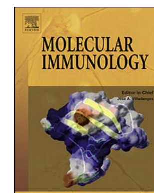
Molecular Immunology 93 (2018) 253–265

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm



Exopolysaccharides from *Lactobacillus delbrueckii* OLL1073R-1 modulate innate antiviral immune response in porcine intestinal epithelial cells

Paulraj Kanmani[a,b,1], Leonardo Albarracin[a,c], Hisakazu Kobayashi[a,b], Hikaru Iida[a,b], Ryoya Komatsu[a,b], A.K.M. Humayun Kober[a,b,d], Wakako Ikeda-Ohtsubo[a,b], Yoshihito Suda[e], Hisashi Aso[b,f], Seiya Makino[g], Hiroshi Kano[g], Tadao Saito[a], Julio Villena[a,c,*], Haruki Kitazawa[a,b,*]

[a] Food and Feed Immunology Group, Laboratory of Animal Products Chemistry, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan
[b] Livestock Immunology Unit, International Education and Research Center for Food Agricultural Immunology (CFAI), Graduate School of Agricultural Science, Tohoku University, Sendai, Japan
[c] Laboratory of Immunobiotechnology, Reference Centre for Lactobacilli (CERELA-CONICET), Tucuman, Argentina
[d] Department of Dairy & Poultry Science, Chittagong Veterinary and Animal Sciences University, Chittagong, Bangladesh
[e] Department of Food, Agriculture and Environment, Miyagi University, Sendai, Japan
[f] Cell Biology Laboratory, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan
[g] Food Science Research Laboratory, Meiji Co., Ltd., Kanagawa, Japan

ARTICLE INFO

Keywords:
Probiotics
Exopolysaccharides
Antiviral activity
Anti-inflammatory activity
Immune modulation
Porcine intestinal epithelial cells

ABSTRACT

Previous studies demonstrated that the extracellular polysaccharides (EPSs) produced by *Lactobacillus delbrueckii* OLL1073R-1 (LDR-1) improve antiviral immunity, especially in the systemic and respiratory compartments. However, it was not studied before whether those EPSs are able to beneficially modulate intestinal antiviral immunity. In addition, LDR-1-host interaction has been evaluated mainly with immune cells while its interaction with intestinal epithelial cells (IECs) was not addressed before. In this work, we investigated the capacity of EPSs from LDR-1 to modulate the response of porcine IECs (PIE cells) to the stimulation with the Toll-like receptor (TLR)-3 agonist poly(I:C) and the role of TLR2, TLR4, and TLR negative regulators in the immunoregulatory effect. We showed that innate immune response triggered by TLR3 activation in porcine IECs was differentially modulated by EPS from LDR-1. EPSs treatment induced an increment in the expression of interferon (IFN)-α and IFN-β in PIE cells after the stimulation with poly(I:C) as well as the expression of the antiviral factors MxA and RNase L. Those effects were related to the reduced expression of A20 in EPS-treated PIE cells. EPS from LDR-1 was also able to reduce the expression of IL-6 and proinflammatory chemokines. Although further *in vivo* studies are needed, our results suggest that these EPSs or a yogurt fermented with LDR-1 have potential to improve intestinal innate antiviral response and protect against intestinal viruses.

1. Introduction

Immunomodulatory lactic acid bacteria (LAB) strains (immunobiotics) are able to impact on human and animal health by modulating antiviral immune responses (Villena et al., 2016). It was demonstrated that immunobiotics, provide protection against viral infection by improving innate and adaptive antiviral immunity that leads to the reduction of the duration of disease, the number of episodes and virus shedding (For recent reviews see Villena et al., 2016; Zelaya et al., 2016). One of the most studied immunobiotic strain regarding its antiviral activity is *Lactobacillus delbrueckii* OLL1073R-1 (LDR-1) that is able to produce immunomodulatory extracellular polysaccharides (EPSs) (Kitazawa et al., 1998; Makino et al., 2006; Makino et al., 2010; Nagai et al., 2011; Makino et al., 2016). EPSs are also molecules that allow the communication of immunobiotics with the host by interacting with pattern recognition receptors (PRRs) expressed in non-immune and immune cells (For a recent review see Laiño et al., 2016).

The EPSs produced by LDR-1 have been studied in detail before. The crude EPS obtained from the culture supernatant of LDR-1 can be fractionated into neutral EPS (NSP) and acidic EPS (APS) by standard chromatographic methods (Kitazawa et al., 1998; Van Calsteren et al., 2015). Earlier studies from Kitazawa et al. (1998) showed that APS produced by LDR-1 induce a significant B-cell dependent mitogenic action on murine lymphocytes and that this mitogenic activity was

* Corresponding authors at: Food and Feed Immunology Group, Laboratory of Animal Products Chemistry, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan
E-mail addresses: jcvillena@cerela.org.ar (J. Villena), haruki.kitazawa.c7@tohoku.ac.jp (H. Kitazawa).
[1] JSPS Postdoctoral Fellow.

http://dx.doi.org/10.1016/j.molimm.2017.07.009
Received 28 February 2017; Received in revised form 3 July 2017; Accepted 18 July 2017
Available online 08 August 2017
0161-5890/ © 2017 Elsevier Ltd. All rights reserved.

completely abolished by dephosphorylation. *In vitro* experiments also revealed the mitogenic activity of APS produced by this strain was mediated through macrophage activation (Nishimura-Uemura et al., 2003). Later, it was reported that APS or EPS were able to significantly increase IFN-γ production by murine splenocytes while no stimulatory effect on IFN-γ production was found for NPS (Makino et al., 2006).

In vivo studies in mice showed that a yogurt fermented with LDR-1 or its purified NPS had the capacity to enhance NK cell activity and IFN-γ production by splenocytes (Makino et al., 2006; Makino et al., 2016). Moreover, the oral administration of yogurt fermented with LDR-1, or its purified EPS or APS were shown to significantly increase the resistance of mice to the respiratory challenge with influenza virus as demonstrated by the improved survival and reduced viral titers in LDR-1-treated animals when compared to controls (Nagai et al., 2011). In addition, Makino et al. (2010) showed in two independent studies in which elderly individuals consumed the immunobiotic LDR-1 yogurt that this dietary treatment augmented NK cell activity and reduced the risk of catching the common cold.

Those mentioned studies have clearly demonstrated that the EPSs produced by LDR-1 are able to improve antiviral immunity, especially in the systemic and respiratory compartments. However, it was not studied before whether EPSs from LDR-1 were able to beneficially modulate intestinal antiviral immunity. In addition, LDR-1-host interaction has been evaluated mainly with immune cells while its interaction with intestinal epithelial cells (IECs) was not addressed before.

We have reported in different studies that the originally established porcine intestinal epithelial cell line (PIE cells) is a useful tool for studying immune responses triggered by different Toll-like receptors (TLR) signaling pathways in IECs (Moue et al., 2008; Hosoya et al., 2011; Villena et al., 2012; Albarracin et al., 2017). TLR3 is highly expressed in PIE cells and the response of these cells to poly(I:C) stimulation resembles the response to rotavirus infection when the production of antiviral factors and inflammatory cytokines and chemokines are evaluated (Hosoya et al., 2011; Ishizuka et al., 2016). Moreover, considering that the porcine gastrointestinal tract has many structural aspects that are more similar to those of the human system than the rodent system (Mair et al., 2014), we have used PIE cells not only for the study of IECs immunobiology in the porcine host (Moue et al., 2008; Hosoya et al., 2011;) but also as a human model (Villena et al., 2012; Albarracin et al., 2017).

In this work, we investigated the capacity of EPS, NPS and APS from LDR-1 to modulate the response of PIE cells to the challenge with the TLR3 agonist poly(I:C). In addition, we evaluated the role of TLR2, TLR4, and TLR negative regulators in the immunoregulatory effects of EPSs produced by LDR-1. These studies could give the scientific basis for the application of EPS, NPS or APS for the prevention of intestinal viral infections in humans or animals.

2. Materials and methods

2.1. Intestinal epithelial cell culture

Porcine intestinal epithelial (PIE) cells were previously established by our laboratory from the intestinal epithelia of unsuckled neonatal swine (Moue et al., 2008). PIE cells were cultured in DMEM medium supplemented with fetal calf serum (FCS 10%), penicillin (100 mg/ml), and streptomycin (100 U/ml). The cultures were grown in 250 ml flask at 37 °C in a humidified atmosphere of 5% CO_2. The cultures were passaged routinely after reaching confluence of 80–90%.

2.2. Extracellular polysaccharides

The EPS from LDR-1 was extracted and fractionated according to the method of Kitazawa et al. (1998). Ion-exchange chromatography was used to fractionate EPS into acidic polysaccharide (APS) and neutral polysaccharide (NPS). The APS and NPS molecular masses and sugar compositions were examined by performing high performance liquid chromatography (Asahi Chemical Industry Co., Ltd., Japan).

2.3. Effect of EPSs on innate antiviral immune response in PIE cells

PIE cells were seeded in 12 well type I collagen-coated plates. Each well contained 1 ml of cells (3.0×10^4 cells), and was cultured by subsequent incubation at 37 °C, 5% CO_2 for three days. PIE cells were treated with EPS, APS, NPS or medium for 48 h. Stock solutions of EPS, APS or NPS were prepared by mixing of 10 mg of each EPSs with 1 ml of DMEM medium. Ten ul of these stock solutions (containing 100 ug of EPS, APS or NPS) were mixed with 1 ml of DMEM medium to stimulate PIE cells (final concentration 100 ug/ml). These doses were selected previously as optimal for immunomodulatory activities (Wachi et al., 2014). After washing three times with fresh medium, the four groups of PIE cells were incubated with poly(I:C) (10 g/ml, Sigma Aldrich, USA) for 3, 6 and 12 h. PIE cells without poly(I:C) stimulation were used as controls. Poly(I:C) dose was previously selected according to its capacity to stimulate TLR3 signaling without producing excessive inflammatory cell damage (Hosoya et al., 2011). The total RNA was isolated to analyze the expressions of type I interferons (IFN-α, IFN-β), cytokines/chemokines (TNF-α, IL-6, CXCL8, CCL4, CXCL10), PRRs (TLR3, RIG-I), and antiviral factors (MxA, RNase L) by quantitative real time-polymerase chain reaction (qPCR).

