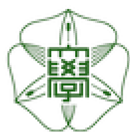

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Lactobacillus rhamnosus GG increases Toll-like receptor 3 gene expression in murine small intestine _ex vivo_ and _in vivo_

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Running header: *L. rhamnosus* GG increases TLR3 gene expression in murine small intestine

Abstract

Administration of *Lactobacillus rhamnosus* GG (LGG) has been reported to be therapeutically effective against acute secretory diarrhoea resulting from the structural and functional intestinal mucosal lesions induced by rotavirus infection; however, the underlying mechanisms remain to be completely elucidated. Because Toll-like receptor 3 (TLR3) plays a key role in the innate immune responses following the recognition of rotavirus, the present study examined whether LGG influences TLR3 gene expression in murine small intestine *ex vivo* and *in vivo*. We employed cultured intestinal organoids derived from small intestinal crypts as an *ex vivo* tissue model. LGG supplementation increased TLR3 mRNA levels in the intestinal organoids, as estimated by quantitative real-time polymerase chain reaction. Likewise, single and 7-day consecutive daily administrations of LGG increased TLR3 mRNA levels in the small intestine of C57BL/6N mice. The mRNA levels of other TLRs were not substantially altered both *ex vivo* and *in vivo*. In addition, LGG supplementation increased the mRNA levels of an antiviral type 1 interferon, interferon-α (IFN-α), and a neutrophil chemokine, CXCL1, upon stimulation with a synthetic TLR3 ligand, poly (I:C) in the intestinal organoids. LGG administration did not alter IFN-α and CXCL1 mRNA levels in the small intestine *in vivo*. Supplementation of other bacterial strains, *Bifidobacterium bifidum* and *Lactobacillus paracasei*, failed to increase TLR3 and poly (I:C)-stimulated CXCL1 mRNA levels *ex vivo*. We propose that upregulation of TLR3 gene expression may play a pivotal role in the therapeutic efficacy of LGG against rotavirus-associated diarrhoea. In addition, we demonstrated that intestinal organoids may be a promising *ex vivo* tissue model for investigating host-pathogen interactions and the antiviral action of probiotics in the intestinal epithelium.

Keywords: rotavirus, intestinal organoids, interferon-α, CXCL1, probiotics

1. Introduction

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill *et al*., 2014). Previous studies have reported the therapeutic efficacy of some *Lactobacillus* strains against intestinal infectious diseases (see review by Liévin-Le Moal and Servin, 2014). In particular, clinical and experimental studies have demonstrated that administration of *Lactobacillus rhamnosus* GG (LGG), a well-documented probiotic strain, is therapeutically effective against acute secretory diarrhoea resulting from the structural and functional intestinal mucosal lesions induced by rotavirus infection. For instance, some clinical trials conducted in children and adults showed that administration of lyophilised powder of LGG shortened the duration of acute diarrhoea associated with rotavirus (Guandalini *et al*., 2000; Majamaa *et al*., 1995; Pant *et al*., 1996; Rautanen *et al*., 1998), whereas there is no clinical evidence that consumption of probiotic strains including LGG prevents the occurrence of intestinal infectious episodes (Liévin-Le Moal and Servin, 2014). Mechanistically, LGG increases mucosal secretory immunoglobulin A and prevents intestinal epithelial surface from viral adhesion, inhibits virus assembly and release, and reduces viral replication in the mucosa (Feng *et al*., 2006; Isolauri, 2004; Kaila *et al*., 1995). LGG also enhances rotavirus-specific interferon (IFN)-γ-producing T cell responses (Sugata *et al*., 2008; Wen *et al.,* 2014). In addition, LGG reduces the rotavirus-induced release of reactive oxygen species in cultured pig and human intestinal epithelial cells (Maragkoudakis *et al*., 2010) and suppresses the rotavirus-induced interleukin (IL)-6 response in a non-transformed porcine intestinal epithelial cell line (Liu *et al*., 2010).

The gastrointestinal tract constantly has direct contact with the outside world and is inhabited by a variety of microbes. The intestinal epithelium senses the microbial environment and produces innate immune responses against pathogenic microbes. Pattern recognition receptors including Toll-like receptors (TLRs) play a key role in innate immune responses via the recognition of specific molecular patterns that are found in a broad range of pathogens (Abreu *et al*., 2005; Kawai *et al*., 2006). Double-stranded RNA (dsRNA), a characteristic by-product

