RESEARCH ARTICLE

A Selected Lactobacillus rhamnosus Strain Promotes EGFR-Independent Akt Activation in an Enterotoxigenic Escherichia coli K88-Infected IPEC-J2 Cell Model

Wei Zhang*, Yao-Hong Zhu*, Jin-Cai Yang, Gui-Yan Yang, Dong Zhou, Jiu-Feng Wang*

Department of Veterinary Clinical Sciences, College of Veterinary Medicine, China Agricultural University, Beijing, China

These authors contributed equally to this work.
* jiufeng_wang@hotmail.com

Abstract

Enterotoxigenic Escherichia coli (ETEC) are important intestinal pathogens that cause diarrhea in humans and animals. Although probiotic bacteria may protect against ETEC-induced enteric infections, the underlying mechanisms are unknown. In this study, porcine intestinal epithelial J2 cells (IPEC-J2) were pre-incubated with and without Lactobacillus rhamnosus ATCC 7469 and then exposed to F4+ ETEC. Increases in TLR4 and NOD2 mRNA expression were observed at 3 h after F4+ ETEC challenge, but these increases were attenuated by L. rhamnosus treatment. Expression of TLR2 and NOD1 mRNA was upregulated in cells pre-treated with L. rhamnosus. Pre-treatment with L. rhamnosus counteracted F4+ ETEC-induced increases in TNF-α concentration. Increased PGE2 concentrations were observed in cells infected with F4+ ETEC and in cells treated with L. rhamnosus only. A decrease in phosphorylated epidermal growth factor receptor (EGFR) was observed at 3 h after F4+ ETEC challenge in cells treated with L. rhamnosus. Pre-treatment with L. rhamnosus enhanced Akt phosphorylation and increased ZO-1 and occludin protein expression. Our findings suggest that L. rhamnosus protects intestinal epithelial cells from F4+ ETEC-induced damage, partly through the anti-inflammatory response involving synergism between TLR2 and NOD1. In addition, L. rhamnosus promotes EGFR-independent Akt activation, which may activate intestinal epithelial cells in response to bacterial infection, in turn increasing tight junction integrity and thus enhancing the barrier function and restricting pathogen invasion. Pre-incubation with L. rhamnosus was superior to co-incubation in reducing the adhesion of F4+ ETEC to IPEC-J2 cells and subsequently attenuating F4+ ETEC-induced mucin layer destruction and suppressing apoptosis. Our data indicate that a selected L. rhamnosus strain interacts with porcine intestinal epithelial cells to maintain the epithelial barrier and promote intestinal epithelial cell activation in response to bacterial infection, thus protecting cells from the deleterious effects of F4+ ETEC.
Introduction

Enterotoxigenic *Escherichia coli* (ETEC) strains are not only the most common cause of travelers’ diarrhea, which can be fatal for children under 5 years of age, they are also the leading cause of post-weaning diarrhea (PWD) in piglets [1,2]. The most prevalent ETEC strain implicated in PWD in piglets expresses F4 (K88)+ fimbriae. Our previous studies have shown that administration of *Lactobacillus rhamnosus* ameliorates F4+ ETEC-induced diarrhea in newly weaned piglets; however, pre-treatment with high doses of *L. rhamnosus* may negate the preventative effects [3–5]. Accumulating evidence suggests that the beneficial effects of *Lactobacillus* strains may be due to their ability to restore the normal microbiota, inhibit pathogen adhesion to the intestinal wall, and maintain the membrane barrier [6–8]. However, the exact mode of action of lactobacilli in this regard remains largely unknown.

Intestinal epithelial cells (IECs) comprise the largest and most important anatomic as well as immunologic barrier against external environmental stimuli. The mucus layer coating the IECs serves as the first line of intestinal defense against infection by physically protecting the cells from direct exposure to bacteria and other antigens [9]. ETEC are capable of gaining access to enterocytes in the small intestine through EatA-induced degradation of MUC2 [10].

Two types of pattern recognition receptors (PRRs), the membrane-bound Toll-like receptors (TLRs) and the cytoplasmic Nod-like receptors (NLRs), provide complementary pathogen surveillance [11]. In general, binding of pathogens to TLRs or NLRs activates nuclear factor-kB (NF-kB) signaling and leads to the production of pro-inflammatory cytokines, chemokines, and antimicrobial peptides, thereby contributing to host defense and inflammation [12]. In addition, various PRRs are involved in regulating intestinal epithelial barrier integrity. Lipopolysaccharide (LPS) increases intestinal tight junction (TJ) permeability both *in vitro* and *in vivo* by inducing enterocyte membrane expression of TLR4 and CD14 [13]. Activation of the phosphatidylinositol-3-kinase (PI3K) pathway as a result of TLR2 signaling strengthens the TJ-associated epithelial barrier [14]. To date, knowledge regarding the mechanism underlying probiotic modulation of the intestinal barrier remains limited, however.

The epithelium maintains its selective barrier function through TJs that mechanically link adjacent cells and seal the intercellular space. The primary proteins thus far identified as TJ-specific integral transmembrane proteins include occludin and the claudins. In addition, the zonula occludens (ZO) may act as a link between the cytoskeleton and other TJ proteins [15]. It has been shown that *L. rhamnosus* GG (LGG, ATCC 53103) promotes expression of ZO-1 and occludin in Caco-2 cells [8,16]. In a piglet diarrhea model, *L. plantarum* inhibited ETEC K88-induced decreases in occludin mRNA and protein levels in the jejunum [17].

Epidermal growth factor receptor (EGFR) signaling is involved in regulating cellular proliferation, differentiation, and survival. Ligation of EGFR by its soluble ligands (EGF, heparin-binding-EGF, transforming growth factor, or amphiregulin) triggers the formation of homodimeric and hetero-dimers with other ErbB family members and the tyrosine auto-phosphorylation of several cytoplasmic proteins [18]. The indirect recruitment of PI3K to tyrosine-phosphorylated EGFR activates the downstream target Akt [19]. A recent study showed that the probiotic LGG transactivates EGFR, leading to suppression of apoptosis of mouse IECs induced by the cytokines TNF-α, IL-1α, and IFN-γ [20]. In a mouse model of colitis induced by 2,4,6-trinitrobenzene sulfonic acid, hirsutone-mediated prevention of down-regulation of ZO-1 and occludin mRNA expression was shown to depend on part on activation of the EGFR/Akt signaling pathway [21]. However, it remains unclear whether *Lactobacillus* mediates this effect and the inhibition of ETEC infection via activation of EGFR and its downstream targets.

In this study, we hypothesized that probiotic *L. rhamnosus* ATCC 7469 regulates the inflammatory response of porcine intestinal epithelial J2 (IPEC-J2) cells and aids in maintaining the...
intestinal barrier through modulating TLR/NLR cooperation and EGFR/Akt signaling to pro-
tect IECs from the deleterious effects of ETEC infection. The aim of this study therefore was to
investigate the effects of Lactobacillus administration on IEC physiology. Our goal is to provide
a rationale for the use of probiotics as therapeutic and preventative agents, at least for infectious
diarrhea, and perhaps also for other diseases associated with mucosal inflammation.

Materials and Methods

Cell line and culture conditions

The porcine intestinal epithelial J2 cell line (IPEC-J2, ACC701, DSMZ) was kindly supplied by
Prof. Yanming Zhang of the Northwest A & F University in China. Cells were continuously
maintained in culture. The IPEC-J2 cells used in this study represented non-transformed, po-
larized-growing porcine jejunal epithelial cells and were isolated from a neonatal, unsuckled
piglet.