2.4. RNA extraction and qPCR

The total RNA was isolated by using TRIzol reagent (Invitrogen). The purity and quantity of RNA was analyzed by Nano drop spectrophotometer ND-1000 UV–vis (NanoDrop Technologies, USA). The quantified RNA (500 ng) was used to synthesize cDNA by Thermal cycler (BIO-RAD, USA). The reaction mixtures (10 μl) were prepared using Quantitect reverse transcription (RT) kit (Qiagen, Tokyo, Japan) according to the manufacturer instructions. The qPCR was performed in a 7300 real-time PCR system (Applied Biosystems, Warrington, UK) with platinum SYBR green (qPCR supermix uracil-DNA glycosylase with 6-carboxyl-X-rhodamine, Invitrogen). The total volume of reaction mixture was 10 μl, which contained 2.5 μl of cDNA and 7.5 μl of master mix that included RT enzyme, SYBR green, forward and reverse primers (1 pmol/μl). The reaction cycles were performed first at 50 °C for 5 min; followed by 95 °C for 5 min; then 40 cycles at 95 °C for 15s, at 60 °C for 30 s and at 72 °C for 30 s. According to the minimum information for publication of qPCR experiments guidelines, β-actin was used as a housekeeping gene because of its high stability across porcine various tissues (Bustin et al., 2009; Nygard et al., 2007). In addition, the NormFinder test with porcine *rpl4*, *gapdh*, *tbp*, and *β-actin* genes were performed to demonstrate the suitability of porcine beta-actin as the housekeeping gene for our experiments. Expression of β-actin was used to normalize cDNA levels for differences in total cDNA levels in the samples.

2.5. TLR negative regulators expression in PIE cells

In order to analyze the expression of TLR negative regulators, PIE cells were cultured (3.0×10^4 cells/well) in 12 well type I collagen-coated plates at 37 °C, 5% CO_2. After 3 days, the confluent cells were incubated with EPS, APS or NPS (100 ug/ml) at 37 °C, 5% CO_2 for 48 h. After stimulation, PIE cells were washed three times with fresh DMEM medium and subsequently incubated with of poly(I:C) (10 ug/ml) for 12 h. The expression of TLR negative regulators (SIGIRR, Tollip, A20, Bcl-3, MKP-1, and IRAK-M) was evaluated by qPCR as described previously (Hosoya et al., 2011).

2.6. Preparation of proteins and western blotting analysis

PIE cells were seeded (1.8×10^5 cells/dish) in 60 mm dishes and

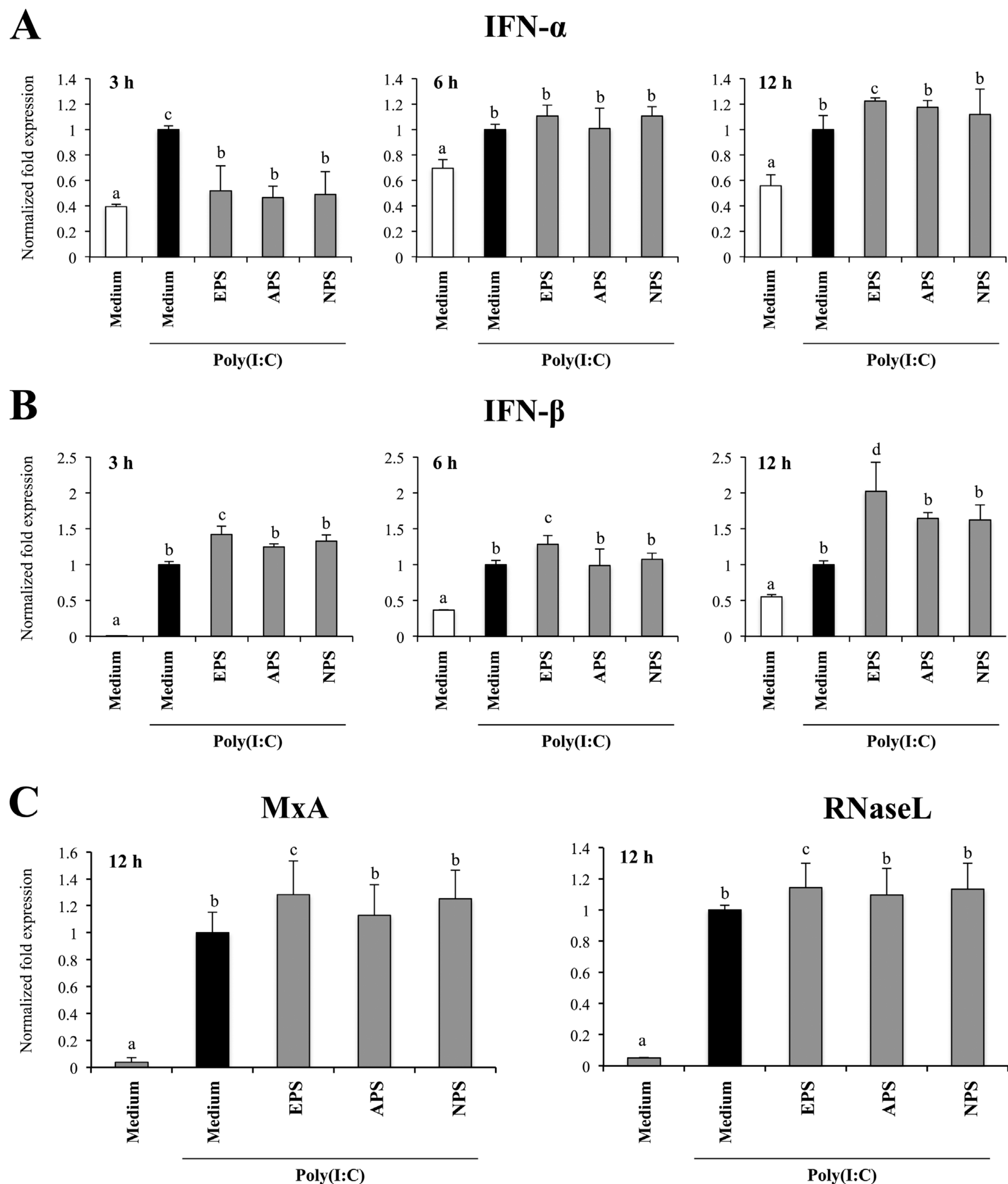


Fig. 1. Antiviral activity of exopolysaccharides (EPS) from *Lactobacillus delbrueckii* OLL1073R-1 (LDR-1) in porcine intestinal epithelial (PIE) cells. EPS was fractionated into neutral EPS (NSP) and acidic EPS (APS) by standard chromatographic methods. PIE cells were treated with EPS, APS, NPS or medium for 48 h and then stimulated with the Toll-like receptor (TLR)-3 agonist poly(I:C). PIE cells without poly(I:C) stimulation were used as controls. The expression of IFN-α (A), IFN-β (B), MxA and RNaseL (C) were estimated by qPCR at the indicated time points after poly(I:C) treatment. The results are expressed as mean ± SD and represent data from three independent experiments (n = 3 in each experiment). Values with different superscripts letters are significantly different within the level $p < 0.05$ (a < b < c).

incubated at 37 °C, 5% CO_2 for 3 days. The confluent PIE cells were stimulated with EPS, APS or NPS (100 ug/ml) for 48 h at 37 °C, 5% CO_2. After stimulation, PIE cells were washed three times with fresh DMEM medium, treated with 10 ug/ml of poly(I:C) and studied in different time intervals (0, 30, 60, 120, 180, and 240 min). Then, PIE cells were washed three times with PBS and the harvested cells were lysed by adding 200 µl of CellLytic M cell lysis reagent (Sigma-Aldrich, St. Louis, MO, USA), containing protease inhibitors (Complete mini, PhosSTOP, Roche, Mannheim, Germany). Afterwards, the lysed cells were transferred to fresh Eppendorf tubes (1.5 ml), kept on ice for 5–10 min and sonicated three times at 50% for 3 s and, centrifuged at 150 × 100 rpm for 5 min. Then, the concentration of protein in the resulting supernatants was estimated by using bicinchoninic acid (BCA) assay kit (Thermo Scientific, Pierce, Rockford, IL).

For western blot analysis, the protein samples (8 µg/sample) were loaded on 10% SDS-polyacrylamide gels and the electrophoretically separated proteins were transferred to a nitrocellulose membrane (Trans-Blot Turbo™, BIO-RAD). The membranes were cut and incubated