of virus infection, is recognised by TLR3, whereas viral single-stranded RNA and CpG DNA are recognised by TLR7 and TLR9, respectively (Kawai *et al*., 2006). Regarding rotavirus, the best-characterised molecular pattern in the virus that is recognised by the innate immune system is dsRNA, and previous studies have shown that TLR3 recognises both rotavirus RNA and intact rotavirus particles (Alexopoulou *et al*., 2001; Sato *et al*., 2006; Zhou *et al*., 2007). However, it remains unknown whether LGG influences TLR3 expression and signaling in the intestinal epithelium for its therapeutic efficacy against rotavirus-associated diarrhoea. The present study examined the effects of LGG on the gene expression of TLRs in murine small intestine *ex vivo* and *in vivo*. In addition, TLR3 activation reportedly induces an antiviral type 1 IFN, IFN-α (Matsumoto and Seya, 2008), and a neutrophil chemokine, CXCL1 (Imaizumi *et al*., 2014). Therefore, the present study also tested whether LGG influences gene expression of IFN-α and CXCL1 in the murine small intestine *ex vivo* and *in vivo*. Moreover, previous clinical studies have shown that *Bifidobacterium bifidum* prevented rotavirus-associated diarrhoea in infants in hospital (Saavedra *et al*., 1994), whereas *Lactobacillus paracasei* strain ST11 was ineffective in children with rotavirus-associated diarrhoea (Sarker *et al*., 2005). Therefore, we tested whether *B. bifidum* and *L. paracasei* influence gene expression of TLR3 and CXCL1 in the murine small intestine *ex vivo*.

Although the small intestinal epithelium has been difficult to model in culture, the establishment of a system for culturing primary stem cell-derived small intestinal organoids has overcome the problem (Sato *et al*., 2009). The small intestinal organoids consist of a polarised epithelium that is patterned into villus-like regions containing differentiated enterocytes, goblet cells, and enteroendocrine cells and crypt-like proliferative zones containing stem cells, transit-amplifying cells, and Paneth cells (Sato *et al*., 2009). Thus, the small intestinal organoids recapitulate critical *in vivo* characteristics, such as the cellular composition and self-renewal kinetics of the small intestinal epithelium (Sato *et al*., 2009). The present study employed the small intestinal organoids as an *ex vivo* tissue model for the small intestinal epithelium. Because rotavirus is known to infect the mature enterocytes of the

villi in the small intestine, which results in epithelial lesions followed by acute secretory diarrhoea (Estes and Morris 1999), employing the small intestinal organoids would be a rational approach for investigating antiviral action of probiotics.

2. Materials and methods

Bacterial strains, cell culture, and growth conditions

LGG (ATCC 53103) was obtained from the American Type Culture Collection (Manassas, VA, USA), while *B. bifidum* (JCM 1254) and *L. paracasei* (JCM 8130T) were obtained from the Japan Collection of Microorganisms (RIKEN, Saitama, Japan). These bacteria were grown in de Man, Rogosa and Sharpe broth (Difco Laboratories; BD Biosciences, Tokyo, Japan) at 37°C for 18 h. *B. bifidum* was cultured under anaerobic condition using the AnaeroPack system (Mitsubishi gas, Tokyo, Japan). Cultured bacteria were washed and resuspended in phosphate-buffered saline (PBS). The bacterial suspensions were lyophilised and stored at -80°C until use.

Animal care

Female C57BL/6N mice (7 weeks old) were purchased from Japan SLC (Shizuoka, Japan) and housed in standard plastic cages in a temperature-controlled (23°C ± 2°C) room under a 12-h light/dark cycle and were allowed free access to tap water and standard laboratory rodent feed (Oriental Yeast, Tokyo, Japan). All study protocols were approved by the Animal Use Committee of Hokkaido University (approval no. 08-0139). Animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

Isolation of small intestinal crypts

Murine small intestinal crypts were isolated according to the protocol of Sato *et al*. (2009). In brief, mice were euthanised by cervical dislocation under sevoflurane anesthesia. A laparotomy was made and the entire length of the small intestine was excised. The intestine

was opened longitudinally and the luminal contents were thoroughly washed out with ice-cold PBS. The tissue was cut into approximately 5-mm pieces and further washed with ice-cold PBS. The tissue fragments were incubated in 2 mM ethylenediaminetetraacetic acid/PBS for 30 min at 4°C. After sedimentation, the supernatant was collected as the villous epithelial cell fraction, while the tissue fragments were resuspended in PBS. After vigorous shaking and sedimentation, the supernatant was passed through a 70-μm cell strainer (BD Biosciences), followed by centrifugation at 200 x *g* for 3 min to separate the crypts from single cells. The resultant precipitate was regarded as the crypt fraction. Aliquots of the villous and crypt fractions were treated with RNAlater RNA stabilisation reagent (Ambion; Life Technologies, Tokyo, Japan) according to the manufacturer's instructions and stored at -80°C until RNA isolation.

