Full Paper Bioscience of Microbiota, Food and Health Vol. 34 (1), 11–23, 2015

In vitro evaluation of immunological properties of extracellular polysaccharides produced by *Lactobacillus delbrueckii* strains

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Received July 16, 2014; Accepted September 30, 2014; Published online in J-STAGE November 8, 2014

We investigated the variation in immunological properties of the extracellular polysaccharides (EPSs) produced by different *Lactobacillus delbrueckii* strains as well as that of their monosaccharide composition. The monosaccharide composition of each EPS produced by *L. delbrueckii* strains, as determined by thin layer chromatography (TLC), showed an appreciable variation in a strain-dependent manner, which could be broadly assigned to 4 TLC groups. Meanwhile, the immunological properties of the EPSs produced by 10 *L. delbrueckii* strains were evaluated in a semi-intestinal model using a Transwell co-culture system, which employed human intestinal epithelial Caco-2 cells on the apical side and murine macrophage RAW264.7 cells on the basolateral side. Each EPS was added to the apical side to allow direct contact with Caco-2 cells and incubated for 6 hr. After incubation, the amounts of TNF-α and several cytokines that had been released by either RAW264.7 or Caco-2 cells were then quantified by cytotoxic activity on L929 cells or the RT-PCR method. It was found that the EPS-stimulated RAW264.7 cells express different profiles of cytokine production via Caco-2 cells but that the profile difference could not be related to the above TLC grouping. The evidence suggests that the EPSs of *L. delbrueckii* strains are diverse not only in their biochemical structure but also in their immunological properties.

Key words: extracellular polysaccharide, *Lactobacillus delbrueckii*, immunological properties, Transwell

INTRODUCTION

Lactic acid bacteria have the ability to produce exocellular polymers called extracellular polysaccharides (EPSs) [1]. EPSs are the main substances involved in biofilm formation and have a role in protecting the microbial community against environmental stress. The structure of EPSs from lactic acid bacteria can be classified into two groups – homopolysaccharides and heteropolysaccharides: the former consists of repeating units of only one type of monosaccharide, while the latter is composed of at least two different sugars at different ratios [2]. For example, the EPSs produced by *Lactobacillus plantarum* C88 is composed of galactose and glucose with a molar ratio of 1:2 [3], whereas that produced by *L. johnsonii* strain 151 has a corresponding ratio of 4:1 [4]. Additionally, the EPS produced by *L. delbrueckii* subsp. *bulgaricus* NCFB2074 is composed of galactose and glucose with a molar ratio of 4:3 [5], whereas that produced by *L. delbrueckii* subsp. *bulgaricus* OLL1073R-1 has a corresponding ratio of 3:2 [6], indicating that the lactobacilli EPS varies not only interspecifically but also intraspecifically.

Over the last 2 decades or so, a number of studies have also demonstrated that EPSs have beneficial effects on the host when orally administered [1]. For example, the EPS produced by *L. rhamnosus* KL37 ameliorated collagen-induced arthritis in mice [7]. The EPS from *L. plantarum* C88 had antioxidant effects that may involve scavenging of reactive oxygen species, upregulation of enzymatic and nonenzymatic antioxidant activities, and reduction of lipid peroxidation [3]. Consumption of yoghurt fermented with a high-EPS producing strain, *L. delbrueckii* OLL1073R-1, reduced the risk of catching the common cold in elderly individuals [8], in which the EPS might enhance type 1 T helper cell (Th1) proliferation, and the

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Table 1. Bacterial strains used in this study

Strain	Origin	Reference
Lactobacillus delbrueckii TU-1	Commercial yogurt	This study
Lactobacillus delbrueckii JCM1002^T	Bulgarian yogurt	JCM*
Lactobacillus delbrueckii JCM1012^T	Sour grain mash	JCM
Lactobacillus delbrueckii JCM1248^T	Emmental (Swiss) cheese	JCM
Lactobacillus delbrueckii JCM15610^T	Indian dairy products	JCM
Lactobacillus delbrueckii KM-1	Commercial yogurt	This study
Lactobacillus delbrueckii KM-2	Lassi (an Indian fermented milk)	This study
Lactobacillus delbrueckii KM-3	Commercial yogurt	This study
Lactobacillus delbrueckii KM-4	Commercial yogurt	This study
Lactobacillus delbrueckii KM-5	Pickled turnip	This study

*Japan Collection of Microorganisms, RIKEN BioResource Center, Japan.

subsequent production of IL-12 and IFN-γ from Th1, eventually leading to higher natural killer cell activity [9, 10]. However, EPSs do not seem to readily make direct contact with host intestinal lymphocytes because no substance, including EPSs, with a molecular weight of more than 10 kDa can cross the gastrointestinal mucosa [11]. Meanwhile, Nishitani et al. [12] provided evidence that a mushroom-derived β-1,3/1,6-glucan, called lentinan, with a molecular weight over 10 kDa can act on the host immune system through interaction with intestinal epithelial cells using a Transwell tissue culture system. Their Transwell tissue culture system was composed of human intestinal epithelial Caco-2 cells and murine macrophage RAW264.7 cells, in which the former cells were placed in the well on the apical side and the latter cells were placed in the well on the basolateral side, and then lentinan was applied into the apical side. In this system, they found that lentinan reduced IL-8 mRNA expression in Caco-2 cells without decreasing of TNF-α production from RAW264.7 cells. In this context, we herein describe indirect and strain-specific immunomodulation of an EPS produced by an *L. delbrueckii in vitro* Transwell tissue culture system using Caco-2 cells and RAW264.7 cells with or without LPS pretreatment.

MATERIALS AND METHODS

Bacterial strains and media used.

The bacterial strains used in this study are listed in Table 1. All strains were stored at −80°C in a de Man-Rogosa-Sharpe (MRS) broth (Oxoid, Basingstoke, UK) until use.

It should be noted that *L. delbrueckii* TU-1, *L. delbrueckii* KM-1, *L. delbrueckii* KM-2, *L. delbrueckii* KM-3, *L. delbrueckii* KM-4 and *L. delbrueckii* KM-5 were isolated in our laboratory from commercial yogurt, lassi and pickled turnip. Briefly, a food sample was a suspended MRS broth for proliferation and 10-fold serial dilutions were prepared. Dilutions up to 10^{-6} of the initial suspension were plated on skimmed milk agar plates. The plates were incubated anaerobically at 37°C for 48 hr. Viscous colonies were subcultured and identified by Gram staining. The identification of these strains was performed by PCRs with the species of *L. delbrueckii* specific primers as described by Tilsala et al. [13]. The primers used for identification of *L. delbrueckii* were as follows: Del I (5′-ACGGATGGATGGAGAGCAG-3′) and Del II (5′-GCAAGTTTGTTCTTTCGAACTC-3′). The amplification profile was as follows: 94°C for 30 sec, 62°C for 30 sec and 72°C for 15 sec, which was repeated for 30 cycles. A preincubation step at 94°C for 15 sec was also included. The PCR assay confirmed that TU-1, KM-1, KM-2, KM-3, KM-4 and KM-5 were *L. delbrueckii*. These strains were cultured on a medium containing 10% (wt/vol) whey powder and 0.5% (wt/vol) yeast extract (Becton, Dickinson and Company, Sparks, MD, USA). The whey powder had been hydrolyzed with proteinase K (Wako Pure Chemical Industries, Osaka, Japan) for 7 hr at 55°C before use.