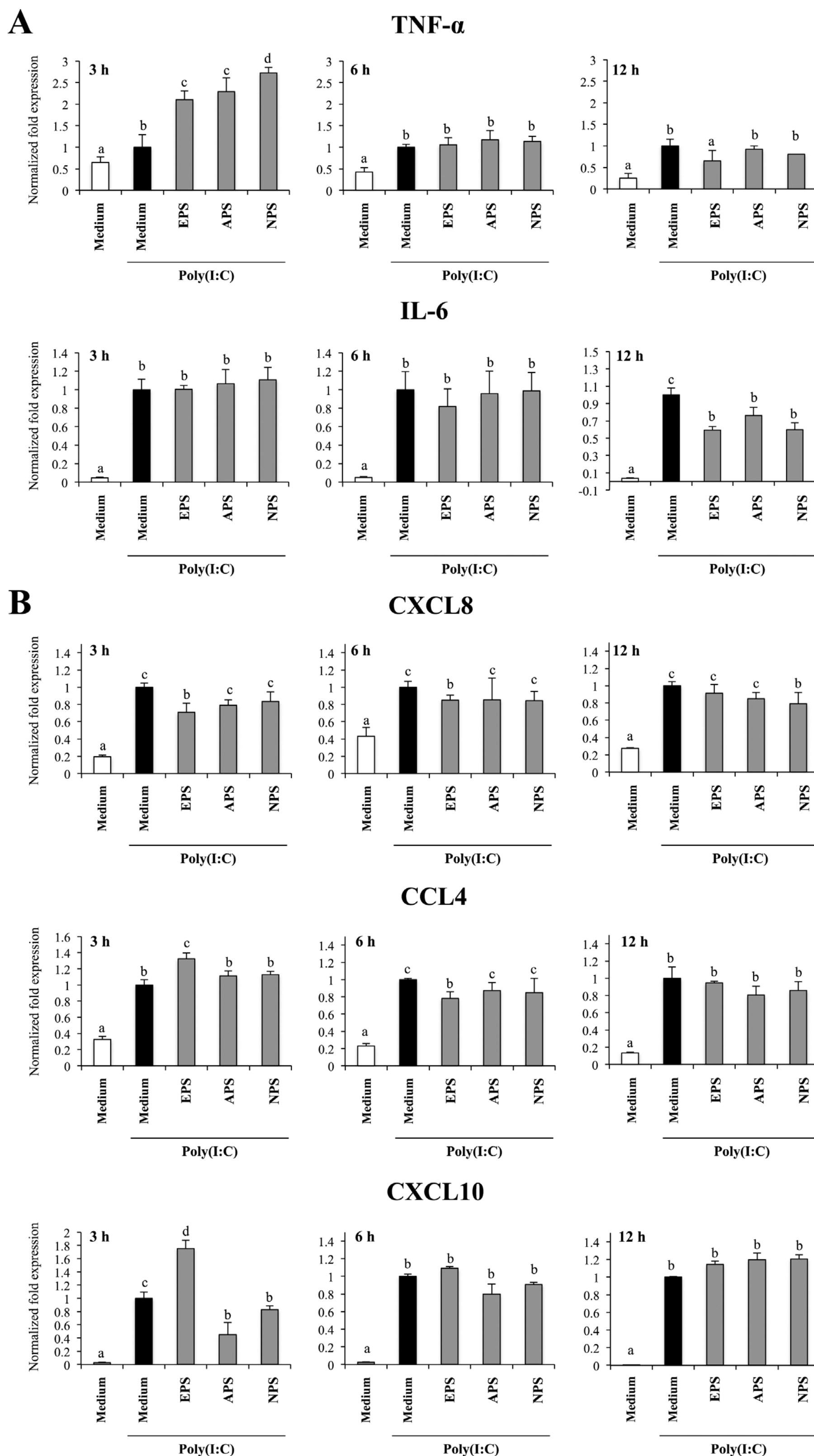


Fig. 2. Immunomodulatory activity of exopolysaccharides (EPS) from *Lactobacillus delbrueckii* OLL1073R-1 (LDR-1) in porcine intestinal epithelial (PIE) cells. EPS was fractionated into neutral EPS (NSP) and acidic EPS (APS) by standard chromatographic methods. PIE cells were treated with EPS, APS, NPS or medium for 48 h and then stimulated with the Toll-like receptor (TLR)-3 agonist poly(I:C). PIE cells without poly(I:C) stimulation were used as controls. The expression of TNF-α, IL-6 (A), CXCL8, CCL4, and CXCL10 (B) were estimated by qPCR at the indicated time points after poly(I:C) treatment. The results are expressed as mean ± SD and represent data from three independent experiments (n = 3 in each experiment). Values with different superscripts letters are significantly different within the level $p < 0.05$ (a < b < c < d).

with blocking buffer for 1–2 h before incubating with primary and secondary antibodies. The phosphorylation of TRAF3 and IRF-3, and the degradation of nuclear factor kappa B inhibitor alpha (IkBα) were evaluated using TRAF3 antibody (Cat. #4729), Phospho-IRF3 (Ser396) (4D4G, Cat. #4947) rabbit antibody, and I kappaB-alpha antibody (IkBa, Cat. #9242) from Cell Signaling Technology (Beverly, MA, USA) at 1000 times dilution of their original concentration, overnight at room temperature. Then, membranes were washed with TBS-T buffer and incubated with anti-rabbit IgG, AP-linked antibody (Cat. #7054) for 1–2 h at room temperature. After washing with TBS-T buffer, the membranes were spread with 200 ul of ECF substrate (GE Healthcare Japan Co., Tokyo, Japan) to detect optical protein bands and

photographed by blot analyzer (Quantity One W409). The exhibited proteins bands were estimated from the peak area of densitogram by using Image J software (National Institute of Health, Bethesda, MD, USA). To detect each total protein, the same membranes were incubated in stripping solution for 10 min (Western Blot Re-Probe Kit, #JZ-008, Jacksun Easy Biotech, Inc., New York, USA), blocked (1–2 h), and subsequently incubated with IRF3 (D83B9, Cat. #4302) antibody, and β-actin (13E5, Cat. #4970) rabbit antibody, from Cell Signaling Technology.

2.7. Role of TLRs in the immunomodulatory activities of EPS

The role of TLR2 and TLR4 in the immunomodulatory activities of EPS, APS, and NPS from LDR-1 were analyzed by using blocking experiments. Unlabeled anti-porcine TLR2 and TLR4-rabbit IgG antibodies (Biolegend, San Diego, CA) were used to block TLRs expression in PIE cells. Briefly, PIE cells were cultured (3.0×10^4 cells/ml) in 12 well type I collagen coated plates at 37 °C, 5% CO_2. After 3 days, the confluent cells were incubated with unlabeled anti-porcine TLR2 or TLR4 IgG antibodies (200 ng/ml) for 12 h. The concentration and time incubation of anti-porcine TLR2 or TLR4 blocking antibodies was selected according to the production of inflammatory factors and intracellular Ca^{2+} flux after stimulation of PIE cells with Pam3CSK4 or LPS respectively (data not shown). Blocking experiments were successfully used in previous works (Wachi et al., 2014). After treatment with blocking antibodies, PIE cells were treated with EPS, APS and NPS (100 ug/ml) for 48 h at 37 °C, 5% CO_2. Finally, the pre-stimulated PIE cells were washed three times with fresh medium and subsequently incubated with Poly(I:C) (10 ug/ml) for 12 h at 37 °C, 5% CO_2. The expression of IFN-β, TNF-α, IL-6, CXCL8, and CCL4 were examined by qPCR as described before.

2.8. Statistical analysis

Statistical analyses were performed using the GLM and REG procedures available in the SAS computer program (SAS, 1994). Comparisons between mean values were carried out using one-way analysis of variance and Fisher's least-significant-difference (LSD) test.

3. Results

3.1. Effect of LDR-1 EPSs on antiviral response in PIE cells

We first determined the effect of EPS, NPS and APS from LDR-1 on the expression of type I IFNs in PIE cells after the treatment with poly (I:C). The stimulation of PIE cells with the TLR3 ligand increased the expression of IFN-α and IFN-β (Fig. 1). PIE cells treated with EPS, APS or NPS before poly(I:C) stimulation showed a significantly lower expression of IFN-α on hour 3 when compared to control. Only the EPS-treated PIE cells showed improved levels of IFN-α on hour 12 after poly (I:C) stimulation (Fig. 1A). Moreover, the incubation of PIE cells with EPS from LDR-1 resulted in a significant increase in the expression of IFN-β in all the studied time points (Fig. 1B). The levels of IFN-β were enhanced only on hour 12 after poly(I:C) stimulation in NPS- and APS-treated PIE cells (Fig. 1B). It is well established that antiviral activity of type I IFNs is associated to their ability to induce the expression of antiviral proteins that can actively reduce the spread of virus by blocking the transcription of the viral genome, and inducing the degradation of viral RNA and DNA. Therefore, we also evaluated whether EPS, APS or NPS were able to induce a differential expression of two antiviral factors, MxA and RNase L, in poly(I:C)-treated PIE cells. As expected, stimulation of PIE cells with poly(I:C) resulted in an increased expression of both MxA and RNase L (Fig. 1C). Incubation of PIE cells with NPS and APS did not induce changes in the expression of these antiviral factors on hour 12 after poly(I:C) stimulation. On the contrary, EPS-treated PIE cells showed a significant increase in the expression of MxA and RNase L (Fig. 1C).

3.2. Effect of LDR-1 EPSs on cytokine/chemokine response in PIE cells

We also evaluated whether EPSs from LDR-1 modulated the expression of inflammatory cytokines and chemokines in PIE cells after poly(I:C) treatment. The stimulation of PIE cells with the TLR3 agonist enhanced the expression of TNF-α and IL-6 (Fig. 2A). PIE cells stimulated with EPS, APS or NPS before poly(I:C) challenge showed a significantly higher expression of TNF-α on hour 3. Only the EPS-treated PIE cells showed reduced levels of TNF-α on hour 12 (Fig. 2A). In addition, EPS-, NPS- and APS-treated cells showed significantly reduced levels of IL-6 on hour 12 (Fig. 2A). Poly(I:C) challenge increased the expression of CXCL8, CCL4 and CXCL10 in PIE cells (Fig. 2B). EPS-treated PIE cells showed significantly reduced levels of CXCL8 on hours 3 and 6 while this chemokine was diminished on hour 12 in NPS-treated cells (Fig. 2B). It was also observed that CCL4 was enhanced on hour 3 in EPS-treated PIE cells and, reduced on hour 6. Interestingly, EPS-treated PIE cells showed a significant increase in the expression of CXCL10 on hour 3 while cells stimulated with NPS or APS showed a reduced expression of this chemokine in this time point (Fig. 2B).

3.3. Effect of LDR-1 EPSs on antiviral PRRs in PIE cells

The expression of the antiviral PRRs, TLR3 and RIG-I was also evaluated in PIE cells after Poly(I:C) stimulation (Fig. 3). The challenge of PIE cells with the TLR3 ligand increased the expression of both TLR3 and RIG-I. PIE cells incubated with EPS, APS or NPS from LDR-1 showed a significantly higher expression of TLR3 on hours 3 and 6 after poly(I:C) stimulation; and a reduced expression of RIG-I on hour 12 when compared to control (Fig. 3).

3.4. Effect of LDR-1 EPSs on TLR negative regulators expressions in PIE cells

We next determined the effect of EPS, NPS and APS from LDR-1 on the expression of the negative regulators of TLRs signaling in PIE after the stimulation with poly(I:C). The treatment of PIE cells with the TLR3 agonist enhanced the expression of A20 and Bcl-3, while it did not affect the expression of SIGIRR, MKP-1, Tollip, or IRAK-M (Fig. 4). EPS- and NPS-treated PIE cells showed significantly increased levels of A20 on hours 3 and 6, respectively. Cells treated with EPS, NPS or APS had a diminished expression of A20 on hour 12 (Fig. 4A). Tollip expression was significantly enhanced in NPS-treated PIE cells on hours 3 and 6 after poly(I:C) stimulation (Fig. 4A). APS- and NPS-treated cells showed a reduced expression of Bcl-3 in the earlier time point studied while its expression on hour 12 was enhanced (Fig. 4A). In addition, NPS-treated PIE cells showed a decreased expression of SIGIRR on hour 3 and increased levels of this TLR negative regulator on hour 6 (Fig. 4B). EPS-, NPS- and APS-treated cells had enhanced levels of IRAK-M on hour 6 and decreased expression of MKP-1 on hours 3 and 12 (Fig. 4B).