IPEC-J2 cells were cultured in Dulbecco’s Modified Eagle medium/Ham’s F-12 (1:1) medi-
um supplemented with 10% heat-inactivated fetal calf serum (FCS) (Invitrogen, Carlsbad, CA)
at 37°C in an atmosphere of 5% CO₂ and 95% air at 95% relative humidity. For bacteria-free
assays, an antibiotic mixture (100 U/ml penicillin and 100 μg/ml streptomycin; Invitrogen) was
added to the culture medium. Undifferentiated cells reached confluence after 1–2 days. The
IPEC-J2 cells were subcultured with PBS containing 0.25% trypsin and 0.5 mM EDTA (Invitro-
gen). For assays described below, IPEC-J2 cells were grown on transwell filters and cultured for
10 d after reaching confluence in medium without FCS to allow for differentiation. Under
these culture conditions, the IPEC-J2 cells differentiated and exhibited enterocytic features, in-
cluding microvilli and TJ’s, when grown on transwell filters. Medium was changed 3 times
per week.

Bacterial strains

Lactobacillus rhamnosus ATCC 7469 was purchased from the Chinese General Microorganism
Culture Collection and grown in De Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Hamp-
shire, UK) for 24 h at 37°C under microaerophilic conditions. After overnight incubation, bac-
teria were diluted 1:100 in fresh MRS broth and grown for about 8 h until reaching mid-log
phase, for all experiments.

The Escherichia coli F4-expressing strain (serotype O149:K91, K88ac) was obtained from
the China Veterinary Culture Collection Center and grown in Luria-Bertani (LB) broth
(Oxoid, Basingstoke, England). After overnight incubation at 37°C with vigorous shaking, bac-
teria were diluted 1:100 in fresh LB and grown for about 3 h until reaching mid-log phase.

Bacterial adhesion assay

IPEC-J2 cells (5 × 10⁵ cells per well) were seeded onto a 6-well transwell collagen-coated PTFE
filter (pore size 0.4 μm; 4.7 cm²; Corning, Corning City, NY). At day 10, confluent monolayers
of cells cultured in medium supplemented with porcine mucin (0.5 mg/ml; Sigma-Aldrich,
Saint Louis, MO) and without were treated under one of three conditions, as follows: (i) F4+
ETEC (10⁷ colony forming units [CFU]/ml) infection alone; (ii) simultaneous incubation with
1 ml of medium containing L. rhamnosus (10⁸ CFU/ml) and F4+ ETEC (10⁷ CFU/ml) infec-
tion; and (iii) pre-incubation with 1 ml of medium containing L. rhamnosus (10⁸ CFU/ml) for
2 h prior to addition of F4+ ETEC (10⁷ CFU/ml). We chose the bacterial concentration and
time of incubation based on preliminary experiments to allow for bacterial adhesion and
membrane damage without disruption of the cell monolayers. At 3 h following F4⁺ ETEC (10⁷ CFU/ml) challenge, the number of F4⁺ ETEC CFU recovered was determined. After incubation, cells were washed with PBS, lysed, and homogenized with 0.1% (v/v) Triton X-100 (Sigma-Aldrich) in ddH₂O and plated on LB agar after serial dilution. Plates were incubated overnight at 37°C, after which the number of CFU was determined. Preliminary experiments confirmed that L. rhamnosus did not form colonies after overnight incubation on LB agar at 37°C under aerobic conditions.

To assay competitive adhesion to mucin, 300 μl of porcine mucin (0.5 mg/ml) in sterile PBS was immobilized passively on Maxisorp microtiter plate wells (Nunc, Roskilde, Denmark) by overnight incubation at 4°C. Wells were then washed twice with PBS to remove unbound mucin. The densities of L. rhamnosus (10⁶ CFU/ml) and F4⁺ ETEC (10⁷ CFU/ml) were adjusted using sterile PBS. Next, 200 μl of a culture of each strain was added to wells coated with mucin as described above and allowed to adhere for 3 h at 37°C. Non-adhering bacteria were then withdrawn, and the wells were washed five times with 300 μl of sterile PBS. Adhered F4⁺ ETEC were released using 300 μl of 0.1% (v/v) Triton X-100 and then enumerated on LB agar.

Mucin production
IPEC-J2 cells (10⁶ cells per filter) differentiated on a 6-well transwell collagen-coated PTFE filter were treated under one of five conditions, as follows: (i) medium; (ii) F4⁺ ETEC (10⁷ CFU/ml) infection alone; (iii) L. rhamnosus (10⁶ CFU/ml) incubation alone; (iv) simultaneous incubation with 1 ml of medium containing L. rhamnosus (10⁶ CFU/ml) and F4⁺ ETEC (10⁷ CFU/ml) infection; and (v) pre-incubation with 1 ml of medium containing L. rhamnosus (10⁶ CFU/ml) for 2 h prior to addition of F4⁺ ETEC (10⁷ CFU/ml). At 3 h after F4⁺ ETEC challenge, IPEC-J2 cells were harvested and fixed with 4% paraformaldehyde at 4°C for 20 min. Acidic mucopolysaccharides were stained with Alcian Blue (AB) at pH 2.5, and neutral mucopolysaccharides were visualized using the periodic acid-Schiff (PAS) reaction (Luoji Biotech, Beijing, China), as described by the manufacturer. AB stains acidic glycoproteins blue and PAS stains neutral glycoproteins pink, whereas mixtures of neutral and acidic mucin glycoproteins appear purple. For semi-quantitative determination of mucin (purple) production, digital images were analyzed using Image Pro Plus 6.0 software (Media Cybernetics, Rockville, MD), allowing quantification of mucin levels as the mean integrated optical density (IOD). Results are presented as the ratio of purple mucin IOD to blue mucin IOD.

Apoptosis assay
Apoptosis of IPEC-J2 cells was assessed using an apoptosis kit with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) for flow cytometry (Invitrogen). At 3 h after F4⁺ ETEC challenge, IPEC-J2 cells were harvested, washed in pre-chilled PBS, and stained with FITC-conjugated annexin V (5 μl) and PI (1 μl) in succession for 10 min at room temperature. Appropriate single-labeled and unlabeled controls were used. After filtering through a 70-μm nylon cell strainer (BD Biosciences, San Jose, CA), cells were assessed for fluorescence using a FACScalibur flow cytometer (BD Biosciences) equipped with FlowJo software. The percentages of early apoptotic (annexin-FITC-positive/PI-negative) and late apoptotic (annexin-FITC/PI-double positive) cells were determined.

Western blotting
At 3 h after F4⁺ ETEC challenge, IPEC-J2 cells were lysed in 0.5 ml of cold RIPA buffer (150 mM sodium chloride, 1.0% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl, pH 8.0) supplemented with complete protease inhibitors.
(104 mM AEBSF, 80 μM aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin, and 1.5 mM pepstatin A) (Sigma-Aldrich). The scraped cell suspensions were centrifuged at 10,000 × g for 15 min at 4°C to remove insoluble debris, and the supernatant was used for Western blot analysis. Protein concentrations were determined using the Bio-Rad DC protein assay kit II (Bio-Rad Laboratories, Hercules, CA). The primary antibodies were rabbit anti-ZO-1 (ab59720), rabbit anti-occludin (ab31721), mouse anti heat shock protein 72 ([Hsp72], ab2787; Abcam, Cambridge, UK), anti-phospho-Ser473 (p)-Akt (ab138726), rabbit anti-phospho-Tyr124 (p)-PKCα (ab32376), rabbit anti-phospho-Tyr1068 (p)-EGFR (ab32430; Epitomics, Burlingame, CA), rabbit anti-total-EGFR (18986-1-AP), anti-total-Akt (10176-2-AP), and mouse anti- glyceraldehyde-3-phosphate dehydrogenase ([GAPDH], 60004-1-Ig) (Proteintech Group, Chicago, IL). Horseradish peroxidase (HRP)-conjugated affinipure goat anti-mouse IgG (H+L) (SA00001-1) or goat anti-rabbit IgG (H+L) (SA00001-2; Proteintech Group) were used as secondary antibodies. GAPDH served as an internal control and exhibited stable expression regardless of treatment. The optical density (OD) of each band was quantified by densitometric analysis using Quantity One software (Bio-Rad Laboratories). Results are presented as the ratio of the p-EGFR or p-Akt band intensity to the total EGFR or total Akt band intensity, respectively, and the ratio of the p-PKCα, ZO-1, and occludin band intensity to the GAPDH band intensity.