Preparation of EPSs

These strains were cultured anaerobically at 37°C for 24 hr on the whey media. After cultivation, bacterial cells and precipitates were removed by centrifugation (14,000 × *g*, 20 min, 4°C). Crude EPSs were precipitated from the supernatants by the addition of 1.5 volumes of cold ethanol, and collected by centrifugation (14,000 × *g*, 20 min, 4°C). They were dissolved in distilled water, and insoluble material was removed by centrifugation (14,000 × *g*, 20 min, 4°C). The crude EPSs were then purified by additional precipitation with 1.5 volumes of cold

ethanol. They were subsequently treated with 10% (wt/vol) trichloroacetic acid (TCA) at 4°C, and the denatured proteins were removed by centrifugation (14,000 × *g*, 20 min, 4°C). The partially purified EPS were obtained by dialysis of the supernatants containing the crude EPSs against distilled water at 4°C for 2 d, followed by lyophilization. The crude EPSs were dissolved in a 0.05 *M* Tris-HCl buffer (pH 8.0) containing 1 m*M* $MgCl_2$, and treated with 2 µg/ml DNase (Roche Applied Science, Basel, Switzerland) and 2 µg/ml RNase (Wako) at 37°C for 6 hr. The proteins in the crude EPS were digested with 0.2 mg/ml of proteinase K for 16 hr at 37°C. The reaction was stopped by heating at 80°C for 10 min. The EPSs were applied to centrifugal filters (Amicon Ultra - 0.5 ml 10 K [Merck Millipore, Darmstadt, Germany]) and centrifuged at 14,500 × rpm for 30 min. Sufficient water was added until the final volume was 500 µl. The EPSs were precipitated with cold ethanol as previously described.

Sugar degradation analysis of EPSs

The EPS samples were hydrolyzed in 1 *N* H_2SO_4 at 90°C for 4 hr. The hydrolysate was neutralized with $BaCO_3$, and the precipitate was removed by filtration using a 0.45-µm filter unit (Merck Millipore). The sugar composition of each EPS was evaluated by thin layer chromatography (TLC) (silica gel 60 plate [Merck]). Briefly, the spent cultures (approximately 2 µl each) were spotted onto different lanes of a TLC plate. The plate was developed in an isopropanol/ethyl acetate/water (3:1:1) solvent. Spots were visualized by spraying the plates with *p*-anisaldehyde (contains acetic acid, H_2SO_4) ethanol solution (Tokyo Chemical Industry, Tokyo, Japan) and heating at 160°C for several minutes.

Cell culture

Cells from the human intestinal epithelial cell line Caco-2, murine macrophage cell line RAW264.7 and murine fibrosarcoma cell line L929 were obtained from American Type culture Collection (ATCC) (Manassas, VA, USA). Human intestinal epithelial cell line, Caco-2, cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, glutamine, high glucose [Wako]) supplemented with 1% MEM Non-Essential Amino Acids (NEAA [Gibco BRL, Grand Island, NY, USA]), 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS [Daiichi Kagaku, Tokyo, Japan]). Murine macrophage cell line, RAW264.7, cells were cultured in DMEM (glutamine, low glucose) supplemented with 100 U/ml penicillin, and 100 µg/ml streptomycin, and 10% (v/v) heat-inactivated FBS. Murine fibrosarcoma cell line, L929, cells were cultured in Eagle's Minimum Essential Medium (MEM [Nissui Pharmaceutical, Tokyo, Japan]) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The cell cultures were incubated in a humidified 5% CO_2 incubator at 37°C.

Transepithelial electrical resistance (TER) measurement

The integrity of the Caco-2 monolayer was determined by measuring the TER value. Tight junctions serve as barriers to paracellular diffusion and the TER reflects the tightness of the junction between epithelial cells. To measure the TER value, Caco-2 cells were grown in Transwell (1.12 cm^2, 0.4 µm pore size [Corning Costar, Cambridge, MA, USA]) inserts with polycarbonate membranes. The Caco-2 cells were seeded at a density 3.0×10^5 cells/well. The medium was changed every 3 days. The monolayer cells were gently rinsed with Hank's Balanced Salts Solution (HBSS: 137 mM NaCl, 5.36 mM KCl, 1.67 mM $CaCl_2$, 1 mM $MgCl_2$, 1.03 mM $MgSO_4$, 0.44 mM KH_2PO_4, and 0.34 mM Na_2HPO_4, pH 7.4) and then equilibrated in the same solution for 30 min in a humidified 5% CO_2 incubator at 37°C (apical side: 200 µl, basolateral side: 800 µl). The integrity of the cell monolayer was evaluated by measuring the TER value with Millicell-ERS equipment (Merck Millipore). The cell monolayer was used when the TER value reached more than 400 $\Omega \cdot cm^2$.

Stimulation of RAW 264.7 cells with EPSs

RAW264.7 cells were seeded at 2.1×10^5 cells/well in 24-well tissue culture plates and incubated overnight to fully adhere to the bottom of the plate. Then, 100 µg/ml of EPSs purified from several strains and 5 ng/ml of LPSs were applied to the same well and incubated at 37°C for 3 hr. As a negative control, 100 µg/ml of a whey sample that had been purified from a purely whey media by the above EPS purification method was used. After incubation, all culture supernatants were collected for TNF-α measurement.

Co-culture system

A Caco-2/RAW264.7 cell co-culture system was used as described by Tanoue et al. [14]. Briefly, the Caco-2 cells were seeded at 3.0×10^5 cells/well onto Transwell insert plates (1.12 cm^2, 0.4 µm pore size [Corning Costar]). The cells were fully differentiated (TER value > 400 $\Omega \cdot cm^2$), and were subjected to the following experiment. RAW264.7 cells were seeded at 2.1×10^5 cells/well in 24-well tissue culture plates and incubated overnight to fully adhere to the wells. After all media had been

replaced with RPMI 1640 (Gibco BRL), the Transwell insert plates with Caco-2 cells were added to the wells of multiple plates preloaded with RAW264.7 cells. Then, the 100 µg/ml of EPSs purified from several strains and whey sample, which were prepared by using the above purification process for EPSs with pure whey media, were applied to the apical side and, incubated at 37°C for 3 hr, and the TER was measured using the method described previously; incubation was then continued at 37°C for 3 hr. Meanwhile LPS (LPS from *Escherichia coli* O127 [Wako]) was added to the basolateral side and not to the apical side as the positive control for the activated RAW264.7 cells and incubated at 37°C for 3 hr. After incubation, all culture supernatants from the basolateral side were collected for TNF-α measurement. The treated Caco-2 cells and the RAW264.7 cells were then harvested to isolate total RNA for real-time polymerase chain reaction assays as described below.