3.5. Modulation of TRAF3, IRF3 and NF-κB activation in PIE cells by LDR-1 EPSs

After TLR3 activation several intracellular signaling molecules including TRAF3, IRF3, and NF-κB participate in the induction of type I IFNs and inflammatory cytokines/chemokines. Then, we next evaluated whether EPS, APS or NPS from LDR-1 were able to modulate the phosphorylation of TRAF3 and IRF3, or the degradation of IκBα in PIE cells stimulated with poly(I:C). TRAF3 was significantly increased at minutes 30 and 120 after the challenge of PIE cells with poly(I:C) (Fig. 5). The incubation of PIE cells with EPS and NPS significantly enhanced TRAF3 between minutes 120 and 240 after the stimulation with poly(I:C) while APS increased TRAF3 during all the studied period (Fig. 5). In addition, phosphorylation of IRF3 was increased between

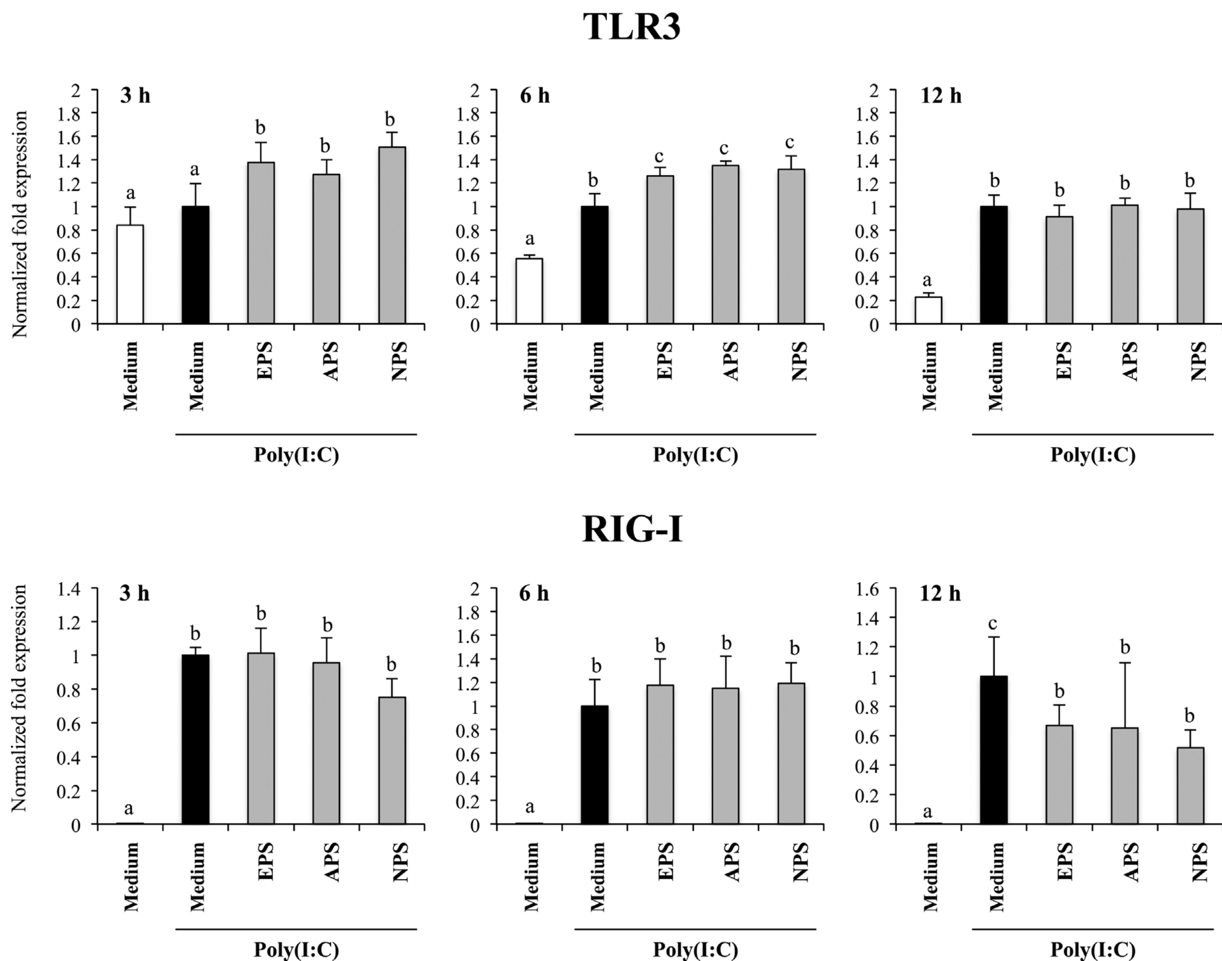


Fig. 3. Effect of exopolysaccharides (EPS) from *Lactobacillus delbrueckii* OLL1073R-1 (LDR-1) in the expression of pattern recognition receptors in porcine intestinal epithelial (PIE) cells. EPS was fractionated into neutral EPS (NSP) and acidic EPS (APS) by standard chromatographic methods. PIE cells were treated with EPS, APS, NPS or medium for 48 h and then stimulated with the Toll-like receptor (TLR)-3 agonist poly(I:C). PIE cells without poly(I:C) stimulation were used as controls. The mRNA level of TLR3 and RIG-I were estimated by qPCR at the indicated time points after poly(I:C) treatment. The results are expressed as mean ± SD and represent data from three independent experiments (n = 3 in each experiment). Values with different superscripts letters are significantly different within the level $p < 0.05$ (a < b < c).

minutes 120 and 240 after the challenge of PIE cells with poly(I:C) (Fig. 6). APS-treated PIE cells showed a significantly increased IRF3 phosphorylation during all the studied period while no modification of this parameter was observed in EPS- or NPS-treated PIE cells (Fig. 6). Finally, we evaluated whether EPSs were able to modulate the NF-κB pathway. The stimulation of PIE cells with poly(I:C) exhibited a reduction in IκBα levels at minute 60 indicating the activation of the NF-κB pathway (Fig. 7). IκBα levels increased later between minutes 120 and 240. PIE cells stimulated with EPS or APS showed a similar kinetics than control cells since IκBα levels were reduced at minute 60, and increased later between minutes 120 and 240 (Fig. 7). On the other hand, NPS-treated PIE cells showed increased levels of IκBα during all the studied period (Fig. 7).

3.6. Role of TLRs on the modulatory activity of LDR-1 EPSs

In order to evaluate the role of TLR2 and TLR4 in the immunomodulatory effects of EPSs from LDR-1 we performed blocking experiments in PIE cells with anti-TLR2 or anti-TLR4 antibodies prior to EPS, APS or NPS treatments. The ability of EPS- and APS-treated PIE cells to increase the expression of IFN-β in response to poly(I:C) stimulation was abolished when cells were treated with anti-TLR2 and anti-TLR4 antibodies, respectively (Fig. 8A). Similarly, the capacity of EPS and APS to induce a reduction in the expression of IL-6 and CCL4 after poly(I:C) stimulation was abolished when PIE cells were treated with anti-TLR2 and anti-TLR4, respectively (Fig. 8B, C). Neither TLR2 nor TLR4 seem to be involved in the capacity of NPS to modulate IFN-β (Fig. 8A), IL-6 (Fig. 8B) or CXCL8 (Fig. 8C) in poly(I:C)-stimulated PIE cells.

4. Discussion

Several research works reported the ability of immunobiotic bacteria to beneficially modulate antiviral response in IECs (For a recent review see Villena et al., 2016). In this regard, we demonstrated in different studies that the PIE cell line is a useful *in vitro* tool for studying type I IFN response triggered by TLR3 activation (Hosoya et al., 2011; Ishizuka et al., 2016; Albarracin et al., 2017). In a previous study, PIE cells were successfully used to screen and select immunobiotic bifidobacteria strains with antiviral effect (Ishizuka et al., 2016). *Bifidobacterium infantis* MCC12 and *Bifidobacterium breve* MCC1274 were selected because they were able to significantly increase IFN-β in response to poly(I:C) challenge. During viral infections, the activation of IFNs signaling increases the expression of a number of IFN-stimulated genes (ISGs) that encode proteins able to counteract virus replication (Sadler and Williams, 2008). Our studies showed that as a result of the enhanced IFN-β levels, there was a concomitant upregulation of the ISGs MxA and RNase L in bifidobacteria-treated PIE cells (Ishizuka et al., 2016). Moreover, both MCC12 and MCC1274 strains significantly enhanced the resistance of PIE cells to rotavirus infection. This and

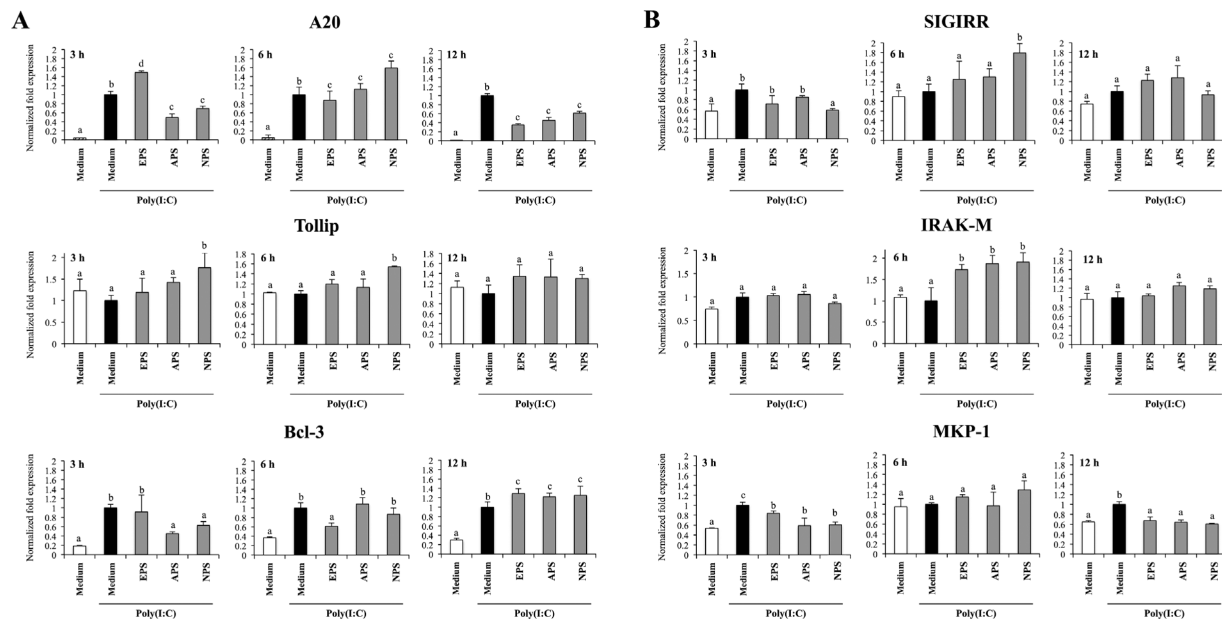


Fig. 4. Effect of exopolysaccharides (EPS) from *Lactobacillus delbrueckii* OLL1073R-1 (LDR-1) in the expression of negative regulators of the Toll-like receptor (TLR) signaling in porcine intestinal epithelial (PIE) cells. EPS was fractionated into neutral EPS (NSP) and acidic EPS (APS) by standard chromatographic methods. PIE cells were treated with EPS, APS, NPS or medium for 48 h and then stimulated with the TLR3 agonist poly(I:C). PIE cells without poly(I:C) stimulation were used as controls. The expression of A20, Tollip, Bcl-3 (A), SIGIRR, IRAK-M and MKP-1 (B) were estimated by qPCR at the indicated time points after poly(I:C) treatment. The results are expressed as mean ± SD and represent data from three independent experiments (n = 3 in each experiment). Values with different superscripts letters are significantly different within the level $p < 0.05$ (a < b < c).

other studies clearly demonstrated the capacity of immunobiotic bacteria to improve innate antiviral immunity in IECs (Hosoya et al., 2011; MacPherson et al., 2014; Ishizuka et al., 2016; Albarracin et al., 2017). However, there are no reports available describing the beneficial effects of immunomodulatory EPSs from LAB on the antiviral immune response of IECs.