Ex vivo study using small intestinal organoids

Small intestinal organoids were cultured according to the protocol of Sato *et al*. (2009). In brief, the crypt fraction obtained as described above was directly resuspended in Matrigel (BD Biosciences) in each well of a 48-well plate (1000 crypts/well) and overlaid with culture medium consisting of Advanced DMEM/F12 supplemented with penicillin/streptomycin, 10 mM HEPES, Glutamax, 1 x N2, 1 x B27 (all from Invitrogen; Thermo Fischer Scientific, Tokyo, Japan), 1 μM *N*-acetylcysteine (Sigma-Aldrich, Tokyo, Japan), 50 ng/mL EGF (PeproTech, Rockey Hill, NJ, USA), 100 ng/mL noggin (PeproTech), and 1 μg/mL R-spondin1 (R&D Systems, Minneapolis, MN, USA). Y-27632 (10 μM; Sigma-Aldrich) was included in the culture medium for the first 2 days to avoid anoikis. The medium was changed every 3 days. Mature small intestinal organoids were obtained after 6 days of incubation. To examine the response of the small intestinal organoids to TLR3 ligand and bacterial strains, intestinal organoids were removed from Matrigel on day 6 of culturing using Cell Recovery Solutions (BD Biosciences) according to the manufacturer's instructions. The organoids were subsequently incubated in the culture medium supplemented with poly (I:C) (10 μg/mL; Enzo Life Sciences, Farmingdale, NY, USA), a TLR3 ligand, or lyophilised powder of cultured

bacteria (4 x 10^6 CFU/mL) for 6 h. The organoids were harvested by centrifugation at 300 x *g* for 3 min at 4°C and treated with RNAlater RNA stabilisation reagent according to the manufacturer's instructions and stored at -80°C until RNA isolation.

In vivo study using mice

To examine the effects of a single intragastric administration of LGG, mice received 0.2 mL of bacterial suspension (2 x 10^9 CFU/mL in PBS) or the vehicle using a stainless steel feeding needle. Six hours after administration, mice were euthanised by cervical dislocation under sevoflurane anesthesia and, after laparotomy, the small intestine was excised and treated with RNAlater RNA stabilisation reagent according to the manufacturer's instructions and stored at -80°C until RNA isolation. To examine the effects of chronic intragastric administration of LGG, *B. bifidum* and *L. paracasei*, mice received a daily dose of 0.2 mL of bacterial suspension (2 x 10^9 CFU/mL in PBS) or the vehicle for 7 days. After 7 days, intestinal samples were obtained as described above.

Isolation and analysis of RNA

Total RNA was isolated from small intestinal organoids and small intestinal tissue samples using the RNeasy Mini Kit (Qiagen, Tokyo, Japan) and first strand cDNA was synthesised using the ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) according to the manufacturers' instructions. Conventional polymerase chain reaction (PCR) was performed in a 25-μL reaction solution containing 2.5 μL of 10× universal buffer, 160 μM dNTPs, 100 nM gene-specific primers (Table 1), 0.25 μL of Gene *Taq* (Nippon Gene, Tokyo, Japan), and 1 μL of the first strand cDNA sample. The PCR conditions were as follows: 95°C for 2 min, followed by 40 cycles at 95°C for 30 s, 60°C for 60 s, and 68°C for 60 s, and a final extension at 68°C for 5 min. The PCR products were separated by electrophoresis through a 2% agarose gel and stained with ethidium bromide. Quantitative real-time PCR (qRT-PCR) was performed using the Thermal Cycler Dice Real-Time System (Takara, Kyoto, Japan). The qRT-PCR reaction was performed in a 25-μL reaction solution containing 12.5 μL of SYBR

Premix Ex *Taq* (Takara), 200 nM gene-specific primers (Table 1), and 2 μL of the first strand cDNA sample. The qRT-PCR conditions were as follows: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 60°C to 65°C for 40 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. The fluorescent products were detected at the last step of each cycle. The relative mRNA expression levels for each sample were normalised to those of hypoxanthine phosphoribosyltransferase.

Statistical analysis

Results are presented as the means and standard deviation (SD)(n = 3 in organoid studies and n = 3 and 8 in single and chronic administration studies, respectively). Unpaired *t*-test was used to compare the means between two groups, while Dunnett's multiple comparisons test following one-way ANOVA was used to compare the means among three groups. Data were analysed using GraphPad Prism for Macintosh (version 6, GraphPad Software, San Diego, CA, USA). *P* values <0.05 were considered to indicate statistical significance.

