Inhibitory effects of *Lactobacillus casei* subsp. *rhamnosus* on *Salmonella* lipopolysaccharide-induced inflammation and epithelial barrier dysfunction in a co-culture model using Caco-2/peripheral blood mononuclear cells

Hsu-Wei Fang,1,2† Shiuh-Bin Fang,3† Jen-Shiu Chiang Chiau,4 Chun-Yan Yeung,5,6 Wai-Tao Chan,5 Chuen-Bin Jiang,5 Mei-Lien Cheng4 and Hung-Chang Lee5,7

1Department of Chemical Engineering and Biotechnology, National Taipei University Technology, Taipei, Taiwan, ROC
2Division of Medical Engineering Research, National Health Research Institutes, Miaoli, Taiwan, ROC
3Centre for Paediatric Gastroenterology, Royal Free Campus, University College London Medical School, London, UK
4Department of Medical Research, Mackay Memorial Hospital, Taipei, Taiwan, ROC
5Department of Paediatrics, Mackay Memorial Hospital, Taipei, Taiwan, ROC
6Mackay Medicine, Nursing and Management College, Taipei, Taiwan, ROC
7Department of Paediatrics, Taipei Medical University, Taipei, Taiwan, ROC

In this study, we investigated the anti-inflammatory and reinforcing barrier effects of *Lactobacillus casei* subsp. *rhamnosus* (Lcr35) on Caco-2 intestinal epithelial cells already exposed to *Salmonella* LPS. Using the Transwell co-culture model, *Salmonella* LPS was apically added to polarized Caco-2 cells co-cultured with peripheral blood mononuclear cells (PBMCs) in the basolateral compartment. LPS-stimulated Caco-2 cells were incubated with Lcr35 for 1, 6, 24 or 48 h. Apical inoculation of Lcr35 after 48 h significantly inhibited the basolateral secretion of interleukin-8 (IL-8) in the Caco-2/PBMC co-culture. The PCR analysis showed that Lcr35 significantly downregulated mRNA expression of monocyte chemoattractant protein 1 (MCP-1) (*P* < 0.05) and had a trend of decreasing mRNA expression of IL-8 (*P* = 0.05), but did not alter mRNA expression of transforming growth factor-β1 in LPS-stimulated Caco-2 cells at 48 h after addition of Lcr35. Compared to non-LPS-pretreated controls, transepithelial electrical resistance (TEER) of the polarized Caco-2 cell monolayers pretreated with LPS for 48 h was decreased by 9.9% (*P* < 0.05). Additionally, compared to those cells only treated with LPS, apical co-incubation with Lcr35 showed biphasic TEER levels increased by 12.1% (*P* < 0.001), 5.7% (*P* < 0.05) and 86.8% (*P* < 0.001) in the Caco-2 cell monolayers compared to those without Lcr35 treatment after 1, 6 and 48 h, respectively. In conclusion, Lcr35 can exert anti-inflammatory effects and ameliorate barrier dysfunction in the *Salmonella* LPS-pretreated inflamed intestinal epithelium *in vitro*.

**INTRODUCTION**

The intestinal epithelium maintains a physical barrier to separate the luminal environment from the host milieu, and acts as a guard of the intestinal tract resisting bacteria, pathogens and other antigens (Sierro *et al.*, 2001). Such intestinal barrier integrity can stabilize the entire intestinal...
In the past decade, the clinical implications and immunomodulatory effects of probiotic bacteria, such as lactobacilli, have been increasingly investigated in both clinical trials and experimental models. In clinical practice, commercial probiotics have been used as therapeutic agents to ameliorate diarrhoea. Treatment with *Lactobacillus rhamnosus* strains can shorten the duration of diarrhoea up to 5 days in patients with rotavirus infections (Szymanski *et al.*, 2006). On the other hand, some *in vitro* studies showed that lactobacilli can not only blunt the pro-inflammatory immune response induced by *Salmonella enterica* serotype Typhimurium (S. Typhimurium) in HT-29 human IECs (O’Hara *et al.*, 2006), but also inhibit the synthesis of interleukin-8 (IL-8) in *Salmonella*-stimulated Caco-2 cells (Nemeth *et al.*, 2006). However, long-duration kinetic impacts of probiotic lactobacilli on pro-inflammatory cytokine responses and integrity of intestinal barrier function after pathogenic stimulation in an enterocyte-leukocyte co-culture model has rarely been discussed. Thus, we conducted this study to assess the inhibitory effects of the commercial probiotic *Lactobacillus casei* subsp. *rhamnosus* (Lcr35), which has been used as a probiotic therapeutic agent (Fang *et al.*, 2009), upon *Salmonella* LPS-induced inflammation and epithelial barrier dysfunction in IECs over a 48 h period *in vitro*.