In this study, we used PIE cells to analyze the immunomodulatory capacity of EPSs from the immunomodulatory LDR-1 strain (Kitazawa et al., 1998; Makino et al., 2006; Makino et al., 2010; Nagai et al., 2011; Makino et al., 2016). Of interest, EPS from LDR-1 was able to induce a significant increase in the expression of IFN-α and IFN-β in PIE cells after the stimulation with poly(I:C) as well as the expression of the ISGs MxA and RNase L (Fig. 9). Several studies reported that the inhibitory activity of MxA proteins is able to protect against the infection with several viruses (Sadler and Williams, 2008; Xiao et al., 2013). In addition, RNase L degrades viral RNA and activates cytoplasmic RIG-I/MDA5 receptors inducing an amplification of type I IFN production and increasing the apoptosis on virus-infected cells (Chakrabarti et al., 2011; Sadler and Williams, 2008). Then, the upregulation of MxA, RNase L, and probably other ISGs induced by EPS from LDR-1 through IFN-β indicate that EPS is able to improve the antiviral state of IECs. Interestingly, although EPS improved IFN-β, MxA, and RNase L expression in PIE cells, this effect was not similar with purified APS or NPS from the OLL1073R-1 strain (Fig. 9). In fact, the increase of IFN-β in APS- and NPS-treated PIE cells was detected later than in EPS-treated cells while the ISGs MxA and RNase L showed no differences when compared to controls during the studied period (12 h after poly(I:C) stimulation). These results indicate that the complete EPS molecule would be necessary for obtaining the highest immunomodulatory/aniviral activity in IECs. These findings contrast with previous studies demonstrating that APS has the same immunomodulatory properties observed for EPS when evaluated in murine splenocytes. In fact, both APS and EPS were equally effective for inducing the improvement of IFN-γ in immune cells from mouse spleen (Makino et al., 2006). These differences could be related to the distinct cells used for experiments (immune versus non-immune cells and mouse versus porcine cells). Therefore, further studies evaluating the immunomodulatory activity of EPS, NPS and APS in mouse intestine and porcine immune cells are necessary in order to fully characterize the beneficial properties of LDR-1 EPSs.

When we evaluated the expression of the zinc-finger protein A20 we observed a significant downregulation after poly(I:C) stimulation in PIE cells previously treated with EPS, APS or NPS from LDR-1. A20 terminates TLR signaling that results in the inhibition of NF-κB activation (Ning and Pagano, 2010). In addition, it was reported that IRF3 activation is suppressed by A20 leading to the reduction of the IFN-mediated immune response (Saitoh et al., 2005). The increase of A20 in EPS-treated PIE cells is in line with the improved IRF3 activation and IFN-β production. In this regard, we reported previously that the immunobiotic strains *B. infantis* MCC12 and *B. breve* MCC1274 significantly reduced the expression of A20 in rotavirus-infected PIE cells, an effect that was related to the enhancement of the antiviral state (Ishizuka et al., 2016). Moreover, it was shown that the stimulation of HT29 cells with poly(I:C) alone increased the expression of A20, but the co-stimulation of those cells with poly(I:C) and immunobiotics significantly reduced A20 expression levels and improved innate immunity (MacPherson et al., 2014).

Activation of TLR3 triggers signaling that leads to not only the production of type I IFNs in IECs but in addition to several and proinflammatory cytokines and chemokines, which alerts host to induce the recruitment and activation of immune cells to combat the invading pathogen (Kondo et al., 2012; Villena et al., 2016). Several cytokines/chemokines are induced in IECs via NF-κB signaling as a result of TLR3 activation by poly(I:C) or viral infection, including the cytokines evaluated in this work: TNF-α, IL-6, CXCL8, CXCL10, and CCL4. Those proinflammatory factors are important for the generation of protective responses mediated by immune cells. However, over-activation of TLR3 signaling and deregulated production of cytokines/chemokines may contribute to the pathogenesis of viral infection (Kondo et al., 2012). It was reported that both purified rotavirus dsRNA and poly(I:C) induce severe mucosal damage in the gut via TLR3 activation including mucosal erosion, villous atrophy, and gut wall attenuation (Zhou et al., 2007). In addition, we have reported that immunobiotic treatments are able to beneficially regulate the inflammatory response induced by

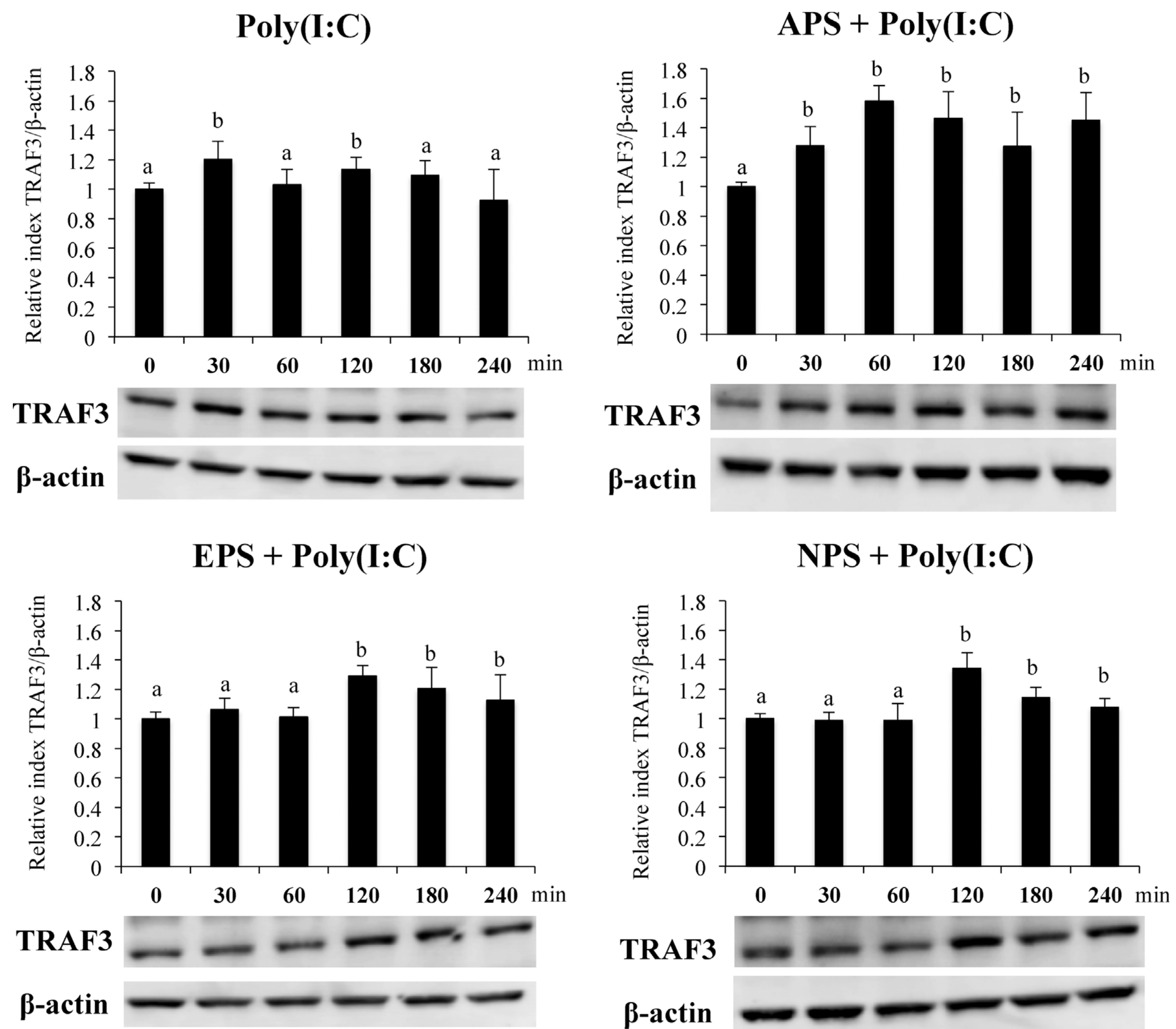


Fig. 5. Effect of exopolysaccharides (EPS) from *Lactobacillus delbrueckii* OLL1073R-1 (LDR-1) in TRAF3 phosphorylation in porcine intestinal epithelial (PIE) cells. EPS was fractionated into neutral EPS (NSP) and acidic EPS (APS) by standard chromatographic methods. PIE cells were treated with EPS, APS, NPS or medium for 48 h and then stimulated with the Toll-like receptor (TLR)-3 agonist poly(I:C). The proteins from lysed cells were extracted and separated by SDS-PAGE, and western-blot was performed to quantify the phosphorylation of TRAF3 at the indicated time points. Intensities of proteins bands were calculated from peak area of densitogram by using image J software. The results are expressed as mean ± SD and represent data from three independent experiments (n = 2 in each experiment). Values with different superscripts letters are significantly different within the level $p < 0.05$ (a < b).

rotavirus or poly(I:C) by modulating the expression of negative regulators of TLR signaling and cytokines/chemokines production (Hosoya et al., 2011; Villena et al., 2014; Ishizuka et al., 2016; Tada et al., 2016). In line with our previous findings, we demonstrated here that EPS-, NPS- and APS-treated PIE cells were able to differentially modulate the expression of TLR negative regulators, TNF-α, IL-6, CXCL8, CXCL10, and CCL4 after poly(I:C) stimulation. The most notable effect was observed in PIE cells stimulated with NPS before poly(I:C) treatment (Fig. 9). NPS-treated cells showed improved expression of TNF-α while IL-6, CXCL8, and CXCL10 were reduced. This effect would be related to the significant reduction in the activation of NF-κB pathway and enhanced expression of four negative regulators of TLR signaling: SIGIRR, Bcl-3, Tollip, and IRAK-M. Of interest, EPS- and APS-treated PIE cells showed diminished levels of IL-6 and CCL4. The lower capacity of those treatments to reduce inflammatory cytokines/chemokines would be explained by their ability to improve only two negative regulators: Bcl-3 and IRAK-M (Fig. 9).

We previously showed that NPS and APS from the immunobiotic strains *L. plantarum* N14 and *L. delbrueckii* TUA4408L are able to activate PRRs expressed in PIE cells, upregulate negative regulators of TLRs signaling and reduce the production of inflammatory mediators after activation of TLR4 by LPS or heat-stable enterotoxigenic *Escherichia coli* (Wachi et al., 2014; Murofushi et al., 2015). Additionally, we found that three PRRs are involved in the activities of those EPSs: TLR2, TLR4 and RP105. Therefore, we also aimed to investigate in this work the potential role of TLR2 and TLR4 in the immunomodulatory effects of EPSs from LDR-1. We observed that the capacity of EPS to modulate the expression of type I IFNs and inflammatory cytokines in poly(I:C)-treated PIE cells was dependent on TLR2.

