Anti-Inflammatory Metabolite Production in the Gut from the Consumption of Probiotic Yogurt Containing Bifidobacterium animalis subsp. lactis LKM512

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There is little evidence for a relationship between probiotic metabolites and host cytokine production. We investigated in the present study the possibility that anti-inflammatory metabolites can be produced in the gut by LKM512 yogurt consumption by using murine macrophage-like J774.1 cells and extracts prepared from the feces of elderly volunteers. These volunteers’ acute inflammation had been inhibited by LKM512 yogurt consumption in a previous test. The tumor necrosis factor (TNF)-α production elicited in J774.1 cells stimulated by lipopolysaccharide (LPS) and in the fecal extracts obtained during the period of LKM512 yogurt consumption was significantly decreased (p < 0.05) than the pre-consumption baseline level. These findings and previous data enable us to conclude that intestinal bacterial metabolites produced by LKM512 yogurt consumption contributed to suppressing the inflammatory cytokine produced by macrophages and that one of the anti-inflammatory metabolites in the fecal extracts was likely to have been a polyamine.

Key words: bacterial metabolite; tumor necrosis factor-α; polyamine; anti-inflammation; probiotics

Probiotic research has advanced in recent years, and effects on disease and the host immune system such as the alleviation of allergic symptoms and of inflammatory bowel disease have been reported. There are many reports on the host’s cytokine pattern being changed by probiotics in vivo; indeed, stimulation of the host cytokine network is one of the important functions of probiotics. This benefit probably depends on two factors, namely the probiotic bacterial cell components and the metabolites produced by probiotics or changed intestinal microbiota. The fact that the former stimulate lymphocytes has been demonstrated by using cell culture systems; for example, Hessle et al. and Karlsson et al. have shown that the lymphocyte recognizes bacterial immunogenicity of cell-wall components through Toll-like receptors; Gram-positive bacteria such as Bifidobacterium and lactic acid bacteria induce type 1 helper T cell (Th1) cytokine production, and Gram-negative bacteria induce Th2 cytokine production using human peripheral blood mononuclear cells. Kimoto et al. have reported that spleen cells from BALB/c mice fed with probiotics, which had been selected as Th1-reaction-inducing bacteria by using a J774.1 cell line, produced more Th1 cytokines, i.e., interleukin (IL)-12 and interferon-gamma, and slightly fewer Th2 cytokines, i.e., IL-4 and IL-6, than the control. These reports seem to show that the cytokine production is directly controlled by the cell components of probiotics. However, the complexity of the intestinal microbiota, which number some 400–500 bacterial species with approximately 10^{11} bacteria per gram of wet feces in the gut, was completely ignored in these experiments with a single culture. The essential probiotic benefits through the complex intestinal microbiota were not addressed by these experiments. It is difficult to believe that probiotics which control the host’s cytokine pattern have a special immunogenicity which influences the immune system of the host, because probiotic strain identified was Bifidobacterium or Lactobacillus, which commensally colonized the human gut. The likelihood that intestinal bacterial metabolites can stimulate the host’s cytokine network cannot be ignored. We reasoned that the host’s lymphocytes may be influenced by intestinal bacterial metabolites more directly than by a change in the intestinal microbiota composition per se, and have focused on those metabolites produced by probiotics or changed intestinal microbiota.

In our previous reports, we have shown that Bifidobacterium animalis subsp. lactis LKM512 had potent acid tolerance and the ability to adhere to intestinal mucus. We provided B. animalis subsp. lactis LKM512-containing yogurt (LKM512 yogurt) to hospitalized elderly volunteers and healthy adults with poor

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LPS, lipopolysaccharide; MIP, macrophage inflammatory protein; Th1, type 1 helper T cell; Th2, type 2 helper T cell; TNF, tumor necrosis factor

defecation. The consumption of LKM512 yogurt resulted in the temporary colonization of LKM512 in the gut\(^\text{15}\) and an increasing frequency of defecation.\(^\text{16}\) Furthermore, the fecal haptoglobin content, which has been used as a marker of acute inflammation,\(^\text{17}\) and the fecal mutagenicity\(^\text{17,18}\) were decreased by LKM512 yogurt consumption. We discussed in these reports the hypothesis that the intestinal polyamines produced by LKM512 yogurt consumption led to suppression of the acute inflammation and antimutagenicity.\(^\text{12,17,18}\)

In the present study, we investigated the possibility that anti-inflammatory metabolites are produced in the gut by LKM512 yogurt consumption. Specifically, we examined the effects on fecal extracts prepared from feces obtained during the pre-consumption period, and of the LKM512 yogurt and placebo consumption on the cytokines produced in the murine macrophage-like cell line, J774.1.