Quantitative real-time PCR

IPEC-J2 cells were collected at 3 h after F4+ ETEC challenge. Total RNA was extracted from the cells using Trizol reagent (Invitrogen). The integrity of extracted RNA was confirmed on agarose gel electrophoresis by staining with ethidium bromide and visualization under UV light. The quantity and purity (OD260/OD280 absorption ratio >1.9) of RNA was determined using a NanoDrop ND-2000C spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). A 2-μg aliquot of total RNA was reverse-transcribed into cDNA with 200 U of M-MLV using 1 μg of oligo (dT)15 primer, 10 mM dNTP mix, M-MLV 5x reaction buffer, and 25 U of rRNasin ribonuclease inhibitor (Promega, Madison, WI) in a final volume of 25 μL. To detect DNA contamination, a negative control (without enzyme) was included. Synthesized cDNA was stored at −20°C prior to real-time PCR analysis.

Quantitative real-time PCR was performed using an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA). Primer sequences are listed in Table 1. The cDNA was amplified in triplicate with SYBR Premix DimerEraser (TakaRa Biototechnology Inc., Shiga, Japan). A non-template control of nuclease-free water was included in each run.

Relative quantification of mRNA expression was assessed by normalizing the cycle threshold (Ct) values of the target genes to the Ct values of the housekeeping gene encoding GAPDH. The results are presented as fold change using the 2−ΔΔCt method. The relative mRNA expression of target genes in each group was calculated using the following equations:

\[
\Delta C_T = C_T (\text{target gene}) - C_T (\text{GAPDH})
\]

or

\[
\Delta \Delta C_T = \Delta C_T (\text{treated group}) - \Delta C_T (\text{control group})
\]

ELISA

The concentrations of IL-10, TNF-α, and PGE2 in supernatants from IPEC-J2 cell cultures at 3 h after F4+ ETEC challenge were determined using commercially available ELISA kits specific for porcine TNF-α, porcine IL-10 (R&D Systems, Minneapolis, MN), and porcine PGE2 (Cayman Chemical Co., Ann Arbor, MI).
Statistical analysis

Statistical evaluations were performed using the SAS statistical software package, version 9.1 (SAS Institute Inc., Cary, NC). Data were also evaluated using ANOVA procedures. With regard to small sample sizes, normal distribution and homogeneity of variance were assumed with the UNIVARIATE (Shapiro-Wilk test) and HOVTEST procedures. Natural logarithm transformation was performed prior to analysis for IL-10 and PGE2 ELISA data to yield a normal distribution. Differences between means were compared using Tukey’s honestly significant difference (HSD) post hoc test. Data were visualized using GraphPad Prism 5 software (Graphpad Software Inc., San Diego, CA). A *P*-value of < 0.05 was considered indicative of statistical significance. All experiments were performed three times.

Results

Pre-incubation with *L. rhamnosus* reduced the adhesion of F4+ ETEC

To investigate the effect of *L. rhamnosus* on adhesion of F4+ ETEC to IPEC-J2 cells and *L. rhamnosus* competition with mucin, we examined the adhesion of F4+ ETEC to IPEC-J2 cells in the absence or presence of mucin coated in microtiter plate wells (Fig 1A and 1B).

In both the absence and presence of mucin, fewer F4+ ETEC CFU were recovered from IPEC-J2 cells pre-incubated with *L. rhamnosus* as compared with IPEC-J2 cells only infected with F4+ ETEC (*P* = 0.003 and *P* = 0.034, respectively, Fig 1A). The number of F4+ ETEC CFU recovered from IPEC-J2 cells pre-incubated with *L. rhamnosus* was lower compared with IPEC-J2 cells co-incubated with *L. rhamnosus* in the absence of mucin (*P* = 0.031). Mucin addition resulted in a decrease in the number of F4+ ETEC CFU recovered from IPEC-J2 cells infected with F4+ ETEC, co-incubated with *L. rhamnosus*, and pre-incubated with *L. rhamnosus* (*P* = 0.008, *P* = 0.006, and *P* = 0.039, respectively). Furthermore, fewer CFU were recovered from IPEC-J2 cells co- or pre-incubated with *L. rhamnosus* in the presence of mucin than from cells only infected with F4+ ETEC (*P* < 0.001). Also, fewer CFU were recovered from IPEC-J2 cells infected with F4+ ETEC or pre-incubated with *L. rhamnosus* in the presence of porcine mucin.

Table 1. Sequences of oligonucleotide primers used for real-time PCR, length of the respective PCR product, and gene accession number.

| Gene product | Primer | Product size (bp) | Accession number |
|--------------|--------|------------------|-----------------|
| **GADPH** | F | CCAGAACATCATCCCTGCTT | 229 | NM_001206359 |
| | R | GTCTTCAGTGTGACCCCGGA |  |  |
| **TLR2** | F | TCATTGTCTAACTTATCATCCTGT | 162 | XM_005653579 |
| | R | TCAGCGAAGGTGTCATTGC |  |  |
| **TLR4** | F | GGCATCGTGGCTAACATC | 108 | NM_001113039 |
| | R | CTGCACTCAGATACCCATCG |  |  |
| **TLR9** | F | GTGGAACGTGTTTGGCATC | 199 | NM_213958 |
| | R | CACAGCCTCTGAGCTTTGT |  |  |
| **NOD1** | F | ACCGATCCAGTGAGCAGATA | 140 | NM_001114277 |
| | R | AAGTCCAAGCTGTAATGAT |  |  |
| **NOD2** | F | GAGCGCATCCTTCAATTTGC | 66 | NM_001105295 |
| | R | AGCTCCTGATCCTTGAAC |  |  |

*aGADPH = glyceraldehyde-3-phosphate dehydrogenase; TLR = toll-like receptor; NOD = nucleotide-binding oligomerization domain.

b F = forward; R = reverse.

DOI:10.1371/journal.pone.0125717.t001
mucin than from cells co-incubated with *L. rhamnosus* in the absence of mucin (*P* = 0.043 and *P* < 0.001, respectively).

In the assay of competitive adhesion to mucin, pre-incubation with *L. rhamnosus* resulted in a decrease in the number of CFU recovered compared with F4+ ETEC challenge alone or co-incubation with *L. rhamnosus* (*P* < 0.001 and *P* = 0.005, respectively; Fig 1B).

**Effect of *L. rhamnosus* on the mucin layer**

Mucin glycoproteins are the main components of the first barrier that bacteria encounter in the intestinal tract. The effect of *L. rhamnosus* on the mucin layer in IPEC-J2 cells with and without F4+ ETEC infection was evaluated using AB-PAS staining (Fig 2). Untreated IPEC-J2 control cells were lined by a continuous, homogeneous, purple mucin layer (Fig 2A). After exposure to F4+ ETEC, the continuous purple mucin layer became disrupted, and mucin production was reduced in IPEC-J2 cells infected with F4+ ETEC alone and in cells co- or pre-incubated with *L. rhamnosus*, compared with untreated IPEC-J2 control cells (*P* < 0.001, *P* < 0.001, and *P* = 0.013, respectively; Fig 2A and 2B). Pre-incubation with *L. rhamnosus*
attenuated F4⁺ ETEC-induced mucin layer disruption (Fig 2A). Although IPEC-J2 cells incubated with *L. rhamnosus* only exhibited lowered mucin production compared with untreated IPEC-J2 control cells (*P* = 0.004), mucin production was higher in cells incubated with *L. rhamnosus*.
**Effect of *L. rhamnosus* on apoptosis of IPEC-J2 cells**

Intestinal epithelial cells commonly undergo apoptosis in response to injurious stimuli (microbial, hypoxic, or chemical), allowing for the dismantling of damaged cells without the release of cellular contents and activation of the immune system. Excessive or inappropriate apoptosis may lead to damage of the intestinal barrier, however. Cells exposed to F4+ ETEC alone had a higher percentage of early and late apoptosis compared with untreated IPEC-J2 controls (*P* = 0.002 and *P* < 0.001, respectively; Fig 3). Pre-incubation (but not co-incubation) with *L. rhamnosus* resulted in a decrease in the percentage of early and late apoptosis during F4+ ETEC infection (*P* = 0.048 and *P* = 0.001, respectively). The percentages of early and late apoptosis were higher in cells co-incubated with *L. rhamnosus* than in untreated IPEC-J2 controls (*P* = 0.002 and *P* = 0.002, respectively) or cells incubated with *L. rhamnosus* alone (*P* = 0.004 and *P* < 0.001, respectively).