TLR2 is among the PRRs that have been widely studied to explain the modulatory effects of immunobiotic bacteria in both immune and IECs. It was reported that *L. amylovorus* DSM 16698T regulated TLR4 activation in Caco-2 cells through modulation of Tollip and IRAK-M and, that this effect was dependent on TLR2 signaling (Finamore et al., 2014). In agreement, our previous studies in PIE cells also revealed that TLR2 plays an important role in the immunoregulatory activity of the immunobiotic strains *L. jensenii* TL2937, *B. longum* BB536, and *B. breve* MCC-117. Our works demonstrated that those strains were able to increase A20 expression and reduce the production of pro-inflammatory cytokines in PIE cells after TLR4 activation in a TLR2-dependent manner (Laiño et al., 2016). We also reported that NPS from *L. plantarum* N14 or *L. delbrueckii* TUA4408L exert their immunomodulatory

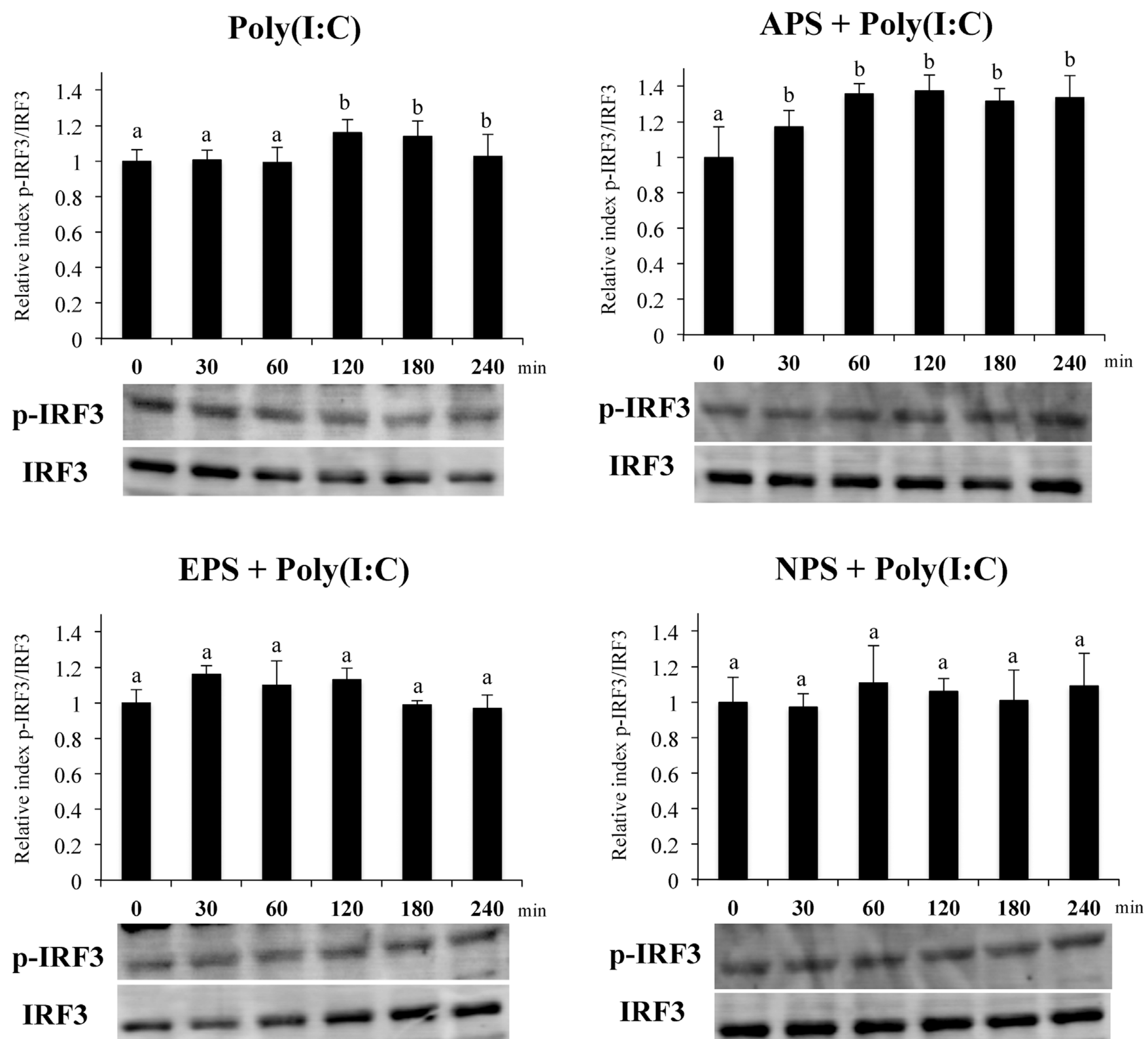


Fig. 6. Effect of exopolysaccharides (EPS) from *Lactobacillus delbrueckii* OLL1073R-1 (LDR-1) in IRF3 phosphorylation in porcine intestinal epithelial (PIE) cells. EPS was fractionated into neutral EPS (NSP) and acidic EPS (APS) by standard chromatographic methods. PIE cells were treated with EPS, APS, NPS or medium for 48 h and then stimulated with the Toll-like receptor (TLR)-3 agonist poly(I:C). The proteins from lysed cells were extracted and separated by SDS-PAGE, and western-blot was performed to quantify the phosphorylation of IRF3 at the indicated time points. Intensities of proteins bands were calculated from peak area of densitogram by using image J software. The results are expressed as mean ± SD and represent data from three independent experiments (n = 2 in each experiment). Values with different superscripts letters are significantly different within the level $p < 0.05$ (a < b).

effects of through TLR2. Both bacteria were able to diminish the expression of pro-inflammatory mediators triggered by TLR4 in PIE cells in a TLR2-dependent manner (Wachi et al., 2014; Murofushi et al., 2015). Here, we did not observe changes in the immunomodulatory activity of NPS from LDR-1 when anti-TLR2 blocking antibodies were used. However, the effect of the complete EPS molecule was abolished by anti-TLR2 treatment. Therefore, EPS from LDR-1 was able to modulate TLR3-triggered antiviral immune response in PIE cells in a TLR2-dependent manner. In line with our results, some works have demonstrated an important role of TLR2 in the resistance against rotavirus infection. The nonstructural protein 4 (NSP4) from rotavirus was identify as a novel agonist of TLR2 since this viral protein triggered the secretion of inflammatory cytokines and chemokines in wild-type immune cells and IECs but not in $TLR2^{-/-}$ cells (Ge et al., 2013). These results indicated that the activation of TLR2 by NSP4 may play an important role in viral pathogenesis and innate immunity in the gut and, therefore, the manipulation of TLR2 activation could offer an alternative to improve antiviral defenses and/or regulate excessive inflammatory responses during infection. In this regard, Liu et al. (2010) showed that TLR2 expression was upregulated in porcine IECs (IPEC-J2 cells) by *L. rhamnosus* GG or peptidoglycan treatments, and that the upregulation of this PRR expression was associated to a protective effect against subsequent rotavirus infection. Further studies employing TLR2 knockdown PIE cells or knockout mice may be valuable in resolving the role of this PRR in the immunomodulatory effect of EPS from LDR-1. In addition, whether EPS from LDR-1 is able to differentially modulate rotavirus induced immune response in IECs or in intestinal infections *in vivo* are opened question, which we propose to address in the near future.

As mentioned before, previous studies evaluating the interaction of NPS and APS from LDR-1 with immune cells demonstrated that the main fraction of EPS with immunomodulatory activity was the APS (Kitazawa et al., 1998; Makino et al., 2006; Makino et al., 2010; Nagai et al., 2011; Makino et al., 2016). Moreover, it was shown that the immunomodulatory activity of APS was completely abolished by dephosphorylation. In this work, we demonstrated that the phosphopolysaccharide APS was able to modulate the response of PIE cells in a TLR4-deppendent manner. APS increased the expression of IRAK-M and Bcl-3, and decreased A20 in a similar way to EPS. However, its immunomodulatory effect was reduced when compared to the complete