Materials and Methods

Preparation of the fecal extract. This study used the feces of six volunteers (three males and three females with an average age of 78.0 years) from whom feces could be collected in all of the designated test periods (pre-consumption, 2 weeks of LKM512 yogurt consumption, after consumption 2 weeks, and 2 weeks of placebo consumption) from a study on elderly volunteers.\(^\text{15,17}\) Frozen feces were diluted 10-fold with 10 mM phosphate-buffered saline (PBS) at pH 7.2 containing 10 mM sodium azide and extracted four times by intensely mixing for 1 min and allowing to stand for 15 min on ice. After this extraction, the precipitate was removed by centrifugation (10,000 \(\times\) g for 20 min at 4 °C), and the supernatant was filtered (0.22 μm) for use as the fecal extract. This extract was stored at −20 °C until needed for analysis. The polyamine concentration of each fecal extract was redetermined by HPLC.\(^\text{17}\)

Cell culture. The murine macrophage-like cell line, J774.1 (= RCB0434), was purchased from the RIKEN Cell Bank (Tsukuba, Japan). The cells were maintained in Falcon 100 × 20 mm plastic culture flasks (Becton Dickinson Labware, Oxnard, CA, USA) in GIBCO RPMI-1640 medium (Invitrogen Corp., Carlsbad, CA, USA) with kanamycin (5 μg/ml; Sigma Chemical Co., St. Louis, MO, USA) and 5% fetal bovine serum. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. The cells harvested by gentle scraping were passaged every 3 days by diluting 1:10 with fresh medium.

Stimulation of J774.1 with the fecal extract. J774.1 cells were suspended in the culture medium at a concentration of 5 × 10⁴ cells/ml, and 1 ml of the cell suspension was plated into a Falcon 24-well culture plate (Becton Dickinson). The cells were incubated for 48 h with LPS of E. coli (Sigma Chemical Co.; a final concentration of 10 ng/ml) and 20 μl of the fecal extract at 37 °C in a humidified atmosphere containing 5% CO₂. After this incubation, the supernatant was harvested and stored at −80 °C until needed for use. The cell survival was analyzed by a Colorimetric (MTT) assay kit (Chemicon International, Temecula, CA, USA).

Measurement of the cytokine content in the culture medium. Tumor necrosis factor (TNF-α) was determined by sandwich ELISA. A serial dilution of recombinant mouse TNF-α (Genzyme, MA, USA; 0–100 ng/ml) was used for ELISA to generate standard curves. In brief, 96-well microplates (Nunc, Roskilde, Denmark) were coated with 50 μl each of rat anti-mouse TNF-α IgM (Bender Medsystem, CA, USA) in a 0.1 M carbonate buffer at pH 9.6 (1 μg/ml) and 4 °C for 12 h. After washing with PBS containing 0.05% Tween-20 (PBS-T), each well was blocked with 150 μl of 1% bovine serum albumin in PBS at 37 °C for 2 h. After washing with PBS-T, 50 μl of the supernatant or a standard mouse TNF-α solution was added, and the culture incubated at 37 °C for 2 h. After washing again with PBS-T, 50 μl of the rabbit anti-mouse TNF-α antibody (Genzyme) diluted at 1:500 in PBS was added, and the culture incubated at 37 °C for 2 h more. After washing with PBS-T to remove the unbound antibodies, the bound antibodies were then reacted for 30 min with 50 μl (1:5,000 dilution) of peroxidase-conjugated anti-rabbit IgG (EY Laboratories, CA, USA). After washing again with PBS-T, 100 μl of substrate buffer (0.4% o-phenylenediamine and 0.003% H₂O₂ in a 0.1 M citrate phosphate buffer at pH 4.0) was added, and the culture incubated at room temperature for 30 min more. The reaction was stopped by adding 50 μl of 2 N H₂SO₄, and the culture quantified spectrophotometrically at 492 nm.