**Effect of *L. rhamnosus* on TLR and NOD mRNA expression**

To understand the interaction between IPEC-J2 inflammatory responses and the effect of *L. rhamnosus* in preventing F4+ ETEC infection, we quantified the relative expression of mRNAs for selected genes encoding TLRs and NODs (Fig 4).

Incubation with *L. rhamnosus* alone increased relative expression of TLR2 and TLR4 mRNAs (*P* = 0.002 and *P* = 0.003, respectively; Fig 4A and 4B). The relative expression of TLR2 mRNA was significantly higher in cells pre-incubated with *L. rhamnosus* during F4+ ETEC infection than in either untreated IPEC-J2 controls or cells only infected with F4+ ETEC (*P* < 0.001). The relative expression of TLR2 mRNA in cells pre-incubated with *L. rhamnosus* was significantly higher than in cells co-incubated with *L. rhamnosus* during F4+ ETEC infection (*P* < 0.001).

As expected, an F4+ ETEC-induced increase (*P* < 0.001) in the relative expression of TLR4 mRNA was observed, and this increase was attenuated (*P* < 0.001) by co-incubation with *L. rhamnosus*. The relative expression of TLR4 mRNA was higher in cells pre-incubated with *L. rhamnosus* than in untreated IPEC-J2 controls or in cells co-incubated with *L. rhamnosus* (*P* = 0.002 and *P* = 0.031, respectively).

The relative expression of TLR9 mRNA was elevated in cells treated with F4+ ETEC alone, cells incubated with *L. rhamnosus* alone, and cells co-incubated with F4+ ETEC and *L. rhamnosus* compared with untreated IPEC-J2 controls, but TLR9 mRNA expression was not elevated...
in cells pre-incubated with *L. rhamnosus* (*P* = 0.025, *P* = 0.009, and *P* < 0.001, respectively; Fig 4C). The relative expression of *TLR9* mRNA was higher in cells co-incubated with *L. rhamnosus* than in cells infected with F4+ ETEC and cells pre-incubated with *L. rhamnosus* (*P* = 0.031 and *P* = 0.013, respectively).

Cells incubated with *L. rhamnosus* alone and those pre-incubated with *L. rhamnosus* exhibited higher relative expression of *NOD1* mRNA than did untreated IPEC-J2 controls (*P* < 0.001 and *P* = 0.004; Fig 4D), but cells infected with F4+ ETEC alone did not. The relative expression of *NOD1* mRNA was higher in cells incubated with *L. rhamnosus* alone than in cells infected with F4+ ETEC and co-incubated with *L. rhamnosus* (*P* = 0.002 and *P* = 0.038, respectively). F4+ ETEC infection led to an increase in the relative expression of *NOD2* mRNA, but *L. rhamnosus* incubation did not (*P* = 0.043; Fig 4E). The F4+ ETEC-induced increase in *NOD2* mRNA expression was attenuated by co- or pre-incubation with *L. rhamnosus* (*P* = 0.002 and *P* = 0.003, respectively).

Concentrations of TNF-α, IL-10, and PGE₂ in IPEC-J2 cell culture supernatants

To test whether *L. rhamnosus* can attenuate the inflammatory response induced by F4+ ETEC, we measured the concentrations of TNF-α, IL-10, and PGE₂ in the supernatant from cultures...
of treated IPEC-J2 cells and untreated controls (Fig 5). TNF-α production was increased both in cells infected with F4+ ETEC alone and in cells co-incubated with F4+ ETEC and L. rhamnosus compared with untreated IPEC-J2 controls ($P < 0.001$; Fig 5A). F4+ ETEC infection resulted in a decrease in the IL-10 supernatant concentration ($P = 0.043$), but pre-treatment with L. rhamnosus prevented this decrease (Fig 5B). Compared with untreated IPEC-J2 controls, F4+ ETEC-infected cells or F4+ ETEC-infected cells pre-incubated with L. rhamnosus secreted a significantly higher amount of PGE2 ($P < 0.001$ and $P = 0.001$, respectively; Fig 5C). However, although the PGE2 concentration in the supernatant of cells co-incubated with L. rhamnosus was elevated, the difference was not significant.

**Effect of L. rhamnosus on p-EGFR, p-Akt, and p-PKCα protein expression**

EGFR affects the survival of epithelial cells through multiple signaling pathways. We therefore examined the effect of L. rhamnosus on activation of EGFR and the downstream regulatory pathway molecules Akt and PKCα (Fig 6).

Expression of p-EGFR was decreased in cells co- or pre-incubated with L. rhamnosus during F4+ ETEC infection compared with untreated IPEC-J2 controls as well as cells infected with F4+ ETEC alone ($P < 0.001$). IPEC-J2 cells only incubated with L. rhamnosus also exhibited lower p-EGFR protein expression compared with cells only infected with F4+ ETEC ($P = 0.005$). In contrast, p-Akt expression was elevated in cells incubated with L. rhamnosus only and cells co- or pre-incubated with L. rhamnosus, compared with untreated IPEC-J2 controls ($P < 0.001$, $P = 0.008$, and $P = 0.022$, respectively; Fig 6A and 6B). Expression of p-Akt was higher in cells incubated with L. rhamnosus only than in cells only infected with F4+ ETEC ($P = 0.006$). No differences in p-PKCα expression were observed among the different groups.

Increased p-EGFR and Hsp72 protein expression was observed at 10 h only in IPEC-J2 cells incubated with L. rhamnosus alone (Fig 6C).

**Effect of L. rhamnosus on ZO-1 and occludin protein expression**

The TJ plays a fundamental role in membrane barrier function and integrity through interaction of the ZO protein with the transmembrane protein occludin and the apical perijunctional actomyosin ring. To investigate the effect of L. rhamnosus on TJ integrity, we examined ZO-1 and occludin protein expression in IPEC-J2 cells with and without pre- or co-incubation with L. rhamnosus during F4+ ETEC infection (Fig 7).
F4+ ETEC infection resulted in a decrease in ZO-1 protein expression ($P = 0.022$), and this decrease was attenuated by co- or pre-incubation with *L. rhamnosus* ($P < 0.001$; Fig 7A and 7B). ZO-1 protein expression was higher in cells only incubated with *L. rhamnosus* than in cells only infected with F4+ ETEC ($P = 0.031$).

Occludin protein expression was elevated in cells only incubated with *L. rhamnosus* and cells pre-incubated with *L. rhamnosus* compared with untreated IPEC-J2 controls ($P = 0.018$ and $P = 0.009$, respectively; Fig 7A and 7C). Compared with cells only infected with F4+ ETEC and cells co-incubated with *L. rhamnosus*, occludin protein expression was higher in cells only incubated with *L. rhamnosus* ($P = 0.004$ and $P = 0.012$, respectively) and cells pre-incubated with *L. rhamnosus* ($P < 0.001$ and $P = 0.007$, respectively).

