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Anti-rotavirus Properties and Mechanisms of Selected Gram-Positive and Gram-Negative Probiotics on Polarized Human Colonic (HT-29) Cells

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Abstract

Probiotics have been investigated to improve the universal rotavirus (RV) vaccination as well as to ameliorate the RV infection. However, underlying mechanisms how probiotics mediate beneficial effects needs more investigation. Thus, in the present study, we used polarized HT-29 cells to assess the anti-RV properties of Gram-positive, (*Lactobacillus acidophilus*, *Lacticaseibacillus rhamnosus* GG, and *Bifidobacterium* subsp. *Lactis* Bb12) and Gram negative, (*Escherichia coli* Nissle 1917) probiotics and study their underlying mechanisms. Our results showed that pre-treatment of HT-29 cells for 4 h with probiotics, significantly reduced ($p < 0.05$) human RV replication and this effect was most pronounced for *E. coli* Nissle followed by *L. acidophilus* and *L. rhamnosus* GG. Strikingly, only pre-treatment with live bacteria or their supernatants demonstrated anti-RV properties. Except Gram negative *E. coli* Nissle, the Gram-positive probiotics tested did not bind to RV. Ingenuity pathway analysis of tight junction (TJ)- and innate immune-associated genes indicated that *E. coli* Nissle or *E. coli* Nissle + RV treatments improved cell–cell adhesion and cell contact, while *L. acidophilus* or *L. acidophilus* + RV treatments also activated cell–cell contact but inhibited cell movement functions. RV alone inhibited migration of cells event. Additionally, *E. coli* Nissle activated pathways such as the innate immune and inflammatory responses via production of TNF, while RV infection activated NK cells and inflammatory responses. In conclusion, *E. coli* Nissle's ability to bind RV, modulate expression of TJ events, innate immune and inflammatory responses, via specific upstream regulators may explain superior anti-RV properties of *E. coli* Nissle. Therefore, prophylactic use of *E. coli* Nissle might help to reduce the RV disease burden in infants in endemic areas.

Keywords Rotavirus · HT-29 cells · *E. coli* Nissle · Probiotics · Tight junction · Innate immune response

Introduction

Infectious gastroenteritis is a leading cause of morbidity and mortality in infants worldwide. Rotavirus (RV)-induced gastroenteritis is one such vaccine preventable disease associated with 215,000 deaths annually worldwide in 2013 [1]. Several factors, including malnutrition, micronutrient deficiencies, breastfeeding, maternal immunity, histo-blood group antigen type, composition of gut microbiota, and medication use have been suggested to reduce efficacy of enteric vaccines in developing countries [2–9]. Typical symptoms include vomiting, watery diarrhea, and fever. The fecal–oral route is the established mode of transmission. Upon ingestion, RV infects mature small intestinal enterocytes and leads to diarrhea via (i) destruction of enterocytes, (ii) downregulation of the absorptive enzymes, (iii) inflammation of the gut, and (iv) compromised gut barrier [10, 11]. Thus, an efficacious and universal treatment to prevent or alleviate RV diarrhea in infants must be capable of (i) regulating immune responses (inflammation), (ii) restoring barrier functions, (iii) stimulating enterocyte proliferation and repair, and (iv) inhibiting RV replication in the gut.

Probiotics represent a potential universal anti-RV treatment, and thus, their effects are being studied widely in

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combination with vaccines, antibiotics, and/or oral rehydration therapies in clinical and animal studies [12–18]. Most of the probiotics are Gram-positive (G+) bacteria; therefore, a wealth of literature regarding probiotic effects on amelioration of RV disease is derived from bacterial species that belong to *Lactobacillus* and *Bifidobacterium* genera [16]. The clinical efficacy of probiotic *Lactobacillus acidophilus* against RV was demonstrated in infants where *L. acidophilus* treatment resulted in decreased severity of RV disease, characterized by improved stool consistency and reduced duration of diarrhea [19–21]. Similarly, in randomized clinical trials, *Lacticaseibacillus rhamnosus* GG administration was shown to shorten the duration of RV diarrhea in children [21–23]. Further, prophylactic supplementation of *L. rhamnosus* LGG reduced the risk of nosocomial diarrhea and rotavirus gastroenteritis in infants [24]. In a clinical study that compared probiotic treatment to oral rehydration therapy, children consuming probiotic *Bifidobacterium* subsp. *lactis* exhibited significantly reduced duration of RV diarrhea [25]. Our group and others have demonstrated that *L. acidophilus*, *L. rhamnosus* GG, and *B. lactis* Bb12 were efficacious in reducing the severity of RV diarrhea in the gnotobiotic piglet model [13, 16, 18, 26]. However, the Gram-negative probiotic *Escherichia coli* Nissle-1917 has not been tested for anti-RV properties in infants [27], although several animal studies from our group highlighted its superior characteristics compared to other Gram-positive probiotics in ameliorating RV disease [13, 14, 18]. A randomized, double-blind clinical trial indicated that administration of *E.coli* Nissle successfully alleviated the idiopathic chronic constipation without any major side effects [28].

Despite cumulative evidence of extensively evaluated Gram-positive and Gram-negative probiotics in clinical and animal studies [13], their anti-RV properties and mechanistic insights are poorly investigated [29]. Therefore, the objectives of this study were to (i) investigate and compare anti-RV properties of Gram-positive probiotics (*L. rhamnosus* GG, *L. acidophilus*, *B. animalis* subsp. *lactis* Bb