IL-1α and IL-10 were measured by commercially available murine IL-1α ELISA and murine IL-10 ELISA Development kits (PeproTech EC, London, UK).

Statistical analysis. The changes in each cytokine and fecal polyamines were analyzed by a paired t-test. Calculations were performed with computer software, STATISTICA (Design Technologies, Tokyo, Japan). \(P < 0.05\) is regarded as significant.

Results and Discussion

The effects of the fecal extracts on TNF-α production due to LPS activation of the J774.1 cells are shown in Fig. 1. The TNF-α production of the J774.1 cells stimulated by the fecal extract prepared from feces obtained during the period of LKM512 yogurt consumption was significantly lower than from the pre-consumption period (\(p < 0.05\)). There was no significant difference in TNF-α production of the J774.1 cells between the period of after placebo consumption and after LKM512 yogurt consumption. The effects of the fecal extracts on IL-1α production due to LPS activation
of the J774.1 cells are shown in Fig. 2. The IL-1α production of the J774.1 cells stimulated by the fecal extract prepared from feces obtained during the period of LKM512 yogurt consumption tended to be lower than that during the pre-consumption period. IL-1α production by stimulating with the fecal extracts obtained from four of six volunteers during the period of LKM512 yogurt consumption was less than that during the pre-consumption period with the fecal extracts obtained from two volunteers exhibiting a dramatically less IL-1α production. The IL-10 production level due to LPS activation of the J774.1 cells was below the detection limit in many of the supernatants tested, so IL-10 data were omitted from this study. These fecal extracts did not have cytotoxicity at this concentration. This finding that the TNF-α and IL-1α production levels due to the LPS activation of J774.1 cells stimulated by a fecal extract were modified by LKM512 yogurt shows that not only bacterial cell components, but also bacterial metabolites influenced the host’s cytokine network.

The feces used in this study were obtained from elderly volunteers whose fecal haptoglobin, which is one of the acute-phase proteins, was decreased by LKM512 yogurt consumption (Table 1). Haptoglobin is produced during inflammation, infection, malignancy and injury. Since this response is mediated by the release of inflammatory cytokines from such macrophages and neutrophilus as IL-1, IL-6, IL-11, and TNF-α, these elderly volunteers were probably relieved of acute inflammation by suppression of the release of these
The polyamines, e.g., putrescine, spermidine, spermine, and cadaverine (some researchers consider cadaverine not to be in the polyamine class), are widely distributed in organisms, and their roles in the synthesis and stabilization of DNA, RNA and proteins, cell proliferation and differentiation, and the regulation of enzymatic activity have all been reported. Polyamines have a large number and a wide range of physiological effects. Research based on an analysis of the intestinal environment indicates that polyamines are indispensable to the maturation of intestinal mucosal tissue; in particular, the secretions of the intestinal mucosa, sIgA and enzymes, are induced by intestinal polyamines. Exogenous polyamines ingested during meals are mainly absorbed in the upper parts of the intestines, and the polyamines in the lower parts of the intestines are thus thought to be synthesized by intestinal microbiota. We have focused on the polyamines as one of the bacterial metabolites that could help explain many health effects, and have confirmed that the intestinal polyamine concentrations were increased by LKM512 yogurt consumption in a test on elderly volunteers. There are reports which show the suppression of inflammation due to polyamines. Szabo has reported that spermine inhibited the induction of nitric oxide synthase, which synthesizes nitric oxide as a cytotoxic free radical, by using J774.2 macrophages. Zhang et al. used human peripheral blood mononuclear cells stimulated with LPS to show that spermine inhibited the synthesis of inflammatory cytokines such as TNF-α, IL-1, IL-6, and macrophage inflammatory proteins (MIP)-1α and MIP-1β. They also clarified the mechanism for the regulation of macrophage activation and inflammation by spermine as follows: in response to an inflammatory stimulus, the activity of the spermine uptake system in activated macrophages is increased, leading to the incorporation of spermine from the surrounding environment. Spermine accumulation at an inflammatory site enhances the intracellular macrophage uptake of spermine, resulting in the down-regulation of macrophage activation and inflammation. The individual changes in the fecal polyamine concentration due to LKM512 yogurt consumption with the elderly volunteers used in this study are shown in Table 1. The spermidine concentration was significantly increased ($p < 0.05$) by LKM512 yogurt consumption on the basis of a paired $t$-test.

