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# Comparison of activity to stimulate mucosal IgA production between *Leuconostoc mesenteroides* strain NTM048 and type strain JCM6124 in mice

**Chiaki MATSUZAKI[1]*, Kenji MATSUMOTO[2], Toshihiko KATOH[1], Kenji YAMAMOTO[1] and Keiko HISA[3]**

[1]*Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, 1-308 Suematsu, Nonoichi, Ishikawa 921-8836, Japan*

[2]*Department of Food Science, Ishikawa Prefectural University, 1-308 Suematsu, Nonoichi, Ishikawa 921-8836, Japan*

[3]*Nitto Pharmaceutical Industries, Ltd., Kamiueno, Muko, Kyoto 617-0006, Japan*

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**The effects of *Leuconostoc mesenteroides* strain NTM048 and type strain JCM6124$^T$ on the murine immune system were characterized. Although the bacterial cells and exopolysaccharides of each strain induced immunoglobulin A production in Peyer's patch cells, the effects of NTM048 were more potent than those of JCM6124$^T$. Oral administration of the cells of each strain increased the fecal immunoglobulin A content in NTM048-treated mice, but not in JCM6124$^T$-treated mice. A flow cytometric analysis showed that the CD4$^+$ T-cell populations in the mouse spleens tended to increase in the NTM048 group. These results suggest that immunomodulating ability is characteristic of strain NTM048.**

**Key words: IgA, exopolysaccharide, systemic immune response, *Leuconostoc mesenteroides***

Lactic acid bacteria (LAB) are generally recognized as safe, and several of them are used as probiotics, which are live bacteria that help to improve human health [1]. However, their probiotic properties, including their beneficial effects on the condition of the gastrointestinal tract and the immune system, are strain dependent, which makes it difficult to supply stable high-quality food with added health-promoting value [2, 3].

Some LAB produce extracellular polysaccharides, called "exopolysaccharides" (EPSs), with attractive biological properties, such as antitumor, antioxidant, cholesterol-lowering, and immunomodulating activities [1]. Kishimoto et al. [4] showed that the EPSs of *Lactobacillus (Lb.) delbrueckii* strains have diverse immunological properties, inducing cytokine production *in vitro*, although the *in vivo* effects of these producer strains have not yet been determined. The relationships between the biochemical activities of an EPS and the immunological properties of the producer strain have not yet been clarified.

We have previously demonstrated that *Leuconostoc* (*Leuc.*) *mesenteroides* subsp. *mesenteroides* strain NTM048 is a probiotic bacterium with an intestinal-IgA-inducing ability in a murine model [5]. Promotion of the host's mucosal IgA secretion by LAB strengthens its first line of defense against various pathogens, including influenza virus and *Streptococcus* spp. [6–8]. We recently showed that the EPS produced by strain NTM048 also induces intestinal IgA secretion [9]. Among the various strains of the same species, *Leuc. mesenteroides* subsp. *mesenteroides* JCM6124$^T$ is the type strain most closely related to strain NTM048, as both strains belong to the same subspecies, and its complete genome sequence is available (GenBank/EMBL/DDBJ accession no. CP000414). Strain JCM6124$^T$ also produces EPS, and application of the strain to food production has been attempted [10]. To examine whether the immunomodulating ability of strain NTM048 is unique among the EPS-producing *Leuc. mesenteroides* subsp. *mesenteroides* strains, we compared the immunological effects of the NTM048 strain and JCM6124$^T$ strain, the latter of which was obtained from the Japan Collection of Microorganisms, RIKEN (Tsukuba, Ibaraki, Japan).

As in our previous study [5], we prepared murine Peyer's patch (PP) cells from 8-week-old male BALB/cA mice, and EPSs were extracted from bacterial cell cultures. All animal experiments were conducted in

*Corresponding author. Mailing address: Chiaki Matsuzaki, Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, Nonoichi, Ishikawa 921-8836, Japan. Phone: +81-76-227-7513. Fax: +81-76-227-7557. E-mail: chiaki@ishikawa-pu.ac.jp.