Gut inflammation system

Briefly, the Caco-2 cells were seeded at 3.0×10^5 cells/well onto Transwell insert plates (1.12 cm^2, 0.4 µm pore size [Corning Costar]). The cells were fully differentiated (TER value > 400 $\Omega \cdot cm^2$), and were subjected to the following experiment. RAW264.7 cells were seeded at 2.1×10^5 cells/well in 24-well tissue culture plates and incubated overnight to fully adhere to the well. After all media had been replaced with RPMI 1640 (Gibco BRL), the Transwell insert plates with the Caco-2 cells were added to the wells of multiple plates preloaded with RAW264.7 cells. Then, 100 µg/ml of EPSs purified by several strains and a whey sample were applied to the apical side and incubated at 37°C for 3 hr. After 3 hr of the incubation, LPS [Wako] was added to the basolateral side. After 3 hr of the incubation, all culture supernatants from the basolateral side were collected for TNF-α measurement. The treated Caco-2 cells and RAW264.7 cells were then harvested to isolate total RNA for real-time polymerase chain reaction assays as described below.

Tumor necrosis factor (TNF)-α measurement

The amounts of TNF-α in the culture medium were quantified based on the cytotoxic activity on L929 cells using murine rTNF-α as the standard as described by Takada et al. [15]. Briefly, L929 cells were plated in 96-well microplates in MEM including 10% FBS and cultured for 3 hr. The medium was replaced with 50 µl of fresh RPMI 1640 medium (supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin) containing 400 µg/ml actinomycin D and 50 µl of each sample. They were cultured for 20 hr at 37°C under 5% CO_2. After the medium was removed, cell lysates were stained with 0.1% crystal violet in ethanol/formaldehyde for 15 min at room temperature. Then, the cells were washed with water and dried. Cell lysates were dissolved in 100 µl of ethanol-PBS (1:1, v/v). The absorbance of the stained solution in wells was measured using a microplate reader. The concentration of TNF-α was calculated using a standard curve.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the Caco-2 cells and the RAW264.7 cells by using Sepasol RNA I Super (Nckalai Tesque, Kyoto, Japan) according to the manufacturer's protocols. Two micrograms of total RNA were transcribed into cDNA in a 20 µl reaction mixture containing 10 µl RNA solution, 4.2 µl diethylpyrocarbonate water, 2.0 µl 10× reverse transcription buffer, 0.8 µl 25 × dNTPs, 2.0 µl oligo $P(dT)_{15}$ primer, and 1.0 µl reverse transcriptase (50 U) using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK). The parameters for the PCR amplification reaction were 5 min at 94°C for denaturation and 5 min at 60°C for annealing, followed by 30 cycles of 90 sec at 72°C, 45 sec at 94°C and 45 sec at 60°C, with a final extension of 10 min at 72°C. The sequences of the PCR primers [16–19] used in this study are shown in Table 2. In this study, mouse β-actin and human peptidylprolyl isomerase A (cyclophilin A) (PPIA) were used as endogenous control genes for RAW264.7 cells and Caco-2 cells, respectively. All PCRs were performed on a DNA thermal cycler (MyCylcler, Bio-Rad, Hercules, CA, USA).

Quantitative RT-PCR

Quantitative PCR was performed with a Thermal Cycler Dice Real Time System (TaKaRa BIO Inc., Ohtsu, Japan) using Premix Ex Taq™ (TaKaRa), and a commercial assay kit, "TaqMan Gene Expression Assays" (Applied Biosystems) for mouse cytokines and β-actin, human cytokines and PPIA according to the manufacturer's protocol. For all panels, the bars represent the ratio of target gene to endogenous gene expression, as determined by the relative quantification method.

Statistical analysis

Statistical analysis was performed with a Student's t-test, and the presence of various asterisks (*, **) indicates statistical differences with significance levels of $p<0.05$ and $p<0.01$ respectively. Data were expressed as the mean ± standard error of triplicate tests.

Table 2. Sequences of the primers used in RT-PCR analysis

Cytokines	Primer	Sequence
mouse β-actin	Sense primer	TGTGATGGTGGGAATGGGTCAG
	Antisense primer	TTTGATGTCACGCACGATTTCC
mouse IL-1α	Sense primer	AAGTTTGTCATGAATGATTCCCTC
	Antisense primer	GTCTCACTACCTGTGATGAGT
mouse IL-10	Sense primer	GTGAAGACTTTCTTTCAAACAAAG
	Antisense primer	CTGCTCCACTGCCTTGCTCTTATT
mouse IL-12	Sense primer	CGTGCTCATGGCTGGTGCAAAG
	Antisense primer	CTTCATCTGCAAGTTCTTGGGC
mouse IL-18	Sense primer	ATGGTACAACCGCAGTAATACGG
	Antisense primer	AGTGAACATTACAGATTTATCCC
mouse IFN-γ	Sense primer	TACTGCCACGGCACAGTCATTGAA
	Antisense primer	GCAGCGACTCCTTTTCCGCTTCCT
mouse IL-15	Sense primer	TTCTCTTCTTCATCCTCCCCCT
	Antisense primer	ATGAAGAGGCAGTGCTTTGA
human PPIA	Sense primer	AATGCTGGACCCAACAC
	Antisense primer	TCCACAATATTCATGCCTT
human IL-12	Sense primer	TTGTGGCTACCCTGGTCCT
	Antisense primer	AGAGTTTGTCTGGCCTTCTGG
human IL-8	Sense primer	TGGCTCTCTTGGCAGCCTTC
	Antisense primer	TCTCCACAACCCTCTGCACC
human TGF-β1	Sense primer	GCTGCTGTGGCTACTGGTGC
	Antisense primer	CATAGATTTCGTTGTGGGTTTC

RESULTS

Sugar degradation analysis by thin layer chromatography (TLC)

The monosaccharide composition of each EPS produced by *L. delbrueckii* strains was evaluated by TLC analysis. A dark oval-shaped glucose spot was seen in the upper part of the plate (Fig. 1, lane 1); a slightly elongated dark galactose spot was seen under the glucose spot (Fig. 1, lane 2); a mixture of glucose and galactose was seen in an area with a combined glucose and galactose spot (Fig. 1, lane 3). The spot patterns of EPSs can be categorized into 4 groups: Group 1, EPSs of *L. delbrueckii* TU-1 and *L. delbrueckii* KM-3, showed a similar pattern to lane 3, but another spot was seen in the upper part of the glucose spot. Lane 11 was not clear compared with lane 4 (Fig. 1, lanes 4 and 11). Group 2, EPSs of *L. delbrueckii* JCM1002^T, *L. delbrueckii* JCM1012^T and *L. delbrueckii* JCM1248^T, showed another spot in the upper part of the glucose spot. A combined glucose and galactose spot was not seen (Fig. 1, lanes 5, 6 and 7), Group 3, EPSs of *L. delbrueckii* JCM15610^T, *L. delbrueckii* KM-1 and *L. delbrueckii* KM-5, showed a similar pattern to lane 2, but the glucose spot was not seen. Another spot was seen in the upper part of the glucose spot (Fig. 1, lanes 8, 9 and 13), and Group 4, EPSs of *L. delbrueckii* KM-2 and *L. delbrueckii* KM-4, showed a similar pattern to lane 3 (Fig. 1, lanes 10 and 12).