**Table 1. Individual Changes in the Fecal Polyamine and Haptoglobin Concentrations Due to LKM512 Yogurt Consumption**

<table>
<thead>
<tr>
<th>Volunteers</th>
<th>Putrescine (µM)</th>
<th>Spermidine (µM)</th>
<th>Spermine (µM)</th>
<th>Cadaverine (µM)</th>
<th>Haptoglobin (ng/g of wet feces)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-consumption</td>
<td>LKM512</td>
<td>Pre-consumption</td>
<td>LKM512</td>
<td>Pre-consumption</td>
</tr>
<tr>
<td>No. 1</td>
<td>61.8</td>
<td>162.4</td>
<td>89</td>
<td>137.2</td>
<td>&lt;5</td>
</tr>
<tr>
<td>No. 2</td>
<td>238.9</td>
<td>125.4</td>
<td>69.6</td>
<td>107.9</td>
<td>&lt;5</td>
</tr>
<tr>
<td>No. 3</td>
<td>18.2</td>
<td>98.2</td>
<td>22.2</td>
<td>47.2</td>
<td>&lt;5</td>
</tr>
<tr>
<td>No. 4</td>
<td>65.2</td>
<td>118.1</td>
<td>26.1</td>
<td>96.8</td>
<td>39.6</td>
</tr>
<tr>
<td>No. 5</td>
<td>&lt;4.5</td>
<td>35.28</td>
<td>8.7</td>
<td>80.7</td>
<td>67.5</td>
</tr>
<tr>
<td>No. 6</td>
<td>20.8</td>
<td>15.7</td>
<td>5.1</td>
<td>3.8</td>
<td>89.5</td>
</tr>
</tbody>
</table>

The spermidine concentration was significantly increased ($p < 0.05$) by LKM512 yogurt consumption on the basis of a paired $t$-test.
stimulation with this fecal extract during the period of LKM512 yogurt consumption in comparison with that during the pre-consumption period. In contrast, the fecal polyamine concentration of volunteer 6 was not increased by LKM512 yogurt consumption, and both the TNF-α and IL-1α production was hardly decreased by stimulation with this fecal extract during the period of LKM512 yogurt consumption in comparison with that during the pre-consumption period. Therefore, the possibility that the intestinal polyamines produced by LKM512 yogurt consumption acted as anti-inflammatory metabolites is high. Since the concentration of the fecal extract in culture medium was only 0.2% feces, the spermidine and spermine concentration in the culture medium was calculated to be 0.1–1 μM. Therefore, the anti-inflammatory activity from a fecal extract might be even greater if the fecal concentration in the culture medium were higher. On the other hand, since an anti-inflammatory effect was induced with such a low polyamine concentration, there were probably other anti-inflammatory materials produced by LKM512 yogurt consumption. For example, an increase in the intestinal short-chain fatty acid concentration and decrease in the LPS content derived from intestinal bacteria may have been involved in anti-inflammatory effect of LKM512 yogurt consumption. The finding that IL-10 was not increased by stimulation with the fecal extract obtained during the period of LKM512 yogurt consumption indicates that anti-inflammatory cytokines did not play a key role in the anti-inflammatory activity due to certain bacterial metabolites.

We conclude from these findings and previous data that the intestinal bacterial metabolites produced by LKM512 yogurt consumption contributed to suppressing the inflammatory cytokine production of macrophages and that one of the anti-inflammatory metabolites in the fecal extracts was likely to have been a polyamine.

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References