**METHODS**

**Bacterial strain and cultivation.** The commercial strain *Lactobacillus casei* subsp. *rhamnosus*, Lcr35 (Antibiophilus), was used in this study. Lcr35 was cultured in Difco Lactobacilli MRS (de Man–Rogosa–Sharpe) broth (Becton Dickinson) at 37 °C with 5 % CO₂ and used in the early stationary phase.

**Cell culture.** Enterocyte-like Caco-2 cells with the structural and functional characteristics of intestinal cells were developed into differentiated intestinal cells (Koninks *et al.*, 1996). The human Caco-2 cells (passage 33–35) were purchased from the Cell Bank at the Bioresource Collection and Research Centre (Hsinchu, Taiwan), seeded at a density of 1 × 10⁵ cells cm⁻² and grown on 0.9 cm² cell culture inserts in the Transwell system (0.4 μm nucleopore size BD Falcon; Milian). The inserts were pre-coated with collagen (Sigma) and placed into 12-well tissue culture plates before cell seeding. Cells were maintained in Dulbecco’s modified Eagle medium (DMEM, 4500 mg glucose 1⁻¹; Amimed) with 20 % (v/v) fetal bovine serum (FBS) and 1 % (v/v) non-essential amino acids (Sigma). Cell culture medium was changed every 2–3 days until 12–14 days later when full polarization of the Caco-2 cell monolayer was achieved.

**Isolation of PBMCs.** The study protocol and consent documents were approved by the Institutional Review Board of Mackay Memorial Hospital. PBMCs were obtained from healthy donors and provided as buffy coats (Blood Transfusion Centre, Taipei, Taiwan). Leukocytes were freshly purified by Ficoll–Hypaque (Pharmacia) centrifugation (400 g, 20 min) and were collected from the interface. The final PBMCs were stored in RPMI 1640 medium (Sigma), supplemented with 1.0 mM HEPES, 1.0 mM sodium pyruvate, 0.05 mM 2-mercaptopethanol, 10 % (v/v) FBS and adjusted to 3 × 10⁶ cells ml⁻¹.

**LPS stimulation of Caco-2/PBMCs in the co-culture model.** Confluent monolayers of polarized Caco-2 cells were developed in tissue culture inserts and transferred to 12-well tissue culture plates. In the Transwell cell culture system, 1 ml fresh purified PBMCs and 1 ml DMEM, respectively, were added to the basal and apical compartments of the culture plates. The apical surface of Caco-2 cell monolayers was challenged with S. Typhimurium LPS (Sigma), at the optimal concentration of 10 ng ml⁻¹, to activate maximal cytokine tumour necrosis factor alpha (TNF-α) secretion (Ménard *et al.*, 2004). In order to trigger an inflammatory response in the whole co-culture system, the Caco-2 cell monolayers were incubated with LPS at 37 °C with 5 % CO₂ for 48 h (Matsumoto & Benno, 2006). After then, the Caco-2 cells and PBMCs were collected and washed once with cold PBS (Fig. 1). After centrifugation (300 g, 5 min), the washed PBMCs were resuspended in the supplemented RPMI 1640 medium, and then were added back to the original basal compartments in the Transwell plates for further experiments (Fig. 1).

**Probiotic anti-inflammatory effects on intestinal cells in vitro.** To investigate the probiotic anti-inflammatory effects on Caco-2 cells, an inoculum of 2 × 10⁶ c.f.u. Lcr35 ml⁻¹ was added onto the apical side of enterocytes after removal of LPS (Fig. 1). The cell survival was assessed using a 3-(4,5-dimethyl-thiazol-2-y1)-2,5-diphenyltetrazolium bromide assay (Matsumoto & Benno, 2006). Compared to the untreated controls, the relative survival rate of polarized Caco-2 cells treated with Lcr35 was still maintained (>95 %) after 48 h. Caco-2/PBMC co-cultures were incubated for 1, 6, 24 or 48 h. The immune responses of Caco-2 cells and PBMCs upon stimulation with Lcr35 were monitored by analysis of cytokine gene transcription including IL-8, monocyte chemotactant protein 1 (MCP-1) and transforming growth factor-β1 (TGF-β1) using RT-PCR, and by determination of IL-8 secretion in cell culture supernatants using ELISA. In all co-culture experiments, IL-8 protein levels were measured in both the...
Lcr35 on gut inflammation and barrier dysfunction