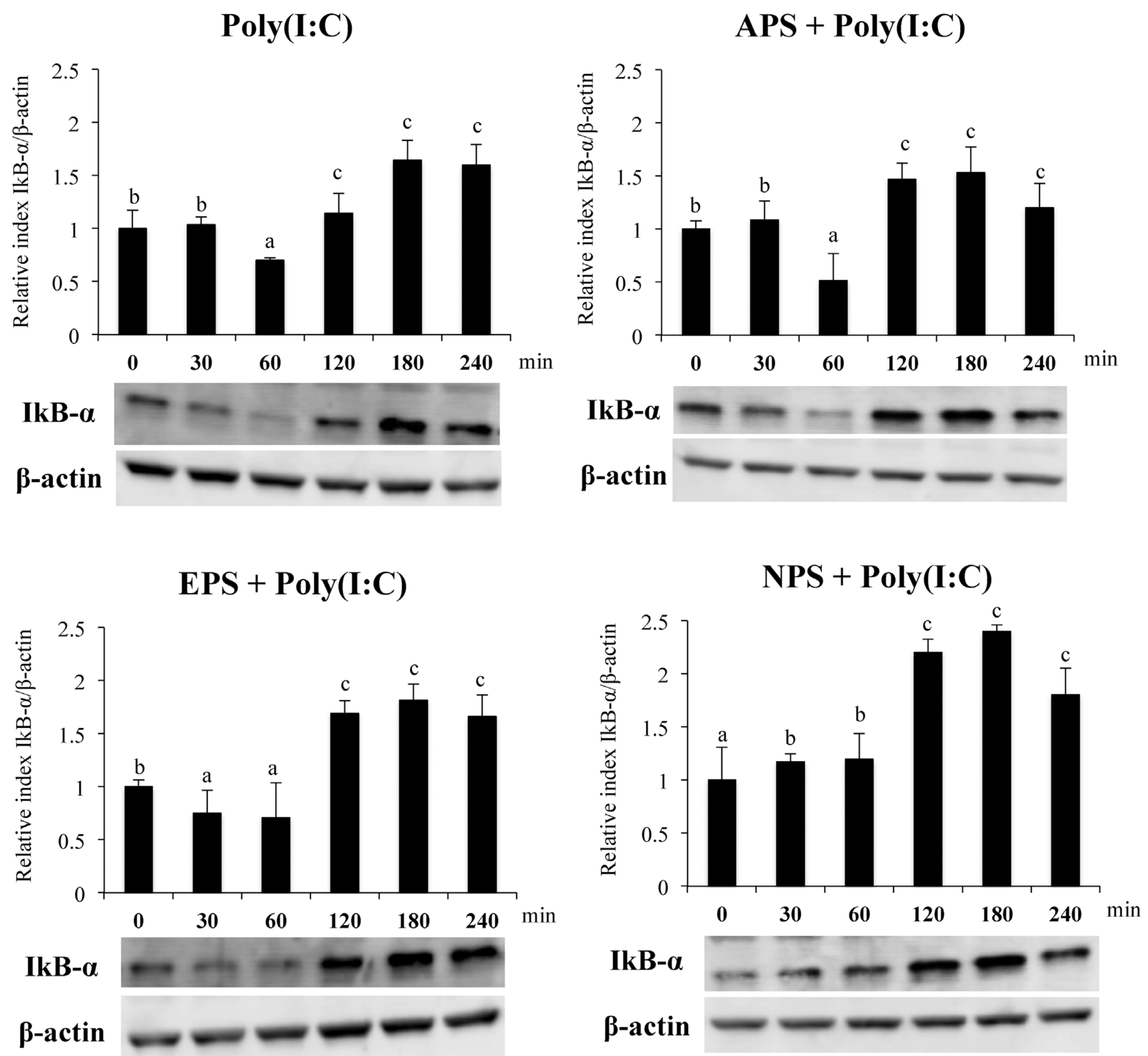


Fig. 7. Effect of exopolysaccharides (EPS) from *Lactobacillus delbrueckii* OLL1073R-1 (LDR-1) on NFκB pathway in porcine intestinal epithelial (PIE) cells. EPS was fractionated into neutral EPS (NSP) and acidic EPS (APS) by standard chromatographic methods. PIE cells were treated with EPS, APS, NPS or medium for 48 h and then stimulated with the Toll-like receptor (TLR)-3 agonist poly(I:C). The proteins from lysed cells were extracted and separated by SDS-PAGE, and western-blot was performed to quantify IκBα at the indicated time points. Intensities of proteins bands were calculated from peak area of densitogram by using image J software. The results are expressed as mean ± SD and represent data from three independent experiments (n = 2 in each experiment). Values with different superscripts letters are significantly different within the level $p < 0.05$ (a < b).

molecule. Therefore, the results of this work indicate that for the modulation of innate antiviral immune response in PIE cells both APS and NPS are necessary. NPS has no effect on immune cells, however it was able to significantly influence the expression of cytokines and chemokines in poly(I:C)-stimulated PIE cells. Of interest, anti-TLR2 or anti-TLR4 blocking antibodies were no able to affect NPS activity on PIE cells indicating that other PRRs would be involved in its effect. One possible PRRs involved in NPS recognition may be the dectin-1 receptor since natural immunomodulatory polysaccharides such as (1,3)-(1,6)-β-glucans have been shown to induce their regulatory effects through their interaction with this receptor (Stier et al., 2014). Identification of the unknown receptor or receptors involved in NSP recognition is another interesting topic for future investigations.

5. Conclusion

In the last decades, EPS produced by beneficial bacteria have received attention because of their contribution to health maintenance (Laiño et al., 2016). Some recent studies demonstrated that EPS produced by probiotic bacteria are able to modulate systemic and mucosal immune responses, and in turn to provide direct health-promoting benefits. In this study, we showed that innate immune response triggered by TLR3 activation in IECs was differentially modulated by EPS from LDR-1. Our results suggest that EPS from LDR-1 have potential to be used for improving intestinal innate antiviral response and protecting against intestinal viruses such as rotavirus. Of interest, the results of this work indicate that for the modulation of innate antiviral immune response in IECs both APS and NPS are necessary. In fact, the most notable improvement of antiviral immunity was observed when the complete molecule EPS was used. To evaluate the ability of EPS, NPS and APS from LDR-1 to modulate the response to poly(I:C) *in vivo* as well as their capacities to improve resistance against intestinal viral infections are interesting topics for future near research.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research (B)(2) (Nos. 24380146, 16H05019) and Challenging Exploratory

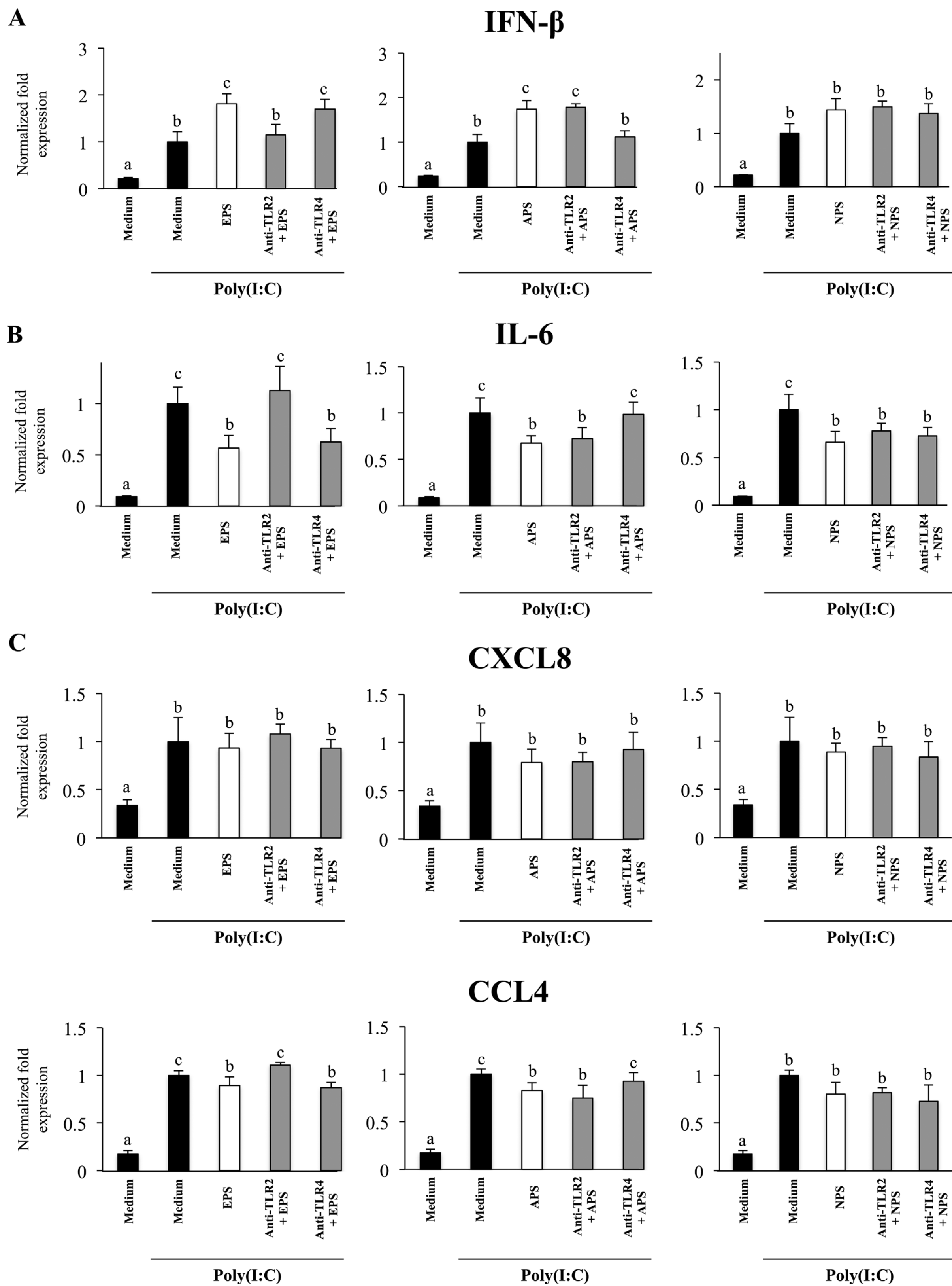


Fig. 8. Role of Toll-like receptor (TLR)-2 and TLR4 in the immunomodulatory effect of exopolysaccharides (EPS) from *Lactobacillus delbrueckii* OLL1073R-1 (LDR-1) in porcine intestinal epithelial (PIE) cells. EPS was fractionated into neutral EPS (NSP) and acidic EPS (APS) by standard chromatographic methods. PIE cells were incubated with anti-TLR2 or anti-TLR4 antibodies, treated with EPS, APS, NPS or medium for 48 h and, then stimulated with the TLR3 agonist poly(I:C). The expression of IFN-β (A), IL-6 (B), CXCL8, and CCL4 (C) were quantified by qPCR 12 h after poly(I:C) treatment. The results are expressed as mean ± SD and represent data from three independent experiments (n = 2 in each experiment). Values with different superscripts letters are significantly different within the level $p < 0.05$ (a < b < c).

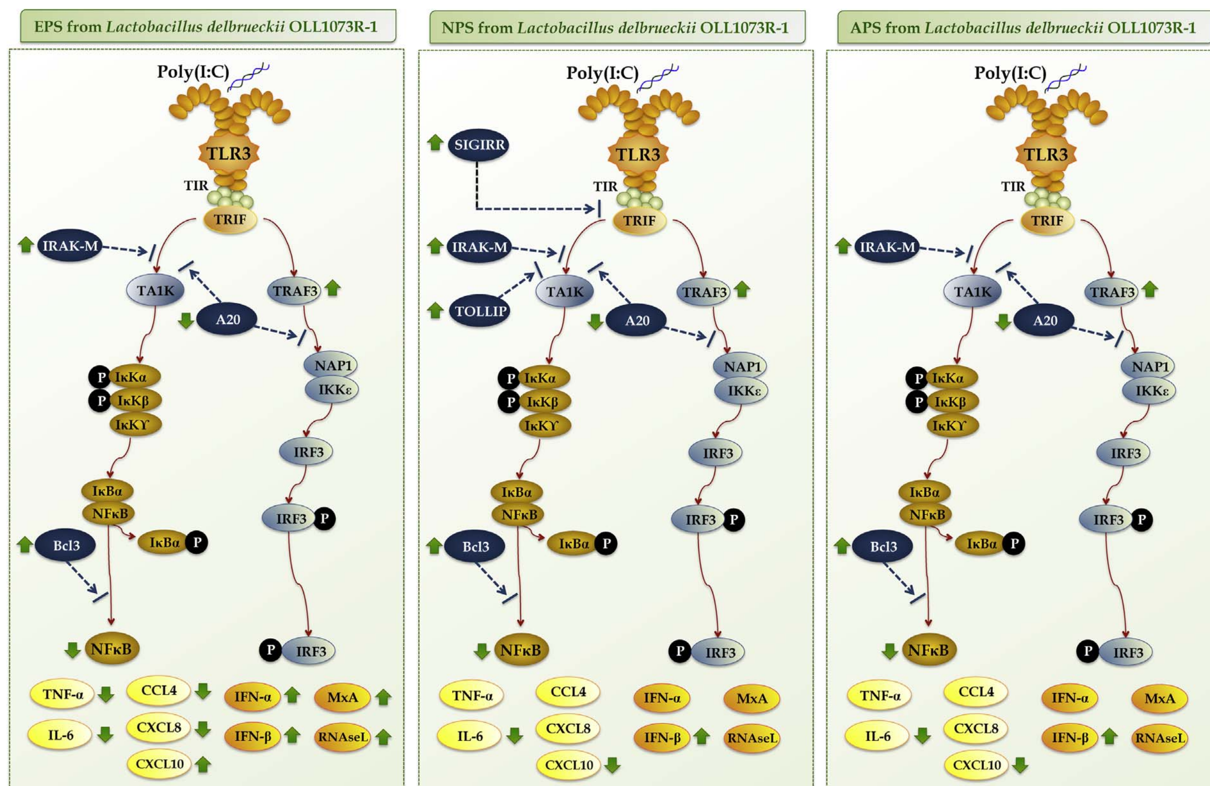


Fig. 9. Proposed mechanisms for the antiviral activity of exopolysaccharides (EPS), acidic extracellular polysaccharide (APS), and neutral extracellular polysaccharides (NPS) from *Lactobacillus delbrueckii* OLL1073R-1 (LDR-1) in porcine intestinal epithelial (PIE) cells after stimulation with the Toll-like receptor (TLR)-3 agonist poly(I:C).

