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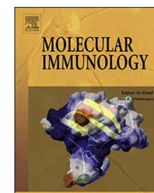

Exopolysaccharides from *Lactobacillus delbrueckii* OLL1073R-1 modulate innate antiviral immune response in porcine intestinal epithelial cells

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*

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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.

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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

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