**Discussion**

Certain probiotics have the potential to serve as alternatives to antibiotics for preventing enteric infections. However, the mechanism underlying the benefits derived from biotherapy with *Lactobacillus* is incompletely understood. In this study, we explored the effects of *L. rhamnosus* on the epithelial barrier and inflammatory response in IPEC-J2 cells. *Lactobacillus rhamnosus* inhibited F4+ ETEC adhesion and ameliorated F4+ ETEC-induced mucin layer destruction in these cells. Moreover, *L. rhamnosus* attenuated F4+ ETEC-induced damage to the IPEC-J2 cell barrier by suppressing F4+ ETEC-induced apoptosis and increasing ZO-1 and occludin protein expression. These data indicated that the dosage of *L. rhamnosus* used in this study was within the effective range for IPEC-J2 cells.

In agreement with a previous study [22], the present study showed that pre-treatment of IPEC-J2 cells with *L. rhamnosus* inhibits the adhesion of F4+ ETEC and that addition of
exogenous mucin enhances the capacity of \textit{L. rhamnosus} to reduce the adhesion of F4+ ETEC to IPEC-J2 cells. The mucus layer is the first barrier pathogens encounter upon contact with epithelial cells. Elaboration of mucus within minutes or hours of insult is considered a major part of IEC innate defenses. Mucin proteins are believed to protect epithelial cells from microbial pathogens by limiting their access to the cells through simple steric hindrance, by providing a physicochemical barrier, or through specific mucin-bacterial interactions [23]. Increased secretion of MUC3 mucin leads to reduced adhesion of enteropathogenic \textit{Escherichia coli} (EPEC) and enterohemorrhagic \textit{Escherichia coli} (EHEC) strains [24,25]. It has been shown that Spac pilin, located at the pilus, is essential for mucus interaction with LGG [26]. A recent study also showed that a C-terminal LPxTG motif-containing protein accounts for the binding of \textit{L. reuteri} ATCC PTA 6475 to Caco-2 cells and mucus [27]. Moreover, the bacterial exopolysaccharide reuteran, synthesized by \textit{Lactobacillus reuteri}, reduces ETEC colonization of piglet jejunal epithelial cells [28]. These data suggest that competition for attachment sites on mucus and amelioration of mucin layer destruction via \textit{L. rhamnosus} play vital roles in preventing F4+ ETEC adhesion.

We found that pre-incubation, rather than co-incubation, with \textit{L. rhamnosus} reduced F4+ ETEC adhesion. Indeed, a previous study found that pre-treatment with probiotics (\textit{Lactobacillus acidophilus} and \textit{Streptococcus thermophilus}) markedly reduced enteroinvasive \textit{Escherichia coli} (EIEC) attachment and spread, whereas simultaneous addition of probiotics with EIEC had a lesser effect, and addition of probiotics 1 hour after EIEC challenge failed to reduce invasion [29]. Probiotics also differ with respect to production of autoinducers that can condition

---

**Fig 7. Western blot detection of tight junction proteins.** (A) Representative panels of zonula occludens-1 (ZO-1) and occludin proteins in IPEC-J2 cells collected from the indicated cultures at 3 h after F4+ ETEC challenge. Expression of GAPDH was measured as an internal control. Results are presented as the ratio of the (B) ZO-1 band intensity and (C) occludin band intensity to the GAPDH band intensity. Data are presented as means ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

doi:10.1371/journal.pone.0125717.g007
the formation of complex biofilms above the epithelial surface, conceivably modulating mucosal responses to the luminal environment [30]. Production of the HSP GroEL in the biofilm was shown to enhance the immunomodulatory effects of *L. casei* ATCC334 [31]. These findings emphasize the importance of investigating a diversity of host-microorganism interactions when profiling probiotics.

In our study, *L. rhamnosus* pre-treatment suppressed F4⁺ ETEC-induced apoptosis of IECs, suggesting that *L. rhamnosus* prevents F4⁺ ETEC-induced apoptosis. Basal epithelial cell extrusion in response to apoptotic stimuli is a normal physiological event in the gastrointestinal tract and does not affect intestinal epithelial barrier integrity; however, an increased epithelial apoptosis rate might compromise the epithelial barrier.

A recent study showed that canonical TLR2 signaling may offer a novel therapeutic approach to barrier recovery in atopic dermatitis and ulcerative colitis [32,33]. In a *Citrobacter rodentium*-induced colitis model, TLR2-deficient mice exhibited 45 to 75% mortality coincident with severe defects in TJ-associated IEC integrity [34]. *Lactobacillus plantarum* enhances TJ function by rearrangement of TJ protein conformation in response to TLR2 signaling [35]. We found that pre-incubation with *L. rhamnosus* following F4⁺ ETEC infection or incubation with *L. rhamnosus* alone increased TLR2 mRNA expression. Activation of TLR2 signaling triggered by *L. rhamnosus* is most likely mediated by lipoteichoic acid, lipoproteins, and peptidoglycans from gram-positive bacteria through formation of a heterodimer with either TLR1 or TLR6 [36]. Invasive *Streptococcus pneumoniae* and *Haemophilus influenzae* were shown to exploit TLR2/TLR4-mediated downregulation of TJ components to facilitate translocation across the epithelium at 24-h post-inoculation [37]. In the present study, *L. rhamnosus* pre-treatment attenuated F4⁺ ETEC-induced TNF-α elevation, indicating that development of a competent TJ may also serve to limit TLR2-mediated inflammation that might be harmful during later stages of F4⁺ ETEC infection. TLR2 expression was enhanced in the early stage of F4⁺ ETEC infection in response to pathogen-induced inflammation in impaired IPEC-J2 cells, as was early ZO-1 and occludin protein expression, which limited interactions between microbial antigens and the mucosal defense system.

Tight junctions are major determinants of epithelial paracellular permeability, and altered TJ permeability contributes to pathogen entry and a net efflux of ions and water. ETEC infection increases transepithelial permeability in early weaned pigs [38]. Heat-stable toxin b from ETEC has been shown to impair intestinal epithelial barrier function by causing the redistribution and/or fragmentation of ZO-1 and occludin [39]. The present data showed that ETEC infection leads to decreased expression of ZO-1 and occludin proteins in IPEC-J2 cells. Furthermore, *L. rhamnosus* enhanced the intestinal barrier related to the increased expression of ZO-1 and an increase in the abundance of occludin protein. We speculate that altered expression of TJ proteins and increased epithelial apoptosis contributed to epithelial barrier dysfunction, and certainly both mechanisms were blocked by probiotic therapy in this IPEC-J2 model.

Two different signaling cascade sequences originating from TLR2 signaling result in intestinal homeostasis and pathogen surveillance: NF-κB and PI3K activation, respectively. In this study, we found that *L. rhamnosus* enhanced Akt activation, thus activating the TLR2/Akt pathway in IECs. Binding of MyD88 adapter-like (Mal) protein to the p85 subunit of PI3K upon activation of the TLR2/TLR6 heterodimer leads to Akt phosphorylation. Activation of the PI3K/Akt pathway as a result of TLR2 activation has also been shown to augment epithelial barrier integrity. TLR2 promotes intestinal barrier function through redistribution of the TJ protein ZO-1 in response to stress-induced damage and suppresses apoptosis under control of the PI3K/Akt pathway [40].
Nod-like receptors are the cytoplasmic counterparts of TLRs, and both receptor types constitute a ‘tour de force’ of cellular defenses against encountered microbial motifs at the plasma membrane and within the cell [41]. In Caco-2 cells, NOD1 prevents IκB kinase and NF-κB activation in response to EIEC infection [42]. The present study showed that pre-incubation with \textit{L. rhamnosus} induces NOD1 mRNA expression, which is accompanied by up-regulation of TLR2 and TLR9 mRNA expression. Interestingly, incubation with \textit{L. rhamnosus} inhibited the F4+ ETEC-induced increase in NOD2 mRNA expression. NOD2 serves as a receptor for MDP, a small molecule derived from bacterial cell wall peptidoglycan [43]. Incubation with \textit{L. rhamnosus} may inhibit internalization of MDP.