3. Results

Small intestinal organoids express TLR genes

In the present study, we successfully isolated murine small intestinal crypts and cultured the organoids (Figure 1; Light-microscopic visualisation of small intestinal organoids). On day 6 of culture, we observed the typical structure of mature intestinal organoids consisting of a central cyst structure and surrounding crypt-like budding structures. We used the organoids on day 6 for the experiments. Ethidium bromide staining of PCR products separated by electrophoresis through a 2% agarose gel showed that both the villous and crypt fractions isolated from murine small intestine contained TLR1-9 mRNAs, whereas the intestinal organoids on day 6 of culture were found to express only TLR1, 3, 5, 6, and 9 mRNAs (Figure 2; Toll-like receptor (TLR) mRNA expression in isolated small intestinal villous and crypt fractions and cultured small intestinal organoids).

LGG increases TLR3 gene expression in small intestinal organoids and murine small intestine

The qRT-PCR analysis showed that LGG supplementation for 6 h increased TLR3 mRNA levels in the intestinal organoids, whereas TLR1, 5, 6, and 9 mRNA levels were unaltered (Figure 3; Effect of *Lactobacillus rhamnosus* GG (LGG) on Toll-like receptor (TLR) mRNA levels in cultured small intestinal organoids (*A*) and murine small intestine (*B* and *C*)). TLR2, 4, 7 and 8 mRNAs remained undetectable when the organoids were cultured with LGG (data not shown). In mice, the TLR3 mRNA levels in the small intestine tended to be higher in the LGG-administered mice than in the vehicle-administered mice 6 h after the single intragastric administration (Figure 3B); however, the level returned to that of the vehicle-administered mice a further 6 h later (data not shown). After the 7-day consecutive daily administration, TLR3 mRNA levels in the small intestine were significantly higher in the LGG-administered mice than in the vehicle-administered mice (Figure 3C). In addition, although no differences were observed in the TLR1, 2, 6, and 9 mRNA levels between the two groups, the TLR4, 5, and 7 mRNA levels were lower in the LGG-administered mice than in the vehicle-administered mice.

LGG increases IFN-α and CXCL1 gene expression in small intestinal organoids stimulated with poly (I:C)

To test whether the increased expression of TLR3 mRNA caused by LGG supplementation plays a role in the protection against viral challenge, the response of IFN-α and CXCL1 mRNAs to poly (I:C), a synthetic TLR3 ligand, was investigated in the intestinal organoids (Figure 4; Effects of poly (I:C), a synthetic Toll-like receptor (TLR) 3 ligand, and *Lactobacillus rhamnosus* GG (LGG) on interferon-α (IFN-α) (*A*) and CXCL1 (*B*) mRNA levels in cultured small intestinal organoids). In the absence of LGG, poly (I:C) tended to increase IFN-α mRNA levels (p=0.194). Similarly, in the presence of LGG, poly (I:C) significantly increased IFN-α mRNA levels (p=0.003). Moreover, this increase was significantly higher than in the absence of LGG (p=0.018). Similarly, in the absence of LGG, poly (I:C) significantly increased CXCL1 mRNA levels (p=0.037), and also in the presence of

LGG, CXCL1 mRNA levels were significantly increased by poly (I:C)(p=0.044). As for IFN-α, this increase tended to be higher than in the absence of LGG (p=0.114). In the absence of poly (I:C) stimulation, LGG did not alter IFN-α and CXCL1 mRNA levels. Likewise, IFN-α and CXCL1 mRNA levels in the small intestine were unchanged after the 7-day consecutive daily administration of LGG *in vivo* (Figure 5; Effects of *Lactobacillus rhamnosus* GG (LGG) on interferon-α (IFN-α) (*A*) and CXCL1 (*B*) mRNA levels in small intestine of mice with 7-days administration).

B. bifidum and L. paracasei failed to increase TLR3 and poly (I:C)-stimulated CXCL1 gene expression in small intestinal organoids

Supplementation of *B. bifidum* and *L. paracasei* for 6 h did not alter TLR3 mRNA levels in the intestinal organoids (Figure 6; Effects of *Bifidobacterium bifidum* and *Lactobacillus paracasei* on Toll-like receptor (TLR) 3 mRNA (*A*) and CXCL1 mRNA upon poly (I:C) stimulation (*B*) in cultured small intestinal organoids). In spite of the presence or absence of *B. bifidum* and *L. paracasei*, poly (I:C) significantly increased CXCL1 mRNA levels (p=0.001 in the absence of bacteria; p=0.027 in the presence of *B. bifidum*; p=0.001 in the presence of *L. paracasei*). The increase of CXCL1 mRNA levels upon poly (I:C) stimulation was significantly lower in the presence of *B. bifidum* and *L. paracasei* than in the absence of bacteria (ANOVA p=0.002). In the absence of poly (I:C) stimulation, *B. bifidum* and *L. paracasei* did not alter CXCL1 mRNA levels.

