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Protective effects of lactic acid bacteria on gut epithelial barrier dysfunction are Toll like receptor 2 and protein kinase C dependent

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Lactic acid bacteria (LAB) are recognized for support of host gut homeostasis but the precise mechanisms remain to be identified. LABs interact with Toll-like receptors (TLRs) which might stimulate barrier function of gut epithelial cells. We previously identified six TLR2-signalling LAB strains. As TLR2 is involved in barrier-function enhancement in gut-epithelium, the epithelial barrier-protective effect of these TLR2-signalling strains was studied by using T84 human colorectal cancer cell monolayer as an *in vitro* gut epithelial barrier model. The protein kinase C (PKC) dependent barrier disruptor A23187 and mitogen-activated protein kinase dependent barrier stressor deoxynivalenol were tested to determine which pathways LAB influenced. We found that exclusively the PKC dependent disruption was prevented by the selected TLR2-signalling LAB strains. This study suggests that TLR2 is a pivotal epithelial barrier modulator, and provides novel insight in the molecular mechanisms by which LAB contribute to intestinal health.

1. Introduction

Lactic acid bacteria (LAB) have traditionally served as starter cultures during the manufacturing of fermented foods.¹ Apart from their role in preservation and fermentation of food ingredients, LAB can offer a wide spectrum of health-promoting properties to the host.² It is suggested that functional properties of LAB largely rely on the interplay between LAB and host cells in the gut mucosa, which is majorly mediated by the binding of LAB ligands to Toll like receptors (TLRs) expressed on host cells.³

Cell wall constituents of LAB cells such as teichoic acid and peptidoglycan are the most identified LAB ligands and are recognized by TLR2.³ Subtle variations in the molecular structure of ligands on LAB cells can evoke divergent host responses, which is most likely attributed to differences in ligand-TLR interactions.³ Very subtle differences in molecular structure may have large consequences for the effects on the host. We previously found that D-alanylation of lactobacilli teichoic acid is essential for immunomodulatory functions.⁴ This underscores the necessity of defining functional performance of individual LAB strains to predict their host effects.

Enforcement of intestinal barrier function has been suggested to be one of the health-promoting properties of LAB.² The gut barrier function is formed by intestinal epithelial cells, which are selective permeable for molecules involved in metabolism but prevent entry of luminal deleterious molecules into the lamina propria.⁵ An adequate epithelial barrier function is of paramount importance for maintaining immunological quiescence in the gut, and is governed by the junctional complex network such as tight junction (TJ) which secures the paracellular space between adjacent epithelial cells.⁵ Epithelial barrier defects are closely associated with various intestinal disorders such as inflammatory bowel disease but also with allergy.⁵ TLR2 signalling is an essential player in regulating intestinal epithelial barrier function.⁶ TLR2 activation is reported to elicit dampened gut inflammation *via* restoring TJ barrier integrity.⁷

Through studying the TLR-signalling capacity of a large number of LAB strains, we previously identified six TLR2-signalling strains, *i.e.*, *Lactobacillus* (*L.*) *acidophilus* CCFM137, *L. fermentum* CCFM381, *L. fermentum* CCFM787, *L. plantarum* CCFM634, *L. plantarum* CCFM734, and *Streptococcus* (*S.*) *thermophilus* CCFM218.⁸ Due to the essential role of TLR2 signalling in gut barrier protection, here these selected TLR2-signalling LAB strains were tested for their capacity to prevent disruption of epithelial barrier function by using T84 colonic carcinoma cell monolayer as an *in vitro* model system. We applied

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protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) dependent barrier stressors to determine in which pathways the LAB strains intervene. Our study therefore contributes to understanding of the molecular mechanisms underlying the modulatory functions exerted by LAB.

2. Materials and methods

2.1. Preparation of bacterial samples

Bacterial strains applied in this study (Table 1) were supplied by Culture Collections of Food Microbiology (CCFM), and cultivated as described earlier.⁸ Preparation of bacterial suspension stocks from stationary-phase bacterial culture was performed as previously described.⁸ Bacterial stocks were stored at -80°C until use.

2.2. Cell line

T84 human colorectal carcinoma cell line (Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands) was grown in DMEM/F-12 (1 : 1) (1 $\times$) medium (Life Technologies Europe B. V., Bleiswijk, the Netherlands) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St Louis, MO USA), 25 mM HEPES (Lonza, Verviers, Belgium), and 60 $\mu\text{g ml}^{-1}$ gentamicin sulfate (Lonza, Verviers, Belgium). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere according to the manufacturer's instructions.