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accordance with the Guidelines for the Proper Conduct of Animal Experiments of the Science Council of Japan (2006). The Animal Experimentation Ethics Committee of Ishikawa Prefectural University approved the study (no. 25-14-2). All statistical analyses were performed with the Ekuseru-Toukei software, version 2010 (SSRI, Tokyo, Japan). P values less than 0.05 were considered statistically significant. We obtained 1.46 g and 2.80 g of EPS from 50 ml cell cultures of NTM048 and JCM6124$^T$, respectively, indicating that the JCM6124$^T$ strain produces more EPS than the NTM048 strain. Bacterial cells ($OD_{600}$ = 0.001, 0.01 and 0.1) or EPS (100 and 250 μg/ml) were incubated with PP cells ($1.25 \times 10^6$ cells/ml) at 37°C in a humidified atmosphere of 5% $CO_2$ for 5 days. The IgA levels in the supernatants were measured with an enzyme-linked immunosorbent assay (ELISA) using a mouse IgA ELISA kit (Bethyl Laboratories, Montgomery, TX, USA). As shown in Fig. 1A, the NTM048 cells dose-dependently induced IgA secretion, and the IgA-inducing activity of the NTM048 cells was significantly higher than that of the JCM6124$^T$ cells ($OD_{600}$ = 0.001, $p<0.05$; $OD_{600}$ = 0.01, $p<0.05$; $OD_{600}$ = 0.1, $p<0.0001$). In contrast, IgA secretion was only induced at the highest concentration of JCM6124$^T$ cells ($OD_{600}$ = 0.1, $p<0.0001$). Like the results for the cells, the IgA-inducing ability of the NTM048 EPS was higher than that of the JCM6124$^T$ EPS. As shown in Fig. 1B, the NTM048 EPS had significantly higher IgA-inducing activity than the JCM6124$^T$ EPS (1.5-fold at 100 μg/ml, $p<0.0001$; 1.3-fold at 250 μg/ml, $p<0.001$). These results strongly suggest that the IgA-inducing activity of EPS is associated with the same activity in the producer cells.

We next examined the intestinal IgA-inducing ability of the NTM048 and JCM6124$^T$ cells and their effects on the systemic immune system in animal experiments using BALB/cA mice. Bacterial cells cultured in 500 l of MRS medium were collected by centrifugation, 514 g of excipient (mainly composed of sucrose and gelatin) was added, and the cells were lyophilized, producing 556 g of LAB condensate powder. The viable cell counts in the LAB condensate were approximately $5.0 \times 10^{11}$ colony-forming units/g. After acclimation to the AIN-76 diet (Research Diets, New Brunswick, NJ, USA) for 2 weeks, 18 BALB/cA mice (8 weeks old, male) were assigned to a control, NTM048, or JCM6124$^T$ group (n = 6 each). In the NTM048 and JCM6124$^T$ groups, the mice were fed AIN-76 diet containing 0.05% of the corresponding LAB condensate powder (containing 0.0038% of lyophilized LAB cells) *ad libitum* for 14 days. In the control group, 0.05% of excipient was added to the AIN-76 diet (Table 1). All the animals were in good health throughout the

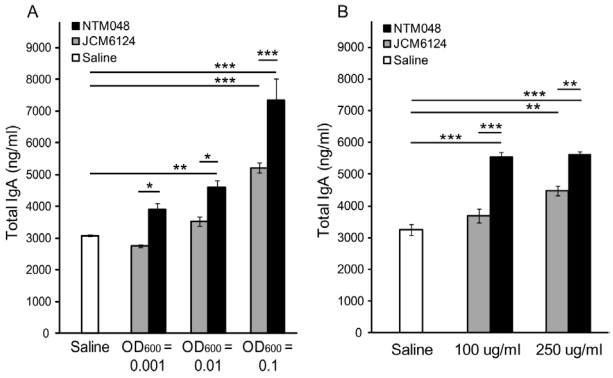


Fig. 1. Comparison of the IgA-inducing abilities of strains NTM048 and JCM6124$^T$ in isolated murine Peyer's patch cells.
(A) The total amount of IgA after treatment with bacterial cells or saline. (B) The total amount of IgA after treatment with exopolysaccharide or saline. Statistical analysis was performed with Tukey's multiple comparison test. Each value is presented as the mean ± SE ($n$ = 5; *p<0.05, **p<0.01, ***p<0.001).

experimental period, with no adverse effects, such as diarrhea. The feces for 1 day were collected every 7 days. The lyophilized feces were homogenized in phosphate-buffered saline containing a protease inhibitor cocktail (Roche, Mannheim, Germany) and allowed to stand for 30 min on ice to extract the IgA. After centrifugation, the supernatant was collected and used as the fecal IgA sample. The IgA content of each sample was analyzed with a mouse IgA ELISA kit, according to our previous study [5]. There were no detectable differences in food intake or body weights among the groups (Table 2). After 14 days, all the experimental mice were killed, and their spleens and blood were harvested.

Blood samples were used to detect liver damage by analyzing the plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels using Transaminase CII Test kits (Wako Pure Chemical Industries, Tokyo, Japan) and to detect kidney disease by analyzing the plasma creatinine (CRE) levels using a LabAssay™ Creatinine kit (Wako Pure Chemical Industries). No adverse effects of LAB intake were observed (Table 2).