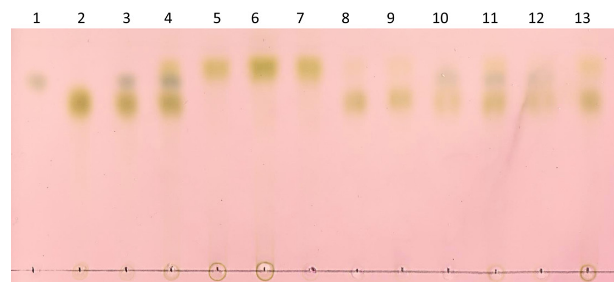

Fig. 1. Thin layer chromatography analysis of sugar compositions of each EPS.

Lane 1, glucose (0.5%, wt/vol); lane 2, galactose (0.5%, wt/vol); lane 3, glucose+galactose (1:1); lane 4, EPS of *L. delbrueckii* TU-1; lane 5, EPS of *L. delbrueckii* JCM1002^T; lane 6, EPS of *L. delbrueckii* JCM1012^T; lane 7, EPS of *L. delbrueckii* JCM1248^T; lane 8, EPS of *L. delbrueckii* JCM15610^T; lane 9, EPS of *L. delbrueckii* KM-1; lane 10, EPS of *L. delbrueckii* KM-2; lane 11, EPS of *L. delbrueckii* KM-3; lane 12, EPS of *L. delbrueckii* KM-4; lane 13, EPS of *L. delbrueckii* KM-5. Development system: isopropanol/ethyl acetate/water (3:1:1) solvent.

TNF-α production by RAW264.7 cells cultured with EPSs

In order to determine whether an EPS itself had any

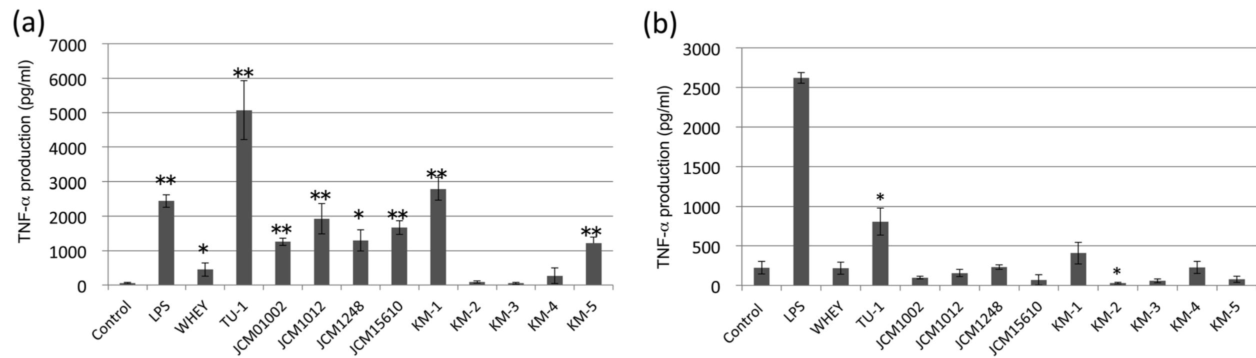

Fig. 2. Direct and indirect effects of EPSs purified by several strains on the production of TNF-α in RAW264.7 cells.

(a) RAW264.7 cells were cultured overnight in a 24-wells plate in 5% CO_2 to allow them to adhere to the bottom of the plate. Then, a 100 µg/ml of EPS sample and 5 ng/ml of LPS were applied to the cells and incubated for 3 hr. LPS-stimulated cultures were used for the positive control. The culture media were then collected for measurement of the TNF-α content. Values represent the means ± SE (n=3). **p<0.01, *p<0.05; there was a significant difference between the EPS-treated group and the control group. (b) RAW264.7 cells were cultured overnight in a 24-wells plate in 5% CO_2 to allow them to adhere to the bottom of the plate. Then, the Transwell inserts on which the Caco-2 cells had been cultured were placed into the 24-well plates preloaded with RAW264.7 cells. Next, 100 µg/ml of EPS was applied to the apical side of the wells and incubated for 3 hr. Then, LPS was added into the basolateral side at the final concentration of 5 ng/ml only as a positive control group, followed by incubation for an additional 3 hr. LPS-stimulated cultures were used for the positive control. The culture media were then collected for measurement of TNF-α content. Values represent means ± SE (n=3). **p<0.01, *p<0.05; there was a significant difference between the EPS-treated group and the control group.

immunological effect on RAW264.7 cells, the production of TNF-α was measured in the supernatants of RAW264.7 cells cultured in direct contact with EPSs produced by *L. delbrueckii* strains. Treatment with the EPS of *L. delbrueckii* TU-1 showed the highest production, followed by the EPS of *L. delbrueckii* KM-1, compared with LPS. In addition, the treatments of RAW264.7 cells with the EPSs of *L. delbrueckii* JCM1002^T, *L. delbrueckii* JCM1012^T, *L. delbrueckii* JCM1248^T, *L. delbrueckii* JCM15610^T, *L. delbrueckii* KM-4 and *L. delbrueckii* KM-5 showed higher production than the control. Moreover, treatment with the whey sample also showed higher production than the control. On the other hand, treatments with the EPSs produced by *L. delbrueckii* KM-2 and *L. delbrueckii* KM-3 showed the same level as the control (Fig. 2a).

TNF-α production by RAW264.7 cells in the co-culture model

In order to determine whether EPSs applied on the apical side of the Caco-2 cell layer had any indirect immunological effect on RAW264.7 cells, TNF-α production in the basolateral side was determined. It should be noted that the TER value of the Caco-2 cells showed no change compared with the control after stimulation with EPSs. Treatment of Caco-2 cells with the EPS of *L. delbrueckii* TU-1 showed approximately four times higher production than the control, while treatment with the EPS of *L. delbrueckii* KM-1 showed two times higher production than the control. On the other hand, treatment with the EPS of *L. delbrueckii* KM-2 showed significantly lower production than the control, and treatments with the EPS of *L. delbrueckii* JCM1002^T, *L. delbrueckii* JCM15610^T, *L. delbrueckii* KM-3 and *L. delbrueckii* KM-5 showed lower production than the control. Treatment with EPSs of other *L. delbrueckii* strains showed the same level as the control (Fig. 2b).

Cytokine production by RAW264.7 cells in the co-culture model

In order to determine the production levels of cytokines including IL-12, IL-15, IL-18, IL-1α, IFN-γ and IL-10, the expression of each cytokine mRNA in the RAW264.7 cells treated with EPSs produced by *L. delbrueckii* strains was examined by the RT-PCR method.