RESULTS AND DISCUSSION

Salmonella LPS-induced inflammation

Our pilot study demonstrated that IL-8 levels in the apical and basal compartment of Caco-2/PBMCs co-culture system were, respectively, 838 pg ml\(^{-1}\) \((P>0.05)\) and 7 046 562 pg ml\(^{-1}\) \((P<0.001)\) after Salmonella LPS challenge for 48 h, compared to 703 and 6729 pg ml\(^{-1}\), respectively, in non-LPS controls. In the present study, Salmonella LPS caused a significant inflammatory response, which was prolonged as late as 6–24 h after removal of the LPS, in polarized Caco-2 cells by triggering the mRNA expression of three cytokine genes, including IL-8 (0 vs 1, 6 and 24 h, Fig. 2). Both IL-8 gene and its post-transcriptional protein expression validate that Salmonella LPS can induce an inflammation in our Caco-2/PBMCs co-culture system. Previous reports demonstrated that Salmonella (Bolton et al., 2000), or its cell wall components (Sierro et al., 2001), may participate in initiating the mucosal inflammation of Caco-2 cells. LPS interacts with the intestinal epithelium and indirectly induces the inflammatory responses of the lymphoid system. Moreover, LPS has been reported to moderately enhance IL-8 secretion in Caco-2 cells (Chen et al., 2006; Wegljarz et al., 2007). Such an LPS challenge to Caco-2 cells can simulate Salmonella enteric infections in vivo because increased IL-8 secretion triggers recruitment of neutrophils to intestinal epithelium, which is the histopathological hallmark of salmonellosis (McCormick et al., 1993).

IL-8 secretion in the presence of Lcr35

Our preliminary studies indicated that the IL-8 secretion in the apical compartment was lower than that in the basal compartment in the Caco-2/PBMCs co-cultures, and also was negligible in the Caco-2 cells without co-cultured PBMCs (data not shown). In a similar Transwell system, the relevant cytokines were only detectable in the basolateral component (Haller et al., 2001). Therefore, the IL-8 concentration was exclusively measured from the medium in the basolateral compartment. Our data showed that the inhibition of IL-8 levels by Lcr35 was initially apparent at 24 h, and the trend reached its maximum at 48 h (Fig. 3). The IL-8 levels in the medium from Lcr35-treated cells were lower than those from non-Lcr35-treated cells at 48 h \((P<0.05)\).
A putative mechanism of the aforementioned phenomenon is that probiotic bacteria could either bind to cell surface receptors on enterocytes, or stimulate the translocation of dendritic cells or secrete mediators diffusing into the cytosols of enterocytes triggering the related signal transduction pathways (Koninkx et al., 1996). IL-8 secretion can be induced through the nuclear factor-kappa B (NF-κB) pathway after challenge of TNF-α or pathogen-associated molecular patterns (PAMPs), such as LPS and flagellin. The signalling pathway of NF-κB activation after Shigella flexneri infection involves the inhibitory protein, IκBα, which can be upregulated by L. casei to block NF-κB activation in Caco-2 cells (Tien et al., 2006). Probiotic Escherichia coli strain Nissle 1917 suppresses TNF-α-induced IL-8 production in human colonic epithelial cells (Kamada et al., 2008). The secretion of IL-8 in the apical compartment was low in both Lcr35-treated and non-Lcr35-treated cells in our study. After Lcr35 treatment, IL-8 secretion was obviously reduced in the basolateral compartment with time compared to the controls. The immune response in Caco-2 cells could be further enhanced by presence of PBMCs after adequate interaction. Furthermore, the inhibition of IL-8 secretion demonstrated that Lcr35 is able to suppress the inflammatory response in vitro.