4. Discussion

dsRNA is a well-characterised rotavirus molecular pattern recognised by the innate immune system. Previous studies have shown that TLR3 recognises rotavirus RNA and intact rotavirus particles (Alexopoulou *et al*., 2001; Sato *et al*., 2006; Zhou *et al*., 2007). We therefore postulated that LGG promotes TLR3-activated antiviral responses through the upregulation of TLR3 expression, resulting in its therapeutic efficacy against rotavirus-associated diarrhoea. As expected, the present study showed that LGG

supplementation upregulated TLR3 gene expression in cultured murine intestinal organoids, an *ex vivo* model for intestinal epithelium. Although statistical significance for the increase in the TLR3 mRNA levels could not be achieved in the murine small intestine 6 h after the single LGG administration, we repeatedly observed the similar results in our preliminary experiments (data not shown). In addition, the present study showed that the 7-day consecutive daily administration of LGG significantly increased the TLR3 mRNA levels in the small intestine. Therefore, we believe that chronic exposure to LGG would be required for stable upregulation of TLR3 gene. It remains to be investigated how quickly TLR3 gene is upregulated following LGG administration and how quickly its effect disappears following cessation of administration. In addition, further studies are necessary to determine the dosage of LGG required for the upregulation of TLR3 gene. A previous clinical study showed that two doses of LGG (10^{10} and 10^{12} CFU) were equally effective to decrease the frequency and duration of rotavirus-associated diarrhoea in children (Basu *et al.*, 2009).

LGG supplementation enhanced poly (I:C)-stimulated gene expression of an antiviral type 1 interferon, IFN-α, and a neutrophil chemokine, CXCL1, in the intestinal organoids. In the absence of poly (I:C) stimulation, however, LGG failed to increase IFN-α and CXCL1 mRNA levels *ex vivo* and *in vivo*. These results suggest that LGG sensitises small intestinal epithelial cells to respond to the TLR3 ligand, at least in part, by the upregulation of TLR3 gene expression. Given that IFN-α and CXCL1 play a role in the protection against rotavirus-associated intestinal mucosa lesions, upregulation of TLR3 gene expression in the small intestinal epithelium would be expected to contribute to the therapeutic efficacy of LGG against rotavirus-associated diarrhoea.

Indeed, Pott *et al*. demonstrated that TLR3 expression levels in the small intestinal epithelium are associated with susceptibility to rotavirus infection (Pott *et al.*, 2012). Because human infants and neonatal mice are highly susceptible to rotavirus infection as compared to adult individuals, the authors of that paper examined TLR3 mRNA levels in the small intestinal

epithelium over the course of postnatal growth in mice. They observed that TLR3 mRNA levels were low in the intestinal epithelium of suckling mice, but that they quickly increased during the postnatal period inversely correlating with rotavirus susceptibility, viral shedding, and histological damage. Likewise, an age-dependent increase in TLR3 mRNA levels was also observed in human small intestinal biopsies. Furthermore, a significantly higher viral shedding rate was seen when TLR3-deficient adult mice were infected with rotavirus. From these findings, Pott *et al*. concluded that upregulation of TLR3 expression might contribute to the age-dependent decrease in susceptibility to rotavirus infection (Pott *et al*., 2012). This previous study supports our idea that upregulation of TLR3 gene expression in the small intestinal epithelium plays a pivotal role in the therapeutic efficacy of LGG against rotavirus-associated diarrhoea.

Although administration of *B. bifidum* reportedly prevented the rotavirus-associated diarrhoea in infants (Saavedra *et al*., 1994), we observed that *B. bifidum* failed to increase TLR3 and poly (I:C)-stimulated CXCL1 gene expression in small intestinal organoids. The results suggest that *B. bifidum* has a different way of working from LGG. Nevertheless, the mechanisms by which LGG promotes TLR3 gene expression remain unclear. TLR2, in concert with TLR1 or TLR6, reportedly recognises various bacterial components, including peptidoglycan, lipopeptide, and lipoprotein of Gram-positive bacteria (Kawai and Akira, 2006). To elicit innate immune responses, Gram-positive bacteria such as LGG need to be recognised via TLR2. Because we failed to detect TLR2 mRNA in the intestinal organoids, however, LGG might stimulate TLR3 gene expression without TLR2 recognition in the intestinal organoids. Further studies will be needed to clarify the molecular mechanisms behind the upregulation of TLR3 gene expression induced by LGG in the intestinal epithelium.