Regarding IL-12, treatments with the EPSs produced by *L. delbrueckii* TU-1 and *L. delbrueckii* KM-4 resulted in upregulated mRNA expression in RAW264.7 cells. In contrast, treatments with the EPSs produced by *L. delbrueckii* JCM1012^T, *L. delbrueckii* JCM15610^T, *L. delbrueckii* KM-2, *L. delbrueckii* KM-3 and *L. delbrueckii* KM-5 resulted in downregulated mRNA expression in RAW264.7 cells (Fig. 3a). Regarding IL-15, treatments with EPS produced by *L. delbrueckii* TU-1 resulted in up-egulated mRNA expression in RAW264.7 cells. In contrast, treatments with EPS produced by

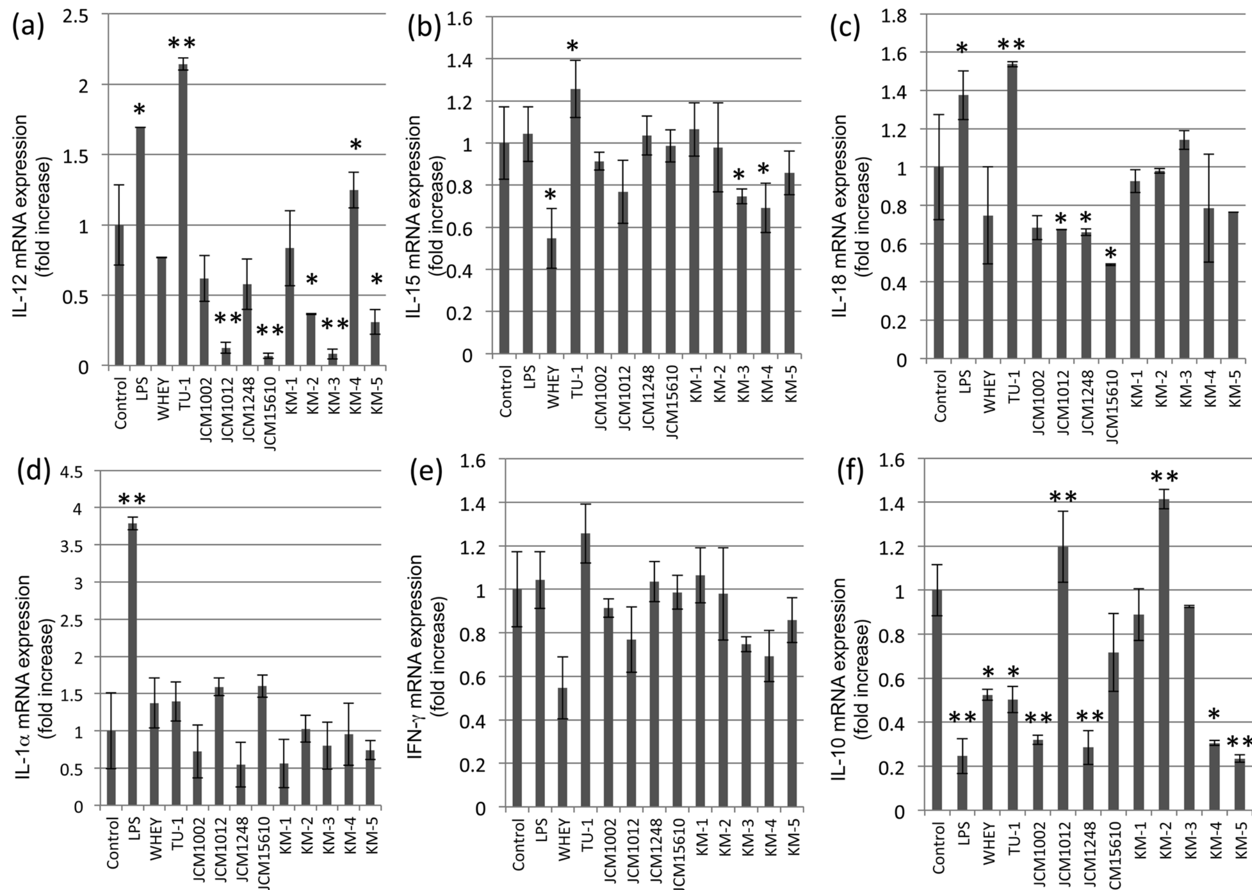

Fig. 3. Effects of EPSs purified by several strains on the production levels of cytokines, including IL-12, IL-15, IL-18, IL-1α, IFN-γ, and IL-10, in the Caco-2/RAW264.7 co-culture model.

First, 100 µg/ml of EPS was applied to the apical side in the Caco-2/RAW264.7 co-culture model and incubated for 3 hr. Then, LPS was added into the basolateral side at the final concentration of 5 ng/ml only as a positive control group, followed by incubation for an additional 3 hr. The mRNA expression of the cytokines in RAW264.7 cells was measured by quantitative RT-PCR as described in the Materials and Methods. Values represent means ± SE (n=3). **p<0.01, *p<0.05; there was a significant difference between the EPS-treated group and the control group. (a) IL-12, (b) IL-15, (c) IL-18, (d) IL-1α, (e), IFN-γ, (f) IL-10.

L. delbrueckii KM-3 and *L. delbrueckii* KM-4 resulted in downregulated mRNA expression in RAW264.7 cells (Fig. 3b). Regarding IL-18, treatments with EPS produced by *L. delbrueckii* TU-1 resulted in upregulated mRNA expression in RAW264.7 cells. In contrast, treatments with EPSs produced by *L. delbrueckii* JCM1012^T, *L. delbrueckii* JCM1248^T and *L. delbrueckii* JCM15610^T resulted in downregulated mRNA expression in RAW264.7 cells (Fig. 3c). Regarding IL-1α, treatments with EPS produced by *L. delbrueckii* strains showed no significant change compared with the control (Fig. 3d). Regarding IFN-γ, treatments with EPS produced by *L. delbrueckii* strains showed no significant change compared with the control (Fig. 3e). Regarding IL-10, treatments with the EPSs produced by *L. delbrueckii* JCM1012^T and *L. delbrueckii* KM-2 resulted in upregulated mRNA expression in RAW264.7 cells. In contrast, treatments with the EPSs produced by *L. delbrueckii* TU-1, *L. delbrueckii* JCM1002^T, *L. delbrueckii* JCM1248^T, *L. delbrueckii* KM-4 and *L. delbrueckii* KM-5 resulted in downregulated mRNA expression in RAW264.7 cells (Fig. 3f).

Cytokine production by Caco-2 cells in the co-culture model

In order to determine the production levels of several cytokines (IL-12, IL-8 and TGF-β1), the expression of cytokine mRNA in Caco-2 cells treated with EPSs produced by *L. delbrueckii* strains was examined by the RT-PCR method.

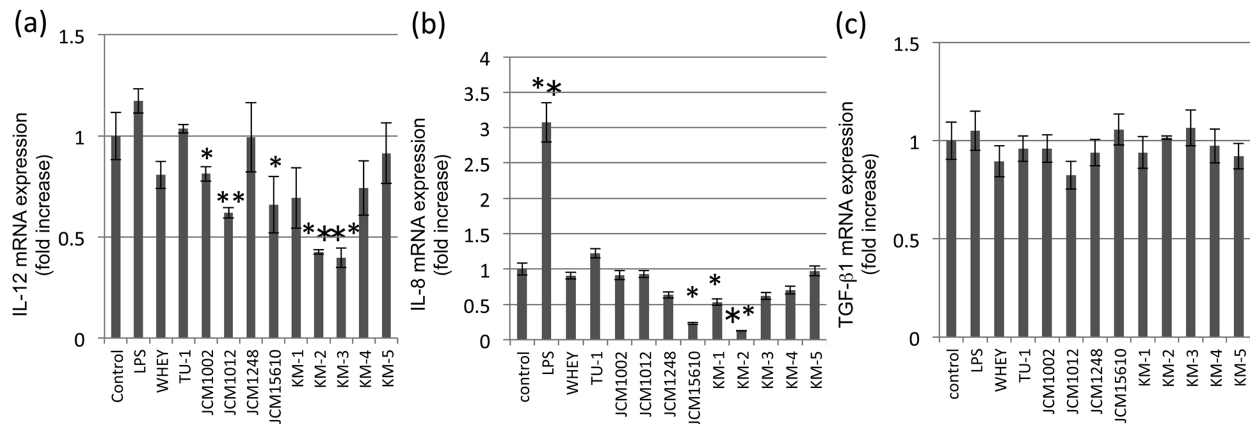

Fig. 4. Effects of EPSs purified by several strains on the production levels of cytokines, including, IL-12, IL-8 and TGF-β1, in the Caco-2/RAW264.7 co-culture model.