It has been reported that Mal contributes to maintenance of the intestinal epithelial barrier via activation of PKC and regulation of TJJs mediated by TLR2, possibly involving translocation of the PKC isoforms PKCα and PKCβ to the TJ region [44,45]. In this study, \textit{L. rhamnosus} had no effect on the expression of PKCα in IPEC-J2 cells. The PKC family consists of 11 isoforms, and these intracellular enzymes are located at or near the ZO complex. TJ assembly is regulated by a network of signaling pathways that may involve different PKC isoforms. A previous study showed that \textit{L. rhamnosus} protection of TJJs and the barrier function from hydrogen peroxide–induced insult is correlated with increased activation of PKCε and PKCβI [46]. In contrast, the probiotic \textit{E. coli} strain Nissle 1917 was shown to prevent EPEC-induced decreases in ZO-2 expression and redistribution by silencing PKCζ activation [47]. Activation of distinct PKC isoforms may differentially affect cellular transport and the barrier function of the epithelium.

EGFR activation was shown to be associated with anti-apoptosis and cell survival [48,49]. In the present study, treatment with \textit{L. rhamnosus} unexpectedly inhibited EGFR activation, which would not otherwise be adversely affected by F4+ ETEC at 3 h after infection. EGFR is predominantly localized along the basolateral sides of polarized IECs [50]. ETEC, however, is an extracellular pathogen that attaches to the apical side of epithelial cells. As shown in a previous study, EPEC-mediated activation of EGFR can be observed as early as 15 min post-infection in EPEC-treated non-polarized Caco-2 cells, whereas EGFR phosphorylation in polarized Caco-2 cells is not evident until 4 h after infection [49]. We found that activation of EGFR by \textit{L. rhamnosus} was time dependent. ETEC-induced defective barrier function may be a pre-requisite for ETEC itself or its secreted proteins to access and activate the basolateral EGFR; \textit{L. rhamnosus} counteracted ETEC-induced basolateral EGFR activation via maintaining the TJ barrier.

A previous study showed that activation of TLR4 by LPS induces Cox-2 expression and PGE2 production via Myd88 signaling, which in turn may stimulate epithelial proliferation via an EGFR-dependent mechanism [51]. \textit{Lactobacillus acidophilus} increases Cox-2 expression and PGE2 secretion in intestinal cell lines [52]. Previous studies showed that \textit{L. amylovorus} and \textit{L. jensenii} suppress TLR4 inflammatory signaling triggered by ETEC through modulation of the negative regulators Tollip and the IL-1R–associated kinases M (IRAK-M), A20, Bcl-3, and MKP-1 [7,53]. Recently, an \textit{in vitro} study using a mouse enterocyte model found that LPS-mediated TLR4 signaling could be inhibited by activation of TLR9 with bacterial DNA via the inhibitory kinase IRAK-M [54]. Dependent on interaction with four different E prostanoid (EP) receptor subtypes, PGE2 regulates many physiological functions of the gut, including mucosal protection, gastrointestinal secretion, and motility [55]. Constitutive PGE2 production via EP4 receptor signaling in the intestine appears to protect the integrity of the epithelial intestinal wall, presumably through enhancement of epithelial cell survival and regeneration of the epithelium. In addition, PGE2 elicits powerful immunosuppressive effects that contribute to the resolution of acute inflammation, facilitating tissue regeneration and the return to homeostasis.

It has been reported that the LGG-derived protein p40 up-regulates \textit{Muc2} gene expression and increases mucus production through transactivation of EGFR/Akt signaling in LS174T...
human colon cancer cells [56]. LGG-derived p40 protein stimulates release of the EGFR ligand HB-EGF to transactivate EGFR, contributing to anti-apoptosis and maintenance of the epithelial barrier [20]. EGFR/Akt pathway activation is required for live LGG or LGG-derived soluble protein p40-stimulated suppression of apoptosis in a concentration-dependent manner [57]. Taken together, these results suggest that L. rhamnosus-mediated EGFR-independent Akt activation may favor IEC activation in response to bacterial infection.

In conclusion, our findings suggest that L. rhamnosus ATCC 7469 protects IPEC-J2 cells from F4+ ETEC infection, partly through reducing the adhesion of F4+ ETEC to the cells and subsequent attenuation of F4+ ETEC-induced mucin layer destruction and suppression of apoptosis of IPEC-J2 cells. Moreover, L. rhamnosus promotes EGFR-independent Akt activation, which may promote activation of IPEC-J2 cells in response to bacterial infection, in turn increasing TJ integrity to optimize the barrier function and restrict pathogen invasion.

**Author Contributions**

Conceived and designed the experiments: WZ YHZ JCY JFW. Performed the experiments: WZ YHZ JCY GYY DZ. Analyzed the data: WZ YHZ JCY. Contributed reagents/materials/analysis tools: JCY GYY. Wrote the paper: WZ YHZ JCY JFW.

**References**

1. Croxen MA, Finlay BB. Molecular mechanisms of *Escherichia coli* pathogenicity. Nat Rev Microbiol. 2010; 8: 26–38. doi:10.1038/nrmicro2265 PMID: 19966814
2. Fairbrother JM, Nadeau É, Gyles CL. *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. Anim Health Res Rev. 2007; 6: 17–39.
3. Li XQ, Zhu YH, Zhang HF, Yue Y, Cai ZX, Lu QP, et al. Risks associated with high-dose *Lactobacillus rhamnosus* in an *Escherichia coli* model of piglet diarrhoea: intestinal microbiota and immune imbalances. PLoS One. 2012; 7: e40666. doi:10.1371/journal.pone.0040666 PMID: 22848393
4. Zhang L, Xu YQ, Liu HY, Lai T, Ma JL, Wang JF, et al. Evaluation of *Lactobacillus rhamnosus* GG using an *Escherichia coli* K88 model of piglet diarrhoea: effects on diarrhoea incidence, faecal microflora and immune responses. Vet Microbiol. 2010; 141: 142–148. doi:10.1016/j.vetmic.2009.09.003 PMID: 19782483
5. Zhu YH, Li XQ, Zhang W, Zhou D, Liu HY, Wang JF. Dose-dependent effects of *Lactobacillus rhamnosus* on serum interleukin-17 production and intestinal T-Cell responses in pigs challenged with *Escherichia coli*. Appl Environ Microbiol. 2014; 80: 1787–1798. doi:10.1128/AEM.03668-13 PMID: 24389928
6. Kleta S, Nordhoff M, Tedin K, Wieler LH, Kolenda R, Oswald S, et al. Role of F1C fimbriae, flagella, and secreted bacterial components in the inhibitory effect of probiotic *Escherichia coli* Nissle 1917 on atypical enteropathogenic *E. coli* infection. Infect Immun. 2014; 82: 1801–1812. doi:10.1128/IAI.01431-13 PMID: 24549324
7. Finamore A, Roselli M, Imbinto A, Seeboth J, Oswald IP, Mengheri E. *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. 2014; 9: e94891. doi:10.1371/journal.pone.0094891 PMID: 24735311
8. Orlando A, Linsalata M, Notarnicola M, Tutino V, Russo F. *Lactobacillus GG* restoration of the gliadin induced epithelial barrier disruption: the role of cellular polyamines. BMC Microbiol. 2014; 14; 19. doi:10.1186/1471-2180-14-19 PMID: 24483336
9. Johansson ME, Sjovall H, Hansson GC. The gastrointestinal mucus system in health and disease. Nat Rev Gastroenterol Hepatol. 2013; 10: 352–361. doi:10.1038/nrgastro.2013.35 PMID: 23478383
10. Kumar P, Luo Q, Vickers TJ, Sheikh A, Lewis WG, Fleckenstein JM. EatA, an immunogenic protective antigen of enterotoxigenic *Escherichia coli*, degrades intestinal mucin. Infect Immun. 2014; 82: 500–508. doi:10.1128/IAI.01078-13 PMID: 24478068
11. Chu H, Mazmanian SK. Innate immune recognition of the microbiota promotes host-microbial symbiosis. Nat Immunol. 2013; 14: 668–675. doi:10.1038/ni.2635 PMID: 23778794
12. Maisonneuve C, Bertholet S, Philpott DJ, De Gregorio E. Unleashing the potential of NOD- and Toll-like agonists as vaccine adjuvants. Proc Natl Acad Sci U S A. 2014; 111: 12294–12299. doi:10.1073/pnas.1400478111 PMID: 25136133
13. Guo S, Al-Sadi R, Said HM, Ma TY. Lipopolysaccharide causes an increase in intestinal tight junction permeability in vitro and in vivo by inducing enterocyte membrane expression and localization of TLR-4 and CD14. Am J Pathol. 2013; 182: 375–387. doi: 10.1016/j.ajpath.2012.10.014 PMID: 23201091