Recent studies have demonstrated that intestinal organoids are a useful *ex vivo* tissue model for investigating intestinal rotavirus infection. It has been reported that intestinal organoids

are susceptible to infection with rotavirus and that rotavirus replication occurs in the intestinal organoids, as evidenced by the detection of nonstructural viral proteins and increased levels of viral RNA (Finkbeiner *et al*., 2012; Foulke-Abel *et al*., 2014; Kovbasnjuk *et al*., 2013; Yin *et al*., 2015). In addition, treatment with antiviral agents, IFN-α and ribavirin, reportedly inhibits rotavirus replication in intestinal organoids (Yin *et al*., 2015). Although we did not perform a rotavirus infection study in the intestinal organoids, we observed that poly (I:C), a synthetic TLR3 ligand, stimulated IFN-α and CXCL1 responses, suggesting that intestinal organoids recapitulate *in vivo* characteristics of antiviral responses to rotavirus molecular patterns in the intestinal epithelium. In the present study, however, cultured intestinal organoids were found to express only TLR1, 3, 5, 6, and 9 mRNAs even though the freshly isolated epithelial cells were found to express TLR1-9 mRNAs. Thus, the TLR mRNA expression profiles were different between the isolated intestinal epithelial cells and the cultured intestinal organoids. In order to make better use of intestinal organoids as an *ex vivo* tissue model for investigating host-pathogen interactions in the intestinal epithelium, the reasons for these discrepancies in the TLR mRNA expression profiles should be clarified. One possibility is that the freshly isolated cells have been *in situ* stimulated to express TLR2, 4, 7 and 8, due to the presence of the endogenous microbiota or other factors in the lumen of the small intestine. The present study found that TLR2, 4, 7 and 8 mRNAs remained undetectable when the organoids were cultured with LGG. Further studies should be performed to explore the factors that stimulate the expression of these TLRs.

Intestinal organoids used in the present study have a three-dimensional closed architecture, with epithelial cells facing their apical surface towards the internal lumen. Because we simply added bacterial strains and synthetic TLR3 ligand to the culture medium of the organoids, these substances should have been exposed inevitably to the basolateral surface of the epithelial cells. In the intestinal epithelial cells, TLR3 is localised to the basolateral plasma membrane as well as endosomal compartments, whereas TLR2 that recognises Gram-positive bacterial components is localised both to the apical and basolateral plasma membranes (Yu

and Gao, 2015). Therefore, on the presumption that the intestinal organoids express TLR2 and 3 as is the case with the small intestine *in vivo*, the organoids could recognise the bacterial strains and synthetic TLR3 ligand through TLR2 and 3 located on the basolateral surface, respectively, even when these substances are added to the culture medium. However, considering that orally ingested probiotics and rotavirus are exposed primarily to the apical surface of the epithelial cells in the intestinal lumen, it would be more natural to apply the bacterial strains and TLR3 ligand to the internal lumen of the intestinal organoids. Recently, Wilson *et al*. reported a model of enteric infection by accessing the apical aspect of the epithelial cells of intestinal organoids using microinjection (Wilson *et al*., 2015). This model would be also useful to study the probiotic actions of beneficial microbes on the intestinal epithelium.

5. Conclusion

The present study revealed that the probiotic LGG increases TLR3 gene expression in murine small intestine *ex vivo* and *in vivo,* and also increases the mRNA levels of an antiviral type 1 IFN, IFN-α, and a neutrophil chemokine, CXCL1, upon stimulation with a synthetic TLR3 ligand, poly (I:C), *ex vivo*. We therefore propose that upregulation of TLR3 gene expression may play a pivotal role in the therapeutic efficacy of LGG against rotavirus-associated diarrhoea. In addition, we demonstrated that intestinal organoids may be a promising *ex vivo* tissue model for investigating host-pathogen interactions and the antiviral action of probiotics in the intestinal epithelium.

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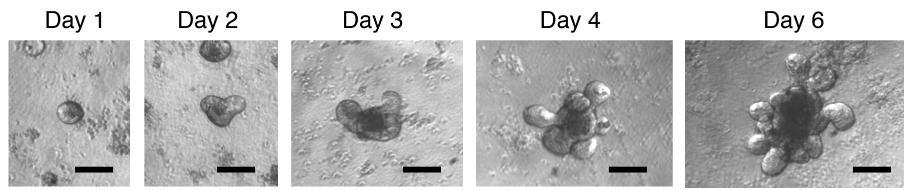

Figure 1 Light-microscopic visualisation of small intestinal organoids. Scale bar indicates 100 μm.

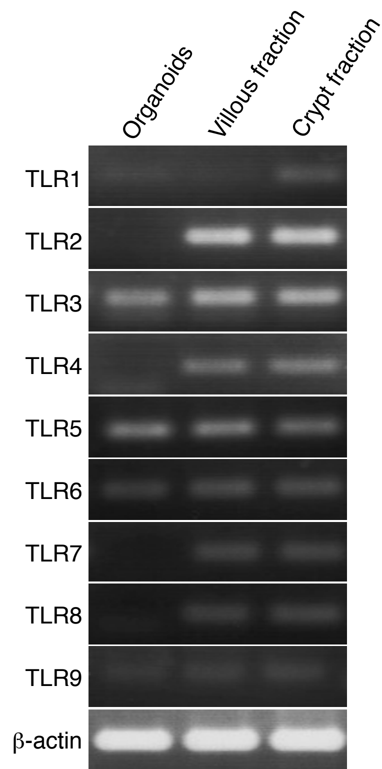

Figure 2 Toll-like receptor (TLR) mRNA expression in isolated small intestinal villous and crypt fractions and cultured small intestinal organoids. β-actin was amplified as a positive control.