2.3. Trans-epithelial electrical resistance (TEER) measurements

Prior to seeding cells, 96-well plates with gold electrodes (96W20idf PET, IBIDI via Applied Biophysics, Germany) were coated as previously described.⁹ Briefly, plates were first coated with 2 mg ml^{-1} L-cysteine (Sigma-Aldrich, St Louis, USA) dissolved in PBS at room temperature for 30 min. After washed with DMEM medium (Lonza, Verviers, Belgium), wells were overnight incubated in DMEM medium containing 0.1% Bovine Serum Albumin (Sigma-Aldrich, St Louis, USA) and 1% PureCol Bovine Collagen Solution (Nutacon, Leimuiden, the Netherlands) at room temperature. At the end of coating, wells were washed twice with complete T84 cell culture medium.

Subsequently, T84 cells were seeded and cultured in pre-coated plates as described earlier.⁹

Before starting stimulation experiments, plates were first installed in the Electric Cell-substrate Impedance Sensing (ECIS) Ztheta instrument (Applied Biophysics) to monitor TEER at multiple frequencies for at least 5 h.¹⁰ Experiments were only performed with cells reaching stable TEER (around 1000 Ohm at 4000 Hz).

2.4. Stimulation of T84 cells

T84 cells were first stimulated with various LAB strains (4×10^6 CFU per well) for 24 h. Afterwards, barrier disruptor calcium ionophore A23187 (Sigma-Aldrich, St Louis, USA) or deoxynivalenol (DON; Sigma-Aldrich, St Louis, USA) was applied in T84 cells to induce epithelial barrier defects. Cells were incubated with A23187 (3 μM) and DON (8.4 μM) in the presence of LAB for another 6 h and 24 h, respectively. Here LAB pretreatment was applied since it was previously found that stimulation of T84 cells with bioactive compounds before disruptor challenge achieved better protection on barrier integrity.¹⁰ TEER was continuously measured with ECIS during the whole treatment period. Untreated cells served as negative control group.

Analysis of TEER measurements at 400 Hz which shows tight junctional resistance started at the moment of disruptor addition.⁹ To quantify the protective effects of LAB against the stressors (A23187 or DON)-elicited T84 barrier injuries, the area under the curve (AUC) was calculated using GraphPad Prism version 6.0 (San Diego, CA, USA). The time point when cells reached the lowest TEER during disruptor challenge was set as the baseline for AUC calculations.⁹

2.5. Statistical analysis

GraphPad Prism version 6.0 (San Diego, CA, USA) was used to conduct statistical analysis. Normal distribution of data was confirmed using the Shapiro-Wilk normality test. Statistical significance was determined using one-way analysis of variance (ANOVA) with Bonferroni multiple comparisons test for *post-hoc* comparison where appropriate. Values of $p < 0.05$ were considered as statistically significant. Data are presented as mean $\pm$ SD. [#],* = $p < 0.05$; ^{##},** = $p < 0.01$; ^{###},*** = $p < 0.001$.

3. Results and discussion

As TLR2 stimulation is essential in modulating gut epithelial barrier function,⁶ we tested the protective potential of the six selected TLR2-signalling strains, *i.e.*, *L. acidophilus* CCFM137, *L. fermentum* CCFM381, *L. fermentum* CCFM787, *L. plantarum* CCFM634, *L. plantarum* CCFM734, and *S. thermophilus* CCFM218⁸ for prevention of epithelial barrier disruption. To this end, T84 human colorectal carcinoma cell monolayers were used here as an *in vitro* model for intestinal epithelial barrier, and were pre-incubated with TLR2-signalling bacteria for 24 h before exposing the epithelial cells to barrier damaging agents. Two barrier disruptors A23187 and DON were applied to evaluate whether LAB-exerted protective effects were

Table 1 Bacterial strains applied in this study

Species	Strain	Source or reference
<i>Lactobacillus acidophilus</i>	CCFM ^a 137	Human feces
<i>Lactobacillus fermentum</i>	CCFM381	Chinese traditional leavened isolate
<i>Lactobacillus fermentum</i>	CCFM787	Not available
<i>Lactobacillus plantarum</i>	CCFM634	Chinese Sichuan pickle; CGMCC ^b 9740
<i>Lactobacillus plantarum</i>	CCFM734	Not available
<i>Streptococcus thermophilus</i>	CCFM218	Kefir

^aCCFM, Culture Collections of Food Microbiology, Jiangnan University, Wuxi, China. ^bCGMCC, China General Microbiological Culture Collection Center, Beijing, China.

specific for certain stressors. Calcium ionophore A23187 was shown to increase tight junction permeability of T84 monolayer through altering intercellular Ca^{2+} levels which is mediated by PKC.¹¹ DON disrupts intestinal barrier function *via* MAPK pathway-involved modulation of tight junction gene expression.¹² AUC of TEER was calculated over a 6 h time period following A23187 addition and over a 24 h period after DON application.