mRNA expression in Caco-2/PBMCs co-cultures with Lcr35

Our preliminary experiment confirmed that IL-8 mRNA expression was strongly increased by LPS stimulation for 48 h, accompanied by corresponding increased IL-8 protein secretion (data not shown). In this study, the LPS-stimulated polarized Caco-2 monolayers, treated with or without Lcr35 for different periods of time, showed that the bands of PCR-amplified IL-8, TGF-β1 and MCP-1 were similarly maintained in their density before 24 h, but only MCP-1 obviously decreased after 48 h after inoculation of Lcr35 (Fig. 2). First, mRNA expression of MCP-1 in the
presence of Lcr35 was significantly lower than non-Lcr35 controls at the 48 h time point (P<0.05, Fig. 2). Second, incubation of Caco-2 cells with Lcr35 for 48 h showed a decreasing trend of IL-8 mRNA expression (P=0.05). Third, there was no significant difference in mRNA expression of TGF-β1 between Lcr35-treated cells and non-Lcr35 controls.

In this study, we used a simplified epithelial-immune cell co-culture system to investigate the anti-inflammatory effects of probiotic Lcr35 by analysis of gene expression in the inflamed IECs. The results demonstrated that IL-8, a chemoattractant for neutrophils (Huber et al., 1991), was significantly downregulated by Lcr35 in the inflamed Caco-2 cells after 48 h duration, but not within 24 h. Similarly, Lactobacillus rhamnosus GG can decrease IL-8 production in TNF-α stimulated Caco-2 cells (Zhang et al., 2005). In addition, TGF-β1 is an important inhibitor of the synthesis of pro-inflammatory cytokines (Parlesak et al., 2004). In the present study, Lcr35 did not significantly affect TGF-β1 gene expression in the inflamed Caco-2 cells over 48 h. Hence, TGF-β1 might not play the key role of inhibiting IL-8 secretion of Caco-2/PBMCs after Salmonella LPS challenge. Interestingly, a previous study revealed that Lactobacillus johnsonii stimulated TGF-β1 mRNA expression but Lactobacillus sakei did not in the Caco-2/PBMCs co-culture model (Haller et al., 2000). Thus, various strains of lactobacilli may exert different regulation upon TGF-β1 gene expression. Moreover, inflammatory stimuli can induce MCP-1, which is a potent chemoattractant for monocytes, memory T lymphocytes and natural killer cells (Gu et al., 2000). Probiotic E. coli Nissle 1917 specifically upregulated MCP-1 mRNA expression in Caco-2 cells within 6 h (Ukena et al., 2005); this is similar to our finding of increasing band density of PCR-amplified MCP-1 mRNA within 1 h (Fig. 2). In contrast, L. sakei and non-pathogenic E. coli upregulated MCP-1 gene expression in Caco-2/PBMCs co-cultures at 16 h (Haller et al., 2000). These data suggested that Lcr35 might initially stimulate host defence mechanisms via the expression of MCP-1 at an earlier stage than other probiotic strains. Strain specificity of lactobacilli or co-existence of PBMCs might explain the varied dynamics in upregulation of MCP-1 gene expression. All of these studies indicated that cytokines or chemokines might be secreted from IECs. Therefore, alteration in gene expression of various cytokines or chemokines in Caco-2 cells could result in subsequent variation of individual protein expression and potentially interact with PBMCs to induce appropriate host immune responses.