To evaluate the effects of LAB on the intestinal immune system, we analyzed the fecal IgA levels. The IgA contents in the feces were analyzed to determine the 1-day intestinal IgA secretion, as follows. The feces from one day were collected from each mouse and lyophilized, and their weight and IgA concentration were measured according to our previous study [5]. As shown in Fig. 2, the IgA levels of the NTM048 group increased throughout the experimental period. On day 7, the IgA

Table 1. Experimental diet used in this study

| Components | Control group | NTM048 group | JCM6124 group |
|---|---|---|---|
| AIN-76 (%, w/w) | 99.95 | 99.95 | 99.95 |
| Excipient (%, w/w) | 0.05 | 0 | 0 |
| NTM048 condensate powder | 0 | 0.05 | 0 |
| [Lyophilized NTM048 cells (%, w/w)] | [0.00] | [0.0038] | [0.00] |
| JCM6124 condensate powder | 0 | 0 | 0.05 |
| [Lyophilized JCM6124 cells (%, w/w)] | [0.00] | [0.00] | [0.0038] |

Table 2. Physiological parameters of mice fed the experimental diets containing LAB condensate powder

| | Control group | NTM048 group | JCM6124 group |
|---|---|---|---|
| Food intake (g/d) | 4.2 ± 0.1 | 4.5 ± 0.1 | 4.0 ± 0.1 |
| Initial body weight (g) | 28.3 ± 0.7 | 28.3 ± 0.8 | 28.2 ± 0.6 |
| Final body weight (g) | 33.3 ± 1.0 | 32.9 ± 1.3 | 32.1 ± 0.7 |
| Body weight gain (g) | 4.9 ± 0.5 | 4.6 ± 0.9 | 3.9 ± 0.5 |
| Blood chemistry | | | |
| IgA (μg/ml) | 246.8 ± 2.1 | 259.2 ± 7.9 | 240.5 ± 7.5 |
| IgG (μg/ml) | 341.6 ± 33.1 | 334.1 ± 13.1 | 321.7 ± 13.6 |
| AST (IU/l) | 28.0 ± 1.8 | 26.0 ± 1.9 | 22.8 ± 1.7 |
| ALT (IU/l) | 22.6 ± 1.6 | 16.9 ± 1.4 | 16.8 ± 3.1 |
| CRE (μg/ml) | 6.1 ± 0.6 | 6.3 ± 0.3 | 6.3 ± 0.3 |

Results are shown as means ± SE ($n$ = 6). AST: aspartate aminotransferase; ALT: alanine aminotransferase; CRE: creatinine

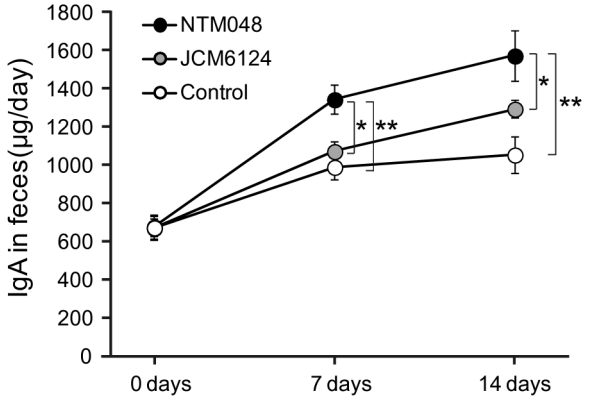


Fig. 2. Fecal IgA content induced by LAB intake.
Mice were administered NTM048 or JCM6124$^T$ cells, and the IgA contents in their feces were compared. Differences were assessed with two-way repeated-measures ANOVA and a *post hoc* analysis with Tukey's multiple comparison test. Each value is presented as the mean ± SE ($n$ = 6; *p<0.05, **p<0.01).

content of the NTM048 group was 1.4-fold higher than that of the control group (p<0.01) and 1.3-fold higher than that of the JCM6124$^T$ (p<0.05) group, and this effect continued until day 14. The JCM6124$^T$ group also showed a slightly higher fecal IgA content than the control group on day 14 (*vs* control group, p=0.07 on day 14). This result is consistent with the *in vitro* results. To elucidate whether the induced IgA was specific to EPS or not, we analyzed the groups for the presence of a specific IgA against NTM048 EPS by ELISA. Purified NTM048 EPSs (0.05 mg/ml) were coated on the wells of a 96-well plate and then reacted to fecal samples. The reacted IgA was analyzed with a mouse IgA ELISA kit. The results showed that the $A_{450}$ was 0.061 ± 0.01 for the control group and 0.060 ± 0.02 for the NTM048 group, indicating that no anti-NTM048 EPS IgA was detected in the NTM048 group. This result suggested that oral administration of NTM048 did not induce an EPS-specific IgA but that it promoted mucosal secretion of IgA against a variety of antigens to strengthen mucosal barrier function. Moreover, we examined the plasma IgA and IgG levels of each groups using a mouse IgA ELISA kit and a mouse IgG ELISA kit (Bethyl Laboratories), respectively. Oral administration of LAB cells did not affect plasma IgA and IgG levels (Table 2).