As shown in Fig. 1, following A23187 exposure AUC of TEER was declined to $41.0 \pm 19.0\%$ of untreated control group which was set as 1 ($p < 0.001$ versus untreated control). This could be prevented with the six TLR2-activating strains. *L. acidophilus* CCFM137, *L. fermentum* CCFM381, *L. fermentum* CCFM787, *L. plantarum* CCFM634, *L. plantarum* CCFM734, and *S. thermophilus* CCFM218 effectively prevented A23187-induced TEER loss ($p < 0.001$ versus A23187 group for all listed strains), resulting in AUC values (compared to untreated control) of $99.1 \pm 10.3\%$, $82.8 \pm 26.9\%$, $95.3 \pm 26.0\%$, $79.1 \pm 15.2\%$, $104.0 \pm 8.8\%$, and $101.0 \pm 12.4\%$, respectively (Fig. 1). DON stimulation also resulted in significant decrease in barrier integrity of T84 monolayers, which was reflected by significantly reduced AUC in DON treatment group ($p < 0.001$ versus untreated control; Fig. 2). Here however we found no preventive effects with pretreatment of any of the tested LAB strains when compared with DON treatment group.

A23187 treatment of T84 cells causes a significant TEER drop without affecting F-actin distribution in cytoskeletal structure and contacts between adjacent cells.¹¹ A23187 causes

Fig. 1 LAB strains suppressed A23187-triggered barrier function defects in T84 cells. T84 cells were pre-stimulated with different LAB strains (4×10^6 CFU per well) for 24 h, after which barrier disruptor A23187 ($3 \mu\text{M}$) was applied in T84 cells for another 6 h. AUC was calculated over the A23187 treatment period. AUC data are presented as percentage of untreated control group. Results shown represent mean and SD of 3 independent experiments. Statistical significance was tested by using one-way ANOVA with Bonferroni multiple comparisons test (* versus untreated control; # versus A23187 treatment group; ** = $p < 0.05$; ### = $p < 0.01$; ###.*** = $p < 0.001$).

Fig. 2 LAB strains could not protect T84 epithelial cells against DON-induced barrier damage. T84 cells were pre-stimulated with different LAB strains (4×10^6 CFU per well) for 24 h, after which barrier damaging agent DON ($8.4 \mu\text{M}$) was applied in T84 cells for another 24 h. AUC was calculated over the DON treatment period. AUC data are presented as percentage of untreated control group. Results shown represent mean and SD of 3 independent experiments. Statistical significance was tested by using one-way ANOVA with Bonferroni multiple comparisons test (* versus untreated control; # versus DON treatment group; ** = $p < 0.05$; ### = $p < 0.01$; ###.*** = $p < 0.001$).

barrier dysfunction in T84 cells *via* increasing intracellular Ca^{2+} concentration, but its barrier-disruptive effect does not depend on calmodulin and phospholipase pathways.¹¹ The PKC family plays a vital role in epithelial barrier regulation,¹³ and is known to be involved in A23187-induced epithelial barrier damage in T84 cells.¹¹ PKC isoforms can influence epithelial barrier function by regulating ion secretion, TJ-related structure, cytoskeletal organization, epithelial cell proliferation, and cell apoptosis.¹³ Notably, distinct PKC isoforms exert differential effects on barrier function.¹³ Moreover, the role of individual PKC isoforms in barrier regulation are largely dependent on the cell line applied, the interactions between different PKC isoforms, and the stressor applied. For instance, bryostatin-1 protected T84 cells from $\text{TNF}\alpha$ -induced barrier damage *via* activating $\text{PKC}\delta$ and $\text{PKC}\epsilon$,¹⁴ while in Caco2 cells $\text{PKC}\delta$ activation mediated hydrogen peroxide-induced barrier disruption.¹⁵ In contrast to the latter, another study showed that in Caco2 cells prebiotic fibers-induced $\text{PKC}\delta$ activation enhanced barrier function during pathogen challenge.¹⁶