**Effect of Lcr35 on integrity of Caco-2 monolayer**

In the treatment of intestinal inflammatory disorders it is fundamental to restore and stabilize physical barrier function. Probiotics have been reported to strengthen gut barrier function (Klingberg et al., 2005). During the first 48 h period in our Caco-2/PBMCs co-cultures, TEER decreased from 420.7 to 414.0 Ω cm² in LPS-treated Caco-2 cells but increased from 420.7 to 455.0 Ω cm² in non-LPS-treated cells (P<0.05). To determine the effect of Lcr35 on LPS-induced epithelial barrier dysfunction of Caco-2 cells, TEER was monitored for 48 h and this showed a significant reinforcement by Lcr35 of the TEER of the Caco-2 cell monolayers with time. Compared to the controls, the TEER of the Lcr35-treated cells increased by 12.1% (598.8 vs 534.2 Ω cm², P<0.001) after 1 h and by 5.7% (517.7 vs 489.7 Ω cm², P<0.05) after 6 h. The TEER levels of the Lcr35-treated Caco-2 cells were 86.7% higher than those of the non-Lcr35-treated cells (874 vs 468 Ω cm², P<0.001) at 48 h (Fig. 4). Such a biphasic booster effect of Lcr35 on the TEER of the Caco-2 monolayers appeared firstly at 1 h with an increase of 64.6 Ω cm², followed by another second surge of 406 Ω cm² at 48 h when compared to non-Lcr35-treated cells (Fig. 4). In this time-dependent phenomenon, withdrawal of LPS and replacement of the medium might account for the first peak; competitive consumption of nutrients in the medium by apical Lcr35 and basal PBMCs might temporarily reduce TEER between 1 and 24 h, but accumulation of released probiotic mediators or their influences upon Caco-2/PBMCs co-cultures taking longer (than 24 h) might exceed the above transitory decrease in TEER to form the second higher peak at 48 h. TEER of polarized Caco-2 monolayers exposed to Lactobacillus salivarius DC5 and Lactobacillus plantarum MF1298 also showed a similar trend (Klingberg et al., 2005). Unlike employing probiotic lactobacilli as a post-infectious therapeutic agent in our experiment, in a different study another probiotic mixture, VSL#3, was applied to prevent a pathogen-induced decrease in TEER (Otto & Podolsky, 2004). To sum up, Lcr35 can retain or even enhance the TEER of Caco-2 cell monolayers, which validates its

---

**Fig. 4.** Lcr35 increased TEER levels of the Caco-2 cell monolayers. The cell monolayers were pre-stimulated with *Salmonella* LPS for 48 h, followed by treatment with Lcr35 that reinforced LPS-suppressed transepithelial resistance. TEER values were measured at various time points (1, 6, 24 and 48 h) and the data are expressed as means ± SEM (error bars) of measurements in triplicate. *, P<0.05 indicates significant difference between the Lcr35-treated group and the control group (one-way ANOVA, n=3); #, P<0.001. ■, Non-Lcr35; ▲, Lcr35.
beneficial role as a post-infectious therapeutic agent in the maintenance and reinforcement of intestinal barrier integrity. This finding is supported by the fact that several probiotics and commensal bacteria, including *L. rhamnosus* GG and *Bifidobacterium infantis*, have been shown to stabilize tight junction-associated protein, zonula occludens-1, or increased zonula occludens-1 expression (Ewaschuk et al., 2008; Johnson-Henry et al., 2008) so as to strengthen epithelial barrier function. Thus, judging from the overall results of inhibition in IL-8 secretion, altered gene expression of MCP-1 and enhancement of TEER in the Caco-2 cell monolayers in our study, Lcr35 could post-infectiously inhibit inflammation of enterocytes caused by enteric pathogens through a putative mechanism involving the integrity of the tight junctions between enterocytes. In other words, Lcr35 functions as a barrier-sustaining agent, or barrier-enhancing agent, to protect the breakdown of intestinal epithelium from enteric pathogens.

In conclusion, our study using the Caco-2/PBMCs coculture model clearly demonstrated the pivotal role of Lcr35 in inhibition of the intestinal anti-inflammatory responses and its beneficial effect upon re-establishment of the intestinal epithelial barrier function after inflammation. Moreover, Lcr35 induced the Caco-2 cells to express various pro-inflammatory cytokines at the gene level. Whether Lcr35 could further signal the PBMCs after such gene regulation to mediate the subsequent responses needs further studies for elucidation. Upon the inhibitory effect of probiotic Lcr35, the secretion of IL-8, a major pathogen-elicted epithelial chemooattractant of the inflammatory responses in salmonellosis, was significantly reduced in the basolateral compartment of a co-culture model where PBMCs predominantly resided and mimicked lymphocytes in the lamina propria. PBMCs have been used elsewhere to study the effect of immunocompetent cells on human IECs (Haller et al., 2000; McKay et al., 1996). Hence, our proposed Caco-2/PBMCs co-culture model is not only a useful *in vitro* system for investigating the modulation of probiotics on immune responses and barrier integrity of the gut after enteric infection or inflammation, but also a feasible platform for screening probiotic therapeutic agents for further animal studies and clinical trials.

**ACKNOWLEDGEMENTS**

This study was financially supported by Mackay Memorial Hospital, National Taipei University of Technology (NTUT 97-140-3), and GenMont Biotech Incorporation, Taiwan.

**REFERENCES**