14. Oppong GO, Rapsinski GJ, Newman TN, Nishimori JH, Biesecker SG, Tukei C. Epithelial cells augment barrier function via activation of the Toll-like receptor 2/phosphatidylinositol 3-kinase pathway upon recognition of Salmonella enterica serovar Typhimurium curli fibrils in the gut. Infect Immun. 2013; 81: 476–486. doi: 10.1128/IAI.00453-12 PMID: 23208603

15. Turner JR. Intestinal mucosal barrier function in health and disease. Nat Rev Immunol. 2009; 9: 799–809. doi: 10.1038/nri2653 PMID: 19855405

16. Mathias A, Duc M, Favre L, Benyacoub J, Blum S, Corthesy B. Potentiation of polarized intestinal Caco-2 cell responsiveness to probiotics complicated with secretory IgA. J Biol Chem. 2010; 285: 33906–33913. doi: 10.1074/jbc.M110.135111 PMID: 20729211

17. Yang K, Jiang Z, Zheng C, Wang L, Yang X. Effect of Lactobacillus plantarum on diarrhea and intestinal barrier function of young piglets challenged with enterotoxigenic Escherichia coli K88. J Anim Sci. 2014; 92: 1496–1503. doi: 10.2527/jas.2013-6619 PMID: 24492550

18. Avraham R, Yarden Y. Feedback regulation of EGFR signalling: decision making by early and delayed loops. Nat Rev Mol Cell Biol. 2011; 12: 104–117. doi: 10.1038/nrm3048 PMID: 21252999

19. Banck MS, Kanwar R, Kulkarni AA, Boora GK, Metge F, Kipp BR, et al. The genomic landscape of small intestine neuroendocrine tumors. J Clin Invest. 2013; 123: 2502–2508. doi: 10.1172/JCI67963 PMID: 23676460

20. Yan F, Liu L, Dempsey PJ, Tsai YH, Raines EW, Wilson CL, et al. A Lactobacillus rhamnosus GG-derived soluble protein, p40, stimulates ligand release from intestinal epithelial cells to transactivate epidermal growth factor receptor. J Biol Chem. 2013; 288: 30742–30751. doi: 10.1074/jbc.M113.492397 PMID: 24043629

21. Seo GS, Jiang WY, Park PH, Sohn DH, Cheon JH, Lee SH. Hirsutenone reduces deterioration of tight junction proteins through EGFR/Akt and ERK1/2 pathway both converging to HO-1 induction. Biochem Pharmacol. 2014; 90: 115–125. doi: 10.1016/j.bcp.2014.05.006 PMID: 24853984

22. Prince T, Mc Bain AJ, O’Neill CA. Lactobacillus reuteri protects epidermal keratinocytes from Staphylococcus aureus-induced cell death by competitive exclusion. Appl Environ Microbiol. 2012; 78: 5119–5126. doi: 10.1128/AEM.00959-12 PMID: 22582077

23. Dharmani P, Srivastava V, Kissoon-Singh V, Chadee K. Role of intestinal mucus in innate host defense mechanisms against pathogens. J Innate Immun. 2009; 1: 123–135. doi: 10.1159/000163037 PMID: 20375571

24. Mack DR, Ahmne S, Hyde L, Wei S, Hollingsworth MA. Extracellular MUC3 mucin secretion follows adherence of Lactobacillus strains to intestinal epithelial cells in vitro. Gut. 2003; 52: 827–833. PMID: 12740338

25. Pan Q, Tian Y, Li X, Ye J, Liu Y, Song L, et al. Enhanced membrane-tethered mucin 3 (MUC3) expression by a tetrameric branched peptide with a conserved TFLK motif inhibits bacteria adherence. J Biol Chem. 2013; 288: 5407–5416. doi: 10.1074/jbc.M112.408245 PMID: 23316049

26. Kankainen M, Paulin L, Tynkkynen S, von Ossowski I, Reunanen J, Partanen P, et al. Comparative genomic analysis of Lactobacillus rhamnosus GG reveals pil containing a human- mucus binding protein. Proc Natl Acad Sci U S A. 2009; 106: 17193–17198. doi: 10.1073/pnas.0908876106 PMID: 19805152

27. Jensen H, Roos S, Jonsson H, Rud I, Grimmer S, van Pijkeren JP, et al. Role of Lactobacillus reuteri cell and mucus-binding protein A (CmbA) in adhesion to intestinal epithelial cells and mucus in vitro. Microbiology. 2014; 160: 671–681. doi: 10.1099/mic.0.073551-0 PMID: 24473252

28. Chen XY, Woodward A, Zijlstra RT, Ganzle MG. Exopolysaccharides synthesized by Lactobacillus reuteri protect against enterotoxigenic Escherichia coli in piglets. Appl Environ Microbiol. 2014; 80: 5752–5760. doi: 10.1128/AEM.01782-14 PMID: 25015886

29. Resta-Lenert S, Barrett KE. Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive Escherichia coli (EIEC). Gut. 2003; 52: 988–997. PMID: 12601956

30. Lebeer S, Verhoeven TL, Perea Velez M, Vanderleyden J, De Keersmaecker SC. Impact of environmental and genetic factors on biofilm formation by the probiotic strain Lactobacillus rhamnosus GG. Appl Environ Microbiol. 2007; 73: 6768–6775. PMID: 17827316

31. Rieu A, Aoudia N, Jego G, Chluba J, Youssi N, Briandet R, et al. The biofilm mode of life boosts the anti-inflammatory properties of Lactobacillus. Cell Microbiol. 2014; 16: 1836–1853. doi: 10.1111/cmi.12331 PMID: 25052472

32. Kuo IH, Carpenter-Mendini A, Yoshida T, McGirt LY, Ivanov AI, Barnes KC, et al. Activation of epithelial toll-like receptor 2 enhances tight junction function: implications for atopic dermatitis and skin barrier repair. J Invest Dermatol. 2013; 133: 988–998. doi: 10.1038/jid.2012.437 PMID: 23223142
33. Ey B, Eyking A, Klepak M, Salzman NH, Gothert JR, Runzi M, et al. Loss of TLR2 worsens spontaneous colitis in MDR1A deficiency through commensally induced pyroptosis. J Immunol. 2013; 190: 5676–5688. doi: 10.4049/jimmunol.1201592 PMID: 23630652

34. Gibson DL, Ma C, Rosenberger CM, Bergstrom KSB, Valdez Y, Huang JT, et al. Toll-like receptor 2 plays a critical role in maintaining mucosal integrity during Citrobacter rodentium-induced colitis. Cell Microbiol. 2007; 10: 388–403. PMID: 17910742