First, 100 µg/ml of EPS was applied to the apical side in the Caco-2/RAW264.7 co-culture model and incubated for 3 hr. Then, LPS was added into the basolateral side at the final concentration of 5 ng/ml only as a positive control group, followed by incubation for an additional 3 hr. The mRNA expression of the cytokines in Caco-2 cells was measured by quantitative RT-PCR as described in the Materials and Methods. Values represent the means ± SE (n=3). **p<0.01, *p<0.05; there was a significant difference between the EPS-treated group and the control group.

Regarding IL-12, treatments with the EPSs produced by *L. delbrueckii* JCM1002^T, *L. delbrueckii* JCM1012^T, *L. delbrueckii* JCM15610^T, *L. delbrueckii* KM-1, *L. delbrueckii* KM-2 and *L. delbrueckii* KM-3 resulted in downregulated mRNA expression in Caco-2 cells (Fig. 4a). Meanwhile, treatments with EPSs produced by *L. delbrueckii* strains showed no significant upregulation. As for IL-8, treatments with EPSs produced by *L. delbrueckii* JCM15610^T, *L. delbrueckii* KM-1 and *L. delbrueckii* KM-2 resulted in downregulated mRNA expression in Caco-2 cells (Fig. 4b). As for TGF-β1, treatments with EPS produced by *L. delbrueckii* strains showed no significant change compared with the control. The expression of cytokines mRNA in Caco-2 cells treated with EPSs produced by *L. delbrueckii* strains was not upregulated (Fig. 4c).

TNF-α and cytokine production by RAW264.7 cells in the gut inflammation model

Treatments of Caco-2 cells with EPSs produced by *L. delbrueckii* showed similar TNF-α production compared to LPS, while the expression of each cytokine mRNA in RAW264.7 cells (Fig. 5) treated with EPSs produced by *L. delbrueckii* strains was examined by the RT-PCR method. Regarding IL-12, the treatments with the EPS produced by *L. delbrueckii* KM-2 resulted in downregulated mRNA expression in RAW264.7 cells. In contrast, treatments with EPSs produced by other strains showed neither up-egulated nor downregulated mRNA

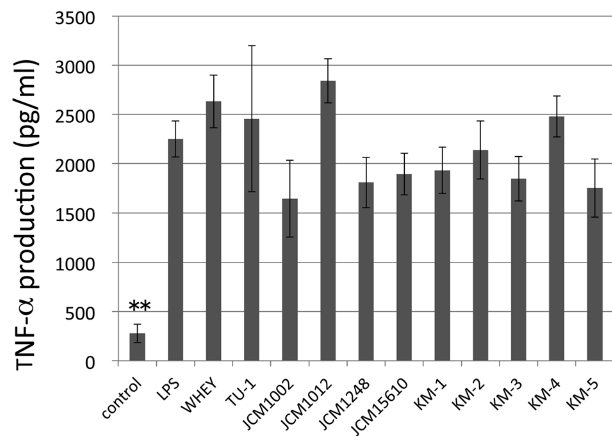

Fig. 5. Effects of EPSs purified by several strains on the production of TNF-α in the Caco-2/RAW264.7 gut inflammation model.

RAW264.7 cells were cultured overnight in a 24-wells plate in 5% CO_2 to allow them to adhere to the bottom of the plate. Then, the Transwell inserts on which the Caco-2 cells had been cultured were placed into the 24-well plates preloaded with RAW264.7 cells. Next, 100 µg/ml of EPS was applied to the apical side of the wells and incubated for 3 hr. Then, LPS was added into the basolateral side at the final concentration of 5 ng/ml only as a positive control group, followed by incubation for an additional 3 hr. LPS-stimulated cultures were used for the positive control. The culture media were then collected for the measurement of TNF-α content. Values represent means ± SE (n=3). **p<0.01, *p<0.05; there was a significant difference between the EPS-treated group and the control group.