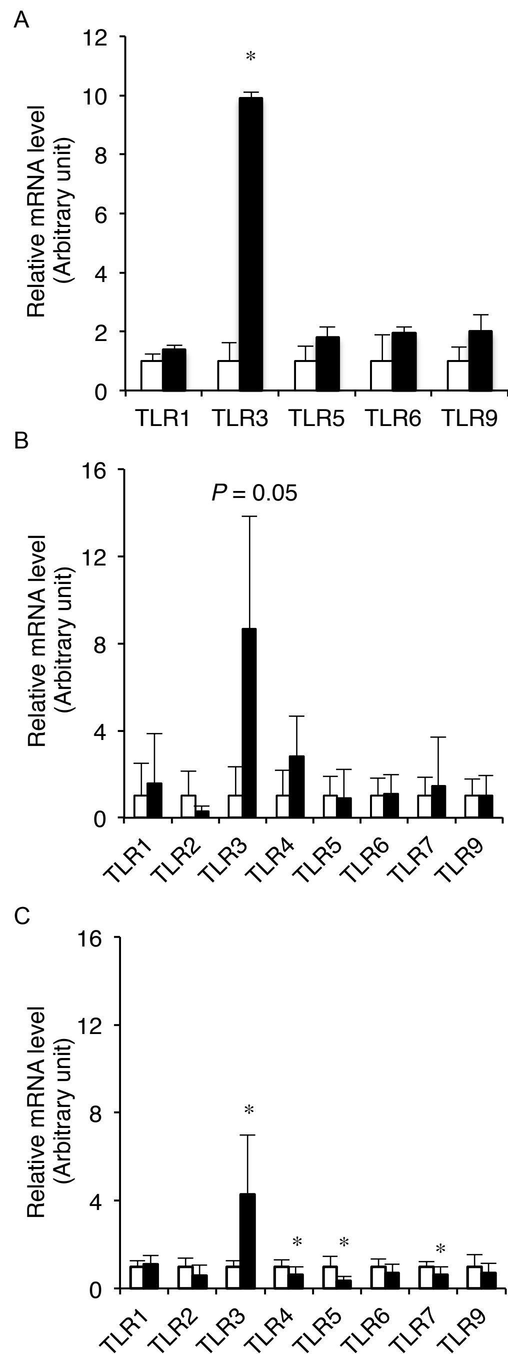

Figure 3 Effect of *Lactobacillus rhamnosus* GG (LGG) on Toll-like receptor (TLR) mRNA levels in cultured small intestinal organoids (*A*) and small intestine of mice with single (*B*) and 7-days (*C*) administrations. White and black bars represent the vehicle- and LGG-treated groups, respectively. Data for the LGG-treated group are shown relative to the levels of the vehicle-treated group, which were set to 1. Data are expressed as the means and SD (n = 3, 3, and 8 in *A*, *B*, and *C*, respectively). *, $p < 0.05$ *vs.* vehicle treatment.