Even though A23187-involved PKC isoforms remain to be identified, it seems plausible that calcium-dependent conventional PKC isozymes are associated with the barrier-disruptive effects of A23187 in T84 cells.¹¹ Despite the cell line-dependency of $\text{PKC}\alpha$ and $\text{PKC}\beta$, in T84 cells these two PKC isozymes were shown to induce barrier damage whereas in Caco2 cells they contributed to barrier protection.^{13,17,18} Therefore, conventional PKC isoforms $\text{PKC}\alpha$ and $\text{PKC}\beta$ are most likely involved in A23187-induced loss of barrier function in T84

cells. Here we showed that TLR2-signalling bacterial strains specifically inhibited A23187-induced TEER reduction. Our finding corroborates a previous study showing that TLR2 signalling receptor Mal regulated epithelial barrier function of T84 cells *via* directly interacting with atypical PKC isoform PKC ζ .¹⁹ Moreover, PKC δ and PKC ϵ activation mediated bryostatin-1-conferred barrier protective effects during TNF α challenge in T84 cells.¹⁴ Therefore, the tested TLR2-stimulating bacteria might attenuate A23187-induced barrier disruption *via* acting on atypical PKC isoform such as PKC ζ or other PKC isoforms such as PKC δ and PKC ϵ , which might counteract the effects of A23187-associated conventional PKC isoforms as discussed above.

DON, a mycotoxin commonly found in cereal products, induces epithelial barrier damage and inflammation *via* acting on MAPK pathway.¹² DON can also induce cell apoptosis and influence cell differentiation.¹² Similar to PKC, MAPK pathway exerts bidirectional effects on intestinal barrier function.²⁰ Among various MAPK subfamilies including c-Jun-N-terminal kinase, extracellular signal-regulated kinase (ERK), and p38, ERK has been mostly recognized to participate in epithelial barrier regulation.²¹ In T84 cells ERK was shown to mediate TGF- β -induced enhancement of barrier integrity,²² while other studies showed that ERK activation in T84 cells contributed to IL-22 or pathogen infection induced barrier disruption.^{23,24} The differential effects of ERK activation on barrier function of T84 cells might be due to the contributions of other involved signalling pathways such as SMAD and PI3 K.^{22,23} Although DON-associated MAPK subfamilies in T84 cells need to be further defined, p44/22 ERK activation was confirmed to mediate DON-induced TJ barrier dysfunction in intestinal porcine epithelial cell lines.^{21,25}

In the current study our strains of different species were observed to confer barrier protection in A23187-mediated barrier damage model. Consistent with our finding, TLR2 activation induced by either probiotic *L. plantarum* WCFS1 or TLR2 ligands derived from bacterial cell wall components was found to strengthen epithelial barrier function in Caco2 cells.^{18,26} Although structural components of bacterial cell wall such as peptidoglycan and teichoic acid have been widely recognized as TLR2 ligands in Gram-positive bacteria,³ the precise effector molecules of individual bacterial strains that serve as TLR2 ligands and mediate the barrier protection are not completely clear. It therefore requires more systematic studies such as applying isogenic mutant bacterial strains deficient in particular structural components to elucidate specific bacterial cell components involved in epithelial barrier modulation. This will provide deeper insights for the molecular mechanisms by which LAB strains confer barrier enhancement.

4. Conclusions

In this study, we demonstrated the efficacy of TLR2-signalling LAB strains in the prevention of epithelial barrier integrity loss

in T84 human colon cancer cells triggered by the PKC-dependent barrier disruptor A23187. LAB was ineffective in abrogating MAPK-induced barrier disruption. Our results corroborate the notion that TLR2 is a pivotal epithelial barrier modulator,⁶ and provide novel insight in the molecular mechanisms by which LAB strains can contribute to intestinal health. Our technology platform of testing TLR signalling LABs combined with barrier dysfunction studies *in vitro* is an effective approach to identify possible LAB strains with beneficial host effects.

Abbreviations

AUC	Area under the curve
DON	Deoxynivalenol
ECIS	Electric cell substrate impedance sensing
ERK	Extracellular signal-regulated kinase
LAB	Lactic acid bacteria
MAPK	Mitogen-activated protein kinase
PKC	Protein kinase C
TEER	Trans-epithelial electrical resistance
TJ	Tight junction
TLR	Toll-like receptor

Author contributions

C. R. and P. d. V. conceived and designed the experiments. C. R. performed the experiments and analysed data. C. R., Q. Z., B. J. d. H., H. Z., M. M. F., and P. d. V. wrote the paper.

Conflicts of interest

There are no conflicts of interest to declare.

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