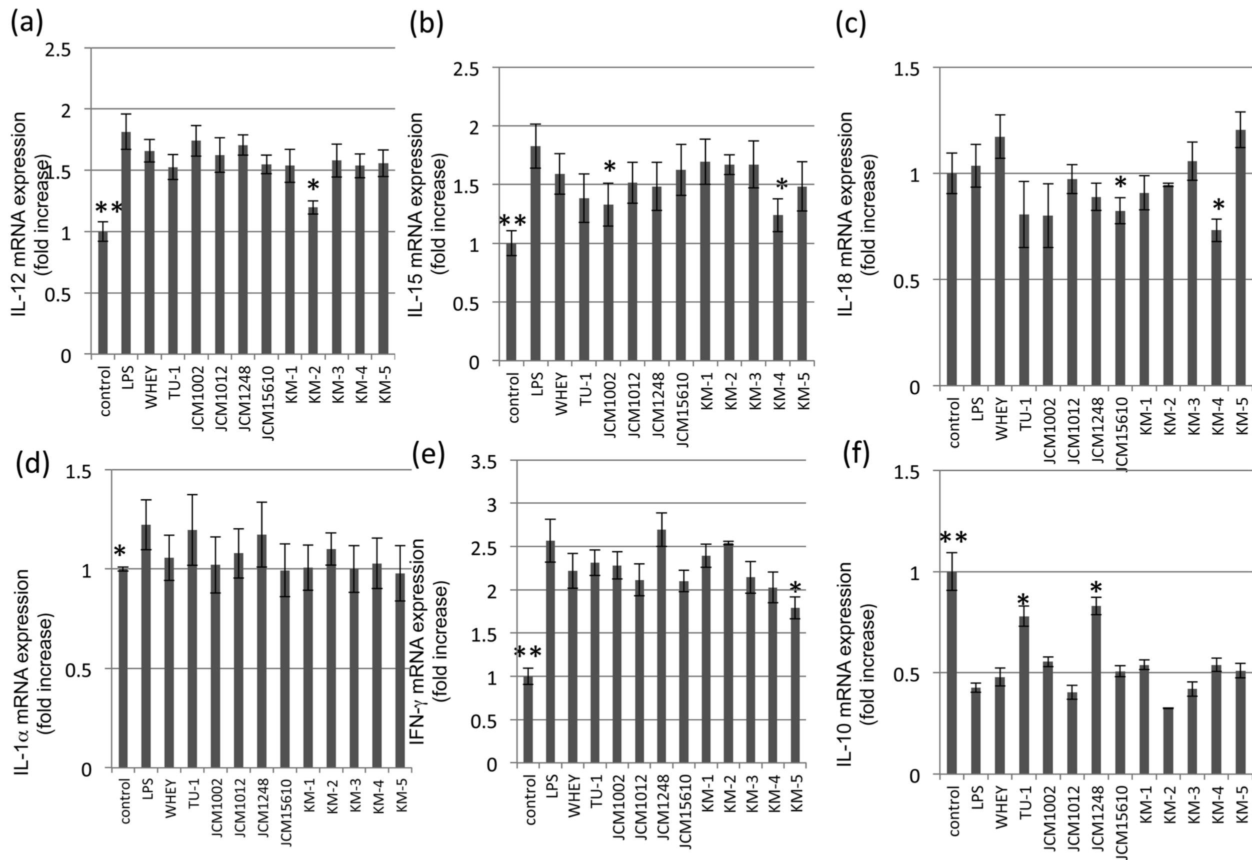

Fig. 6. Effects of EPSs purified by several strains on the production levels of cytokines, including IL-12, IL-15, IL-18, IL-1α, IFN-γ, and IL-10, in the Caco-2/RAW264.7 gut inflammation model.

First, 100 µg/ml of EPS was applied to the apical side in the Caco-2/RAW264.7 co-culture model and incubated for 3 hr. Then, LPS was added into the basolateral side at the final concentration of 5 ng/ml only as a positive control group, followed by incubation for an additional 3 hr. The cytokines mRNA expression in RAW264.7 cells was measured by quantitative RT-PCR as described in the Materials and Methods. Values represent means ± SE (n=3). **$p<0.01$, *$p<0.05$; there was a significant difference between the EPS-treated group and the control group. (a) IL-12, (b) IL-15, (c) IL-18, (d) IL-1α, (e) IFN-γ, (f) IL-10.

expression in RAW264.7 cells (Fig. 6a). Regarding IL-15, the treatments with the EPSs produced by *L. delbrueckii* JCM1002^T and *L. delbrueckii* KM-4 resulted in downregulated mRNA expression in RAW264.7 cells (Fig. 6b). Regarding IL-18, the treatments with the EPSs produced by *L. delbrueckii* JCM15610^T and *L. delbrueckii* KM-4 resulted in downregulated mRNA expression in RAW264.7 cells (Fig. 6c). The production level of IL-1α of RAW264.7 cells, treated with EPSs produced by *L. delbrueckii* strains, was comparable to that of those treated with LPS (Fig. 6d). As for IFN-γ, treatments with EPS produced by *L. delbrueckii* KM-5 resulted in down-regulated mRNA expression in RAW264.7 cells (Fig. 6e). Regarding IL-10, treatments with the EPS produced by *L. delbrueckii* TU-1 and *L. delbrueckii* JCM1248^T resulted in upregulated mRNA expression in RAW264.7 cells (Fig. 6f).

Cytokine production by Caco-2 cells in the gut inflammation model

The expression of cytokine mRNA in Caco-2 cells treated with EPSs produced by *L. delbrueckii* strains was examined by the RT-PCR method. Regarding IL-12, the treatments with EPSs produced by *L. delbrueckii* JCM15610^T, *L. delbrueckii* KM-2, *L. delbrueckii* KM-4 and *L. delbrueckii* KM-5 resulted in downregulated mRNA expression in Caco-2 cells (Fig. 7a). Regarding IL-8, the treatments with EPSs produced by *L. delbrueckii* JCM15610^T, *L. delbrueckii* KM-2 and *L. delbrueckii* KM-3 resulted in downregulated mRNA expression in Caco-2 cells (Fig. 7b). As for TGF-β1, the treatments with EPS produced by *L. delbrueckii* TU-1, *L. delbrueckii*

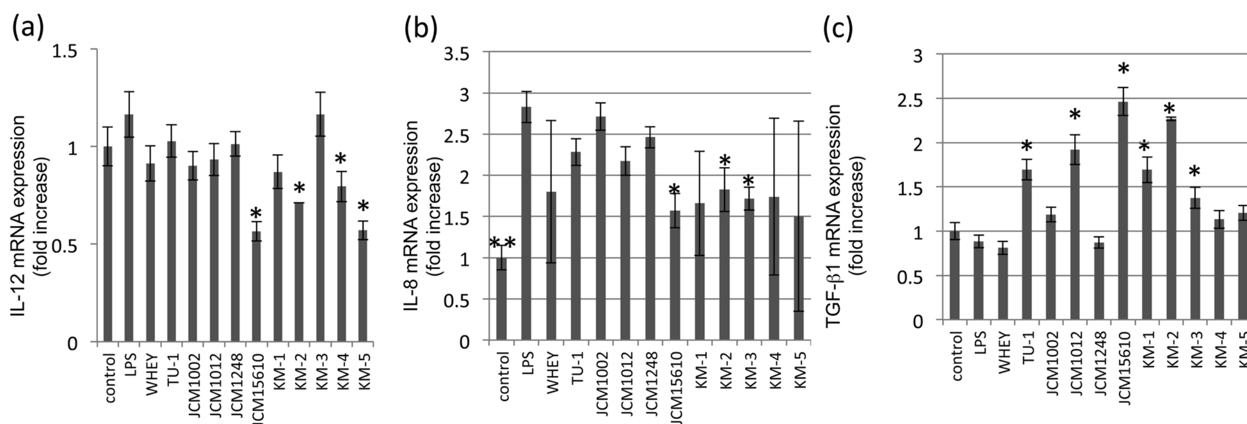

Fig. 7. Effects of EPSs purified by several strains on the production levels of cytokines, including, IL-12, IL-8 and TGF-β1, in the Caco-2/RAW264.7 gut inflammation model.

First, 100 μg/ml of EPS was applied to the apical side in the Caco-2/RAW264.7 co-culture model and incubated for 3 hr. Then, LPS was added into the basolateral side at the final concentration of 5 ng/ml only as a positive control group, followed by incubation for an additional 3 hr. The mRNA expression of the cytokines in Caco-2 cells was measured by quantitative RT-PCR as described in the Materials and Methods. Values represent the means ± SE (n=3). $^{**}p<0.01$, $^{*}p<0.05$; there was a significant difference between the EPS-treated group and the control group.

JCM1002^{T}, *L. delbrueckii* JCM15610^{T}, *L. delbrueckii* KM-1, *L. delbrueckii* KM-2 and *L. delbrueckii* KM-3 resulted in up-egulated mRNA expression in Caco-2 cells (Fig. 7c).