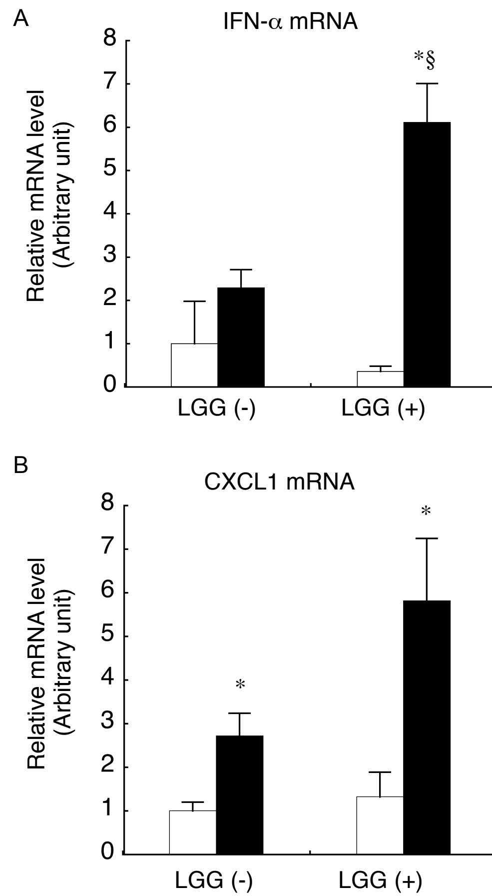

Figure 4 Effects of poly (I:C), a synthetic Toll-like receptor (TLR) 3 ligand, and *Lactobacillus rhamnosus* GG (LGG) on interferon-α (IFN-α) (*A*) and CXCL1 (*B*) mRNA levels in cultured small intestinal organoids. White and black bars represent the groups treated without or with poly (I:C), respectively. Data are shown relative to the levels of the group treated without LGG and poly (I:C), which were set to 1. Data are expressed as the means and SD (n = 3). *, $p < 0.05$ *vs.* without poly (I:C). §, $p < 0.05$ *vs.* without LGG.

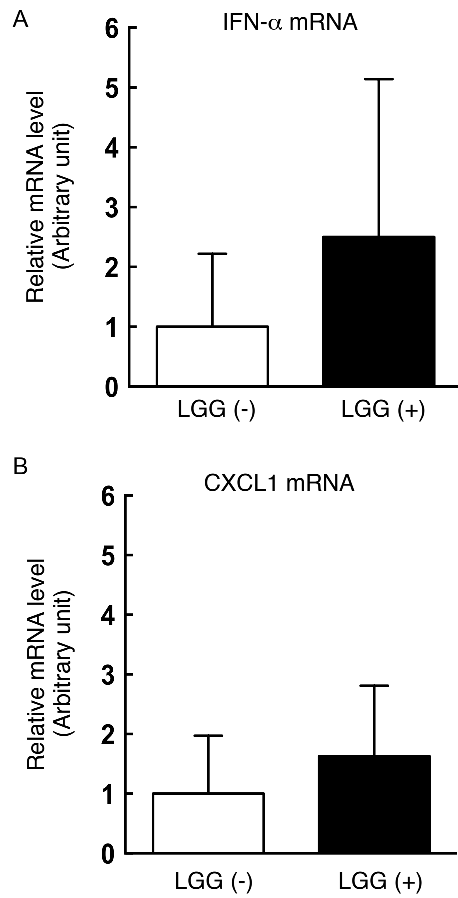

Figure 5 Effects of *Lactobacillus rhamnosus* GG (LGG) on interferon-α (IFN-α) (*A*) and CXCL1 (*B*) mRNA levels in small intestine of mice with 7-days administration. White and black bars represent the vehicle- and LGG-treated groups, respectively. Data for the LGG-treated group are shown relative to the levels of the vehicle-treated group, which were set to 1. Data are expressed as the means and SD (n = 8).

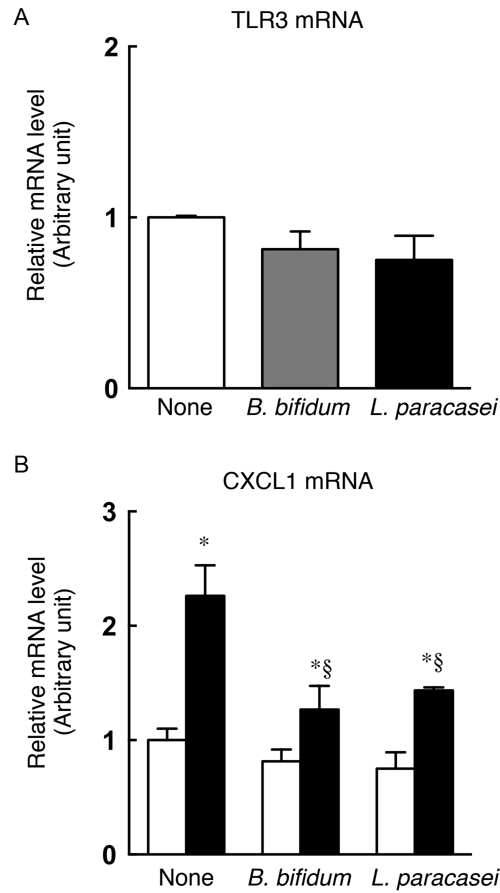

Figure 6 Effects of *Bifidobacterium bifidum* and *Lactobacillus paracasei* on Toll-like receptor (TLR) 3 mRNA (*A*) and CXCL1 mRNA upon poly (I:C) stimulation (*B*) in cultured small intestinal organoids. In chart *B*, white and black bars represent the groups treated without or with poly (I:C), respectively. Data are shown relative to the levels of the group treated without bacterial strains and poly (I:C), which were set to 1. Data are expressed as the means and SD (n = 3). *, $p < 0.05$ *vs.* without poly (I:C). §, $p < 0.05$ *vs.* without bacterial strains.