35. Karczewski J, Troost FJ, Konings I, Dekker J, Kleerebezem M, Brummer R-JM, et al. Regulation of human epithelial tight junction proteins by Lactobacillus plantarum in vivo and protective effects on the epithelial barrier. Am J Physiol Gastrointest Liver Physiol. 2010; 298: G851–G859. doi: 10.1152/ajpgi.00327.2009 PMID: 20224007

36. Trinchieri G, Sher A. Cooperation of Toll-like receptor signals in innate immune defence. Nat Rev Immunol. 2007; 7: 179–190. PMID: 17318230

37. Clarke TB, Francella N, Huegel A, Weiser JN. Invasive bacterial pathogens exploit TLR-mediated downregulation of tight junction components to facilitate translocation across the epithelium. Cell Host Microbe. 2011; 9: 404–414. doi: 10.1016/j.chom.2011.04.012 PMID: 21575911

38. McLamb BL, Gibson AJ, Overman EL, Stahl C, Moeser AJ. Early weaning stress in pigs impairs innate mucosal immune responses to enterotoxigenic E. coli challenge and exacerbates intestinal injury and clinical disease. PLoS One. 2013; 8: e59838. doi: 10.1371/journal.pone.0059838 PMID: 23637741

39. Yan F, Cao H, Chaturvedi R, Krishna U, Hobbs SS, Dempsey PJ, et al. Epidermal growth factor receptor. Nature. 2003; 422: 317–322. doi: 10.1038/nature0163558080237265 PMID: 17110941

40. Kim JG, Lee SJ, Kagnoff MF. Nod1 is an essential signal transducer in intestinal epithelial cells infected with bacteria that avoid recognition by Toll-like receptors. Infect Immun. 2004; 72: 1487–1495. PMID: 14977954

41. Fritz JH, Ferrero RL, Philpott DJ, Girardin SE. Nod-like proteins in immunity, inflammation and disease. Nat Immunol. 2006; 7: 1250–1256. doi: 10.1038/nri1850 PMID: 16785072

42. Shimazu T, Villena J, Tohno M, Fujie H, Hosoya S, Shimosato T, et al. Immunobiotic Lactobacillus rhamnosus in F4+ ETEC Infection – Lactobacillus rhamnosus therapeutics for gastrointestinal infections. Curr Opin Microbiol. 2011; 14: 571–576. doi: 10.1016/j.copmi.2011.04.003 PMID: 21575911

43. Seth A, Yan F, Polk DB, Rao RK. Probiotics ameliorate the hydrogen peroxide-induced epithelial barrier disruption by a PKC- and MAP kinase-dependent mechanism. Am J Physiol Gastrointest Liver Physiol. 2008; 294: G1060–G1069. doi: 10.1152/ajpgi.00202.2007 PMID: 18292183

44. Rubino SJ, Selvanantham T, Girardin SE, Pilipott DJ. Nod-like receptors in the control of intestinal inflammation. Curr Opin Immunol. 2012; 24: 398–404. doi: 10.1016/j.coi.2012.04.010 PMID: 22677577

45. Corr SC, Palsson-McDermott EM, Grishina I, Barry SP, Aviello G, Bernard NJ, et al. MyD88 adaptor-like (Mal) functions in the epithelial barrier and contributes to intestinal integrity via protein kinase C. Mucosal Immunol. 2014; 7: 57–67. doi: 10.1038/mi.2013.24 PMID: 23612054

46. Cario E, Gerken G, Podolsky DK. Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function. Gastroenterology. 2007; 133: 1359–1374. PMID: 17408640

47. Rose J, Koutsouris A, Viewanathan VK. Enteropathogenic Escherichia coli-induced epithelial growth factor receptor activation protects gastric epithelial cells from Helicobacter pylori-induced apoptosis. Gastroenterology. 2009; 136: 1297–1307. doi: 10.1053/j.gastro.2008.12.059 PMID: 19250983

48. Zavala MM, Wiesmuller LL, Wiesmuller LL, et al. Lactobacillus jensenii elicits anti-inflammatory activity in porcine intestinal epithelial cells by modulating negative regulators of the Toll-like receptor signaling pathway. Infect Immun. 2012; 80: 276–288. doi: 10.1128/IAI.05729-11 PMID: 22083706

49. Zyrek AA, Cichon C, Helms S, Enders C, Sonnenborn U, Schmidt MA. Molecular mechanisms underlying the probiotic effects of Escherichia coli Nissle 1917 involve ZO-2 and PKCζ redistribution resulting in tight junction and epithelial barrier repair. Cell Microbiol. 2007; 9: 804–816. PMID: 17087734

50. Yan F, Cao H, Chaturvedi R, Krishna U, Hobbs SS, Dempsey PJ, et al. Epidermal growth factor receptor activation protects gastric epithelial cells from Helicobacter pylori-induced apoptosis. Gastroenterology. 2009; 136: 1297–1307. doi: 10.1053/j.gastro.2008.12.059 PMID: 19250983

51. Jamora C, DasGupta R, Kocieniewski P, Fuchs E. Segregation of receptor and ligand regulates activation of epithelial growth factor receptor. Nature. 2003; 422: 317–322. PMID: 12646922

52. Fukata M, Chen A, Klepper A, Krishnareddy S, Vamadevan AS, Thomas LS, et al. Cox-2 is regulated by toll-like receptor-4 (TLR4) signaling and is important for proliferation and apoptosis in response to intestinal mucosal injury. Gastroenterology. 2006; 131: 868–877. PMID: 16952555

53. Roxas JL, Koutsouris A, Viewanathan VK. Enteropathogenic Escherichia coli-induced epidermal growth factor receptor activation contributes to physiological alterations in intestinal epithelial cells. Infect Immun. 2007; 75: 2316–2324. PMID: 17339360

54. Fukata M, Chen A, Klepper A, Krishnareddy S, Vamadevan AS, Thomas LS, et al. Cox-2 is regulated by toll-like receptor-4 (TLR4) signaling and is important for proliferation and apoptosis in response to intestinal mucosal injury. Gastroenterology. 2006; 131: 868–877. PMID: 16952555

55. Otte JM, Mahjurian-Namari R, Brand S, Werner I, Schmidt WE, Schmitz F. Probiotics regulate the expression of COX-2 in intestinal epithelial cells. Nutr Cancer. 2009; 61: 103–113. doi: 10.1080/0163558080237265 PMID: 19116880

56. Shimazu T, Villena J, Tohno M, Fujie H, Hosoya S, Shimosato T, et al. Immunobiotic Lactobacillus jensenii elicits anti-inflammatory activity in porcine intestinal epithelial cells by modulating negative regulators of the Toll-like receptor signaling pathway. Infect Immun. 2012; 80: 276–288. doi: 10.1128/IAI.05729-11 PMID: 22083706
54. Gribar SC, Sodhi CP, Richardson WM, Anand RJ, Gittes GK, Branca MF, et al. Reciprocal expression and signaling of TLR4 and TLR9 in the pathogenesis and treatment of necrotizing enteroocolitis. J Immunol. 2009; 182: 636–646. PMID: 19109197

55. Dey I, Lejeune M, Chadee K. Prostaglandin E2 receptor distribution and function in the gastrointestinal tract. Br J Pharmacol. 2006; 149: 611–623. PMID: 17016496

56. Wang L, Cao H, Liu L, Wang B, Walker WA, Acra SA, et al. Activation of epidermal growth factor receptor mediates mucin production stimulated by p40, a Lactobacillus rhamnosus GG-derived protein. J Biol Chem. 2014; 289: 20234–20244. doi:10.1074/jbc.M114.553800 PMID: 24895124

57. Yan F, Cao H, Cover TL, Washington MK, Shi Y, Liu L, et al. Colon-specific delivery of a probiotic-derived soluble protein ameliorates intestinal inflammation in mice through an EGFR-dependent mechanism. J Clin Invest. 2011; 121: 2242–2253. doi: 10.1172/JCI44031 PMID: 21606592