Research (Nos. 23658216, 26660216, 16K15028) and Open Partnership Joint Projects of JSPS Bilateral Joint Research Projects from the Japan Society for the Promotion of Science (JSPS) to H. Kitazawa. Dr. Paulraj Kanmani and AKM Humayun Kober were supported by JSPS (Postdoctoral Fellowship for Foreign Researchers, Program Nos. 25-03397 and 15F15401 respectively). This work was also supported by the JSPS Core-to-Core Program Advanced Research Networks “Establishment of international agricultural immunology research-core for a quantum improvement in food safety”.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2017.07.009.

References

Albarracin, L., Kobayashi, H., Iida, H., Sato, N., Nochi, T., Aso, H., Salva, S., Alvarez, S., Kitazawa, H., Villena, J., 2017. Transcriptomic analysis of the innate antiviral immune response in porcine intestinal epithelial cells: influence of immunobiotic lactobacilli. Front. Immunol. 8, 57.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., et al., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622.

Chakrabarti, A., Jha, B.K., Silverman, R.H., 2011. New insights into the role of RNase L in innate immunity. J. Interface Cytokine Res. 31 (1), 49–57.

Finamore, A., Roselli, M., Imbinto, A., Seeboth, J., Oswald, I.P., Mengheri, E., 2014. *Lactobacillus amylovorus* inhibits the TLR4 inflammatory signaling triggered by Enterotoxigenic *Escherichia coli* via modulation of the negative regulators and involvement of TLR2 in intestinal Caco-2 cells and pig explants. PLoS One 9, e94891.

Ge, Y., Mansell, A., Ussher, J.E., Brooks, A.E., Manning, K., Wang, C.J., Taylor, J.A., 2013. Rotavirus NSP4 triggers secretion of proinflammatory cytokines from macrophages via toll-like receptor 2. J .Virol. 87 (20), 11160–11167.

Hosoya, S., Villena, J., Shimazu, T., Tohno, M., Fujie, H., Chiba, E., Shimosato, T., Aso, H., Suda, Y., Kawai, Y., Saito, T., Alvarez, S., Itoh, H., Kitazawa, H., 2011. Immunobiotic lactic acid bacteria beneficially regulate immune response triggered by poly (I: C) in porcine intestinal epithelial cells. Vet. Res. 42 (1), 1.

Ishizuka, T., Kanmani, P., Kobayashi, H., Miyazaki, A., Soma, J., Suda, Y., Aso, H., Nochi, T., Iwabuchi, N., Xiao, J.Z., Saito, T., Villena, J., Kitazawa, H., 2016. Immunobiotic bifidobacteria strains modulate rotavirus immune response in porcine intestinal epitheliocytes via pattern recognition receptor signaling. PLoS One 11 (3), e0152416.

Kitazawa, H., Harata, T., Uemura, J., Saito, T., Kaneko, T., Itoh, T., 1998. Phosphate group requirement for mitogenic activation of lymphocytes by an extracellular phosphopolysaccharide from *Lactobacillus delbrueckii* ssp. *bulgaricus*. J. Food Microbiol. 40 (3), 169–175.

Kondo, T., Kawai, T., Akira, S., 2012. Dissecting negative regulation of toll-like receptor signaling. Trend Immunol. 33 (9), 449–458.

Laiño, J., Villena, J., Kanmani, P., Kitazawa, H., 2016. Immunoregulatory effects triggered by lactic acid bacteria exopolysaccharides: new insights into molecular interactions with host cells. Microorganisms 4 (3), 27.

Liu, F., Li, G., Wen, K., Bui, T., Cao, D., Zhang, Y., Yuan, L., 2010. Porcine small intestinal epithelial cell line (IPEC-J2) of rotavirus infection as a new model for the study of innate immune responses to rotaviruses and probiotics. Viral Immunol. 23 (2), 135–149.

MacPherson, C., Audy, J., Mathieu, O., Tompkins, T.A., 2014. Multistrain probiotic modulation of intestinal epithelial cells immune response to a double-stranded RNA ligand, Poly (I:C). Appl. Environ. Microbiol. 80 (5), 1692–1700.

Mair, K.H., Sedlak, C., Käser, T., Pasternak, A., Levast, B., Gerner, W., et al., 2014. The porcine innate immune system: an update. Dev. Comp. Immunol. 45, 321–343.

Makino, S., Ikegami, S., Kano, H., Sashihara, T., Sugano, H., Horiuchi, H., Saito, T., Oda, M., 2006. Immunomodulatory effects of polysaccharides produced by *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1. J. Dairy Sci. 89 (8), 2873–2881.

Makino, S., Ikegami, S., Kume, A., Horiuchi, H., Sasaki, H., Orii, N., 2010. Reducing the risk of infection in the elderly by dietary intake of yoghurt fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1. Br. J. Nutr. 104 (07), 998–1006.

Makino, S., Sato, A., Goto, A., Nakamura, M., Ogawa, M., Chiba, Y., Hemmi, J., Kano, H., Takeda, K., Okumura, K., Asami, Y., 2016. Enhanced natural killer cell activation by exopolysaccharides derived from yogurt fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1. J. Dairy Sci. 99 (2), 915–923.

Moue, M., Tohno, M., Shimazu, T., Kido, T., Aso, H., Saito, T., Kitazawa, H., 2008. Toll-like receptor 4 and cytokine expression involved in functional immune response in an originally established porcine intestinal epitheliocyte cell line. Biochim. Biophys. Acta (BBA)-Gen. Sub. 1780 (2), 134–144.

Murofushi, Y., Villena, J., Morie, K., Kanmani, P., Tohno, M., Shimazu, T., Aso, H., Suda, Y., Hashiguchi, K., Saito, T., Kitazawa, H., 2015. The toll-like receptor family protein RP105/MD1 complex is involved in the immunoregulatory effect of exopolysaccharides from *Lactobacillus plantarum* N14. Mol. Immunol. 64 (1), 63–75.

Nagai, T., Makino, S., Ikegami, S., Itoh, H., Yamada, H., 2011. Effects of oral administration of yogurt fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1 and its exopolysaccharides against influenza virus infection in mice. Int. Immunopharmacol. 11 (12), 2246–2250.

Ning, S., Pagano, J.S., 2010. The A20 deubiquitinase activity negatively regulates LMP1 activation of IRF7. J. Virol. 84 (12), 6130–6138.

Nishimura-Uemura, J., Kitazawa, H., Kawai, Y., Itoh, T., Oda, M., Saito, T., 2003. Functional alteration of murine macrophages stimulated with extracellular polysaccharides from *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1. Food Microbiol. 20 (3), 267–273.

Nygard, A.B., Jorgensen, C.B., Cirera, S., Fredholm, M., 2007. Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR. BMC Mol. Biol. 8, 67.

Sadler, A.J., Williams, B.R., 2008. Interferon-inducible antiviral effectors. Nat. Rev. Immunol. 8 (7), 559–568.

Saitoh, T., Yamamoto, M., Miyagishi, M., Taira, K., Nakanishi, M., Fujita, T., Akira, S., Yamaoka, S., Yamaoka, S., 2005. A20 is a negative regulator of IFN regulatory factor 3 signaling. J. Immunol. 174 (3), 1507–1512.

Stier, H., Ebbeskotte, V., Gruenwald, J., 2014. Immune-modulatory effects of dietary yeast beta-1, 3/1, 6-D-glucan. Nutr. J. 13, 38.

Tada, A., Zelaya, H., Clua, P., Salva, S., Alvarez, S., Kitazawa, H., Villena, J., 2016. Immunobiotic Lactobacillus strains reduce small intestinal injury induced by intraepithelial lymphocytes after Toll-like receptor 3 activation. Inflamm. Res. 65 (10), 771–783.

Van Calsteren, M.R., Gagnon, F., Nishimura, J., Makino, S., 2015. Structure determination of the neutral exopolysaccharide produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL1073R-1. Carbohydr. Res. 413, 115–122.

Villena, J., Chiba, E., Vizoso-Pinto, M.G., Tomosada, Y., Takahashi, T., Ishizuka, T., Aso, H., Salva, S., Alvarez, S., Kitazawa, H., 2014. Immunobiotic *Lactobacillus rhamnosus* strains differentially modulate antiviral immune response in porcine intestinal epithelial and antigen presenting cells. BMC Microbiol. 14, 126.

Villena, J., Vizoso-Pinto, M.G., Kitazawa, H., 2016. Intestinal innate antiviral immunity and immunobiotics: beneficial effects against rotavirus infection. Front. Immunol. 7, 563.

Wachi, S., Kanmani, P., Tomosada, Y., Kobayashi, H., Yuri, T., Egusa, S., Shimazu, T., Suda, Y., Aso, H., Sugawara, M., Saito, T., Mishima, T., Villena, J., Kitazawa, H., 2014. Lactobacillus delbrueckii TUA4408L and its extracellular polysaccharides attenuate enterotoxigenic *Escherichia coli*-induced inflammatory response in porcine intestinal epitheliocytes via Toll-like receptor-2 and 4. Mol. Nutr. Food Res. 58 (10), 2080–2093.

Xiao, H., Killip, M.J., Staeheli, P., Randall, R.E., Jackson, D., 2013. The human interferon-induced MxA protein inhibits early stages of influenza A virus infection by retaining the incoming viral genome in the cytoplasm. J. Virol. 87 (23), 13053–13058.

Zelaya, H., Alvarez, S., Kitazawa, H., Villena, J., 2016. Respiratory antiviral immunity and immunobiotics: beneficial effects on inflammation-coagulation interaction during influenza virus infection. Front. Immunol. 7, 633.

Zhou, R., Wei, H., Sun, R., Tian, Z., 2007. Recognition of double-stranded RNA by TLR3 induces severe small intestinal injury in mice. J. Immunol. 178 (7), 4548–4556.