To evaluate the effects of the LAB on the systemic immune system, we then flow cytometrically analyzed the frequency of T cells ($CD4^+$ and $CD8^+$) at the lymphocyte gate ($FSC^{low}$, $SSC^{low}$) in mouse splenocytes (Fig. 3) using a FACSAria flow cytometer and the FACSDiva™

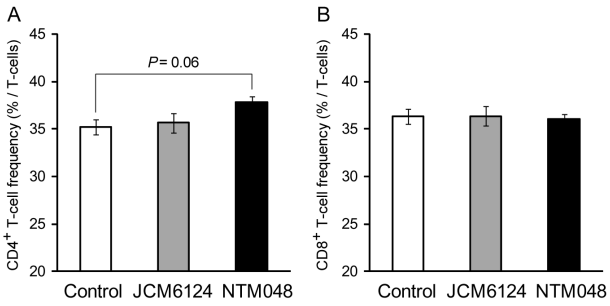


Fig. 3. T-cell stimulation in the spleens of mice fed NTM048 or JCM6124$^T$ cells.

(A) Ratio of $CD4^+CD3^+$ cells/$CD3^+$ cells. (B) Ratio of $CD8^+CD3^+$ cells/$CD3^+$ cells. Statistical analysis was performed with Tukey's multiple comparison test, and the results are shown in the graph. Each value is presented as the mean ± SE ($n$ = 6).

4.1 software (BD Biosciences, San Jose, CA, USA). The spleen cells were dispersed through a 70-µm cell strainer to produce single-cell suspensions. After centrifugation (800 rpm, 8 min), red blood cell lysis buffer ($NH_4Cl$ 0.83%, $KHCO_3$ 0.1%, EDTA-2Na 0.00372%) was added to the cell pellet. The cells were incubated for 5 min, washed twice, resuspended in phosphate-buffered saline with 2% fetal bovine serum, and used as splenocytes. The splenocytes were simultaneously stained with phycoerythrin (PE)–Cy7-conjugated anti-mouse CD3e, PE-conjugated anti-mouse CD4, and APC-conjugated anti-mouse CD8a antibodies using Mouse T Lymphocyte Subset Antibody Cocktail (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The CD4:CD8 ratio is generally used as a fundamental measure of the immune response [11]. The $CD4^+$ T-cell ($CD4^+CD3^+$ cells) population (generally associated with helper/inducer functions) in T-cells ($CD3^+$ cells) of the NTM048-treated mice tended to be larger than that in the control mice (p=0.06), whereas treatment with JCM6124$^T$ did not affect the T-cell population in the lymphocytes. No effect on the $CD8^+$ T-cell ($CD8^+CD3^+$ cells) population (usually associated with cytotoxic/suppressor activities) was observed. The spleen contains various immune cells, including T and B cells, dendritic cells, and macrophages, and regulates the systemic immune system to protect the body against blood-borne infections [12]. Therefore, oral administration of NTM048 cells may have some effect on the systemic immune system.

Tok et al. [13] reported that differences in the quantity of EPS production influence the cholesterol-lowering activity of *Lb. delbrueckii* subsp. *bulgaricus* strains. However, the biochemical properties of EPSs are also known to affect the immunological properties of LAB. Xu et al. [14] showed that the acidic EPS produced by *Bifidobacterium animalis* RH had higher antioxidant activity than the neutral EPS produced by cells of the same strain. Makino et al. [15] showed that the acidic EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* OLL1073R-1 induced interferon-γ production in mouse splenocytes more strongly than the neutral EPS produced by cells of the same strain. In the present study, *in vitro* analysis showed that the NTM048 EPS had significantly higher IgA-inducing activity than the JCM6124$^T$ EPS. Moreover, the IgA-inducing ability of strain NTM048 *in vivo* was significantly higher than that of strain JCM6124$^T$. The variations in the EPSs present in the same subspecies could affect their immunological activities *in vivo* and might change the immunological properties of the producer strains. A precise analysis of the differences between the NTM048 EPS and JCM6124 EPS is currently underway in our laboratory. Our results suggest that immunomodulating activity is a specific characteristic of NTM048 cells and the NTM048 EPS.

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