate an anti-inflammatory cytokine response. L. acidophilus TMC 0356 also induce the secretion of much proinflammatory cytokine IL-6 and IL-12. The final in vivo immune response remains therefore to be determined. This is also indicated by observations that L. rhamnosus GG is able to reduce the inflammation in Crohn’s disease,\textsuperscript{25} although we did not find IL-10 production here. Other factors are also likely to be involved, such as modification of the intestinal microflora, production of secretory IgA, and improvement of the intestinal barrier function.\textsuperscript{25}

Our results indicate that the immunoregulatory effects of lactic acid bacteria are heterogeneous, and strain-dependent, and suggest that some specific commensal lactic acid bacteria may able to suppress inflammatory responses and that other can intensify such responses. This difference makes it possible to guide the immune response in the desired direction.

Acknowledgment

Financial support was obtained from the Research Association for New Food Creation of the Japanese Ministry of Agriculture and Forestry.

References

8) He, F., Morita, H., Hashimoto, H., Hosoda, M., Kurisaki, J., Ouwehand, A. C., Isolauri, E., Benno,