DISCUSSION

In the present study, the levels of TNF-α were measured in two different systems: 1) a direct stimulation of macrophage RAW264.7 cells with EPSs and 2) a co-culture model using a combination of intestinal epithelial Caco-2 cells and RAW264.7 cells. Although the two systems showed a similar TNF-α production pattern, the level of TNF-α production was much lower in the co-culture system compared with direct stimulation. One of the explanations for this result may be that EPSs penetrated the Caco-2 monolayer on the basolateral side of the well, thereby interacting with the macrophage. However, this is not likely based on the following two grounds. Firstly, in the present study, the TER value showed no change after adding EPSs to the apical side of the Caco-2 cell layer when compared with the control (without addition of EPSs). TER is a highly sensitive parameter for membrane permeability, and the changes in TER largely reflects an indicator of the tightness of the intercellular junctions: a decrease in TER indicates an increase in the paracellular permeability, and vice versa. The present study indicates that the monolayer of Caco-2 cells was intact throughout the experiments. Secondly, it is known that Caco-2 cells form tight junctions (TJs) after differentiation [20] and that TJs control paracellular transport by preventing any macromolecule over 10 kDa from passing through the epithelial layer [21]. It is thus unlikely that an EPS with a molecular weight of more than 10 kDa crossed the TJs of the Caco-2 cells in the present study. On this basis, it is suggested that some EPSs activated the macrophages indirectly, possibly through their interaction with the Caco-2 cells.

EPSs produced by different *L. delbrueckii* strains showed different profiles of cytokine production by RAW264.7 cells in the co-culture system. Regarding pro-inflammatory cytokine TNF-α that was used as an indicator of macrophage activation, the EPS produced by *L. delbrueckii* TU-1 showed an enhanced production of TNF-α, whereas the EPS produced by *L. delbrueckii* KM-2 showed a suppressed production of TNF-α. Regarding IL-12, IL-15 and IL-18, which might activate directly NK cells [22], the EPS produced by *L. delbrueckii* TU-1 resulted in upregulation of all 3 cytokines mRNA expressions. In contrast, treatments with the EPSs produced by *L. delbrueckii* JCM1012^{T}, *L. delbrueckii* JCM15610^{T} and *L. delbrueckii* KM-3 resulted in downregulation of 2 of the 3 mRNA expressions. It should be noted that IL-12 is involved in the differentiation of naïve T cells into the Th1 cells and plays a critical role in promoting Th1 responses, which is essential for a successful defense against intracellular pathogen infections [23]. Regarding IL-1α and IFN-γ, which might activate the Th1 cell, thereby activating NK cell [24, 25], EPSs produced by *L. delbrueckii* strains did not upregulate or downregulate

any cytokine expression. IL-10 is an anti-inflammatory cytokine that inhibits the synthesis of pro-inflammatory cytokines such as IL-1α, IL-8, IL-12 and TNF-α in activated macrophages, thereby shifting the host's immune responses toward the Th2 type [26]. The EPSs produced by *L. delbrueckii* JCM1012^T and *L. delbrueckii* KM-2 resulted in upregulated mRNA expression. In contrast, the treatments with the EPSs produced by *L. delbrueckii* TU-1, *L. delbrueckii* JCM1002^T, *L. delbrueckii* JCM1248^T, *L. delbrueckii* KM-4 and *L. delbrueckii* KM-5 resulted in downregulated mRNA expression.

The evidence from the present study suggests that oral administration of the EPS produced by *L. delbrueckii* TU-1 exerts a Th1 type immune response, while the EPSs produced by *L. delbrueckii* JCM1012^T and *L. delbrueckii* KM-2 exerts a Th2 type immune response. In fact, the EPS produced by *L. delbrueckii* TU-1 increased TNF-α production from RAW264.7 cells and exerted excessive Th1 cytokines (IL-12, IL-15 and IL-18) mRNA expression, while the mRNA expression of IL-10, a Th2 cytokine, was downregulated in RAW264.7 cells. In the present study, treatment with LPS upregulated the IL-1α cytokine, but the EPS produced by *L. delbrueckii* TU-1 did not upregulate the IL-1α cytokine. The evidence suggests that the EPS produced by *L. delbrueckii* TU-1 exerts a Th1 type immune response that is not identical to that exerted by LPS. Furthermore, the treatments with EPSs produced by different *L. delbrueckii* strains did not affect TNF-α production of the LPS-pretreated RAW264.7 cells. The evidence suggests that EPSs promote NK cell activity but do not aggravate inflammation. This, in turn, points to the prospect that EPSs produced by *L. delbrueckii* can be an ideal biogenics against microbial infections (i.e., influenza, norovirus).

In the present study, IL-12, IL-8 and TGF-β1 mRNA expression in Caco-2 cells was not affected by the presence of EPSs. In a similar co-culture system, Parlesak et al. [27] reported that nonpathogenic *Escherichia coli* induced high IL-8 and IFN-γ mRNA expression but very little IL-12 mRNA expression in Caco-2 cells. It is thus suggested that some unknown signal was released from Caco-2 cells to act on the RAW264.7 cells, which then upregulated Th1 or Th2 profile cytokines. More studies are necessary to evaluate the above possibility and identify the unknown signals.

Recently, it has been reported that EPSs produced by *L. delbrueckii* are polymerized repeating units mainly composed of glucose and galactose, exhibiting strain-dependent variation [28]. For example, the EPS produced by *L. delbrueckii* subsp. *bulgaricus* NCFB2074 has a heptasaccharide repeat unit, and the repeat unit is highly branched [5], on the other hand, the EPS produced by *L. delbrueckii* ssp. *bulgaricus* LBB.B332 has a pentasaccharide repeat unit, and the repeat unit is not branched [29]. Our TLC analysis also showed such strain-dependent variation in EPSs, and it was possible to separate the sugar compostion of the EPSs into four groups. It should be noted that there were some extra spots other than these sugars, and we tried to identify what sugars these spots represented using other mono- and polysaccharides as references without success. These unidentifiable spots may be undigested units of the sugars or impurities of the samples. Further study is thus necessary to determine a more precise sugar alignment for EPSs and their tertiary structure through use of high-performance liquid chromatography (HPLC).

As described above, the EPSs produced by *L. delbrueckii* TU-1 and *L. delbrueckii* KM-4 seem to promote a Th1 type immune response, while the EPSs produced by *L. delbrueckii* JCM1012^T and *L. delbrueckii* KM-2 seem to have promoted a Th2 type immune response. This immunological classification failed to correspond to our TLC grouping of EPS. More studies are thus necessary to determine a more precise sugar alignment for EPSs and their tertiary structure using more sophisticated tools such as HPLC and nuclear magnetic resonance spectroscopy so that we can evaluate the possible link between the configurations of EPSs and their immune-regulating properties. Meanwhile, our initial attempt to use human cells alone for the intestinal model induced marked inflammation of the cells (date not shown). We thus employed another model using cells of different species origins, in which human intestinal epithelial Caco-2 cells and murine macrophage RAW264.7 cells did not induce any apparent disorder of the cells. We thus considered that the system was a model of normal intestinal immunity.

In conclusion, EPSs produced by *L. delbrueckii* strains are structurally diverse not only in their biochemical structure but also in their immune-regulating properties. Such properties are likely to be exerted through a mere contact with the epithelial cells of the host intestine. A further investigation is in progress to evaluate the above hypothesis through *in vivo* experiments.

ACKNOWLEDGEMENTS

This work was supported by the Special Coordination Fund for Promoting Science and Technology, Creation of Innovation Centers for Advanced Interdisciplinary Research Areas (Innovative Bioproduction Kobe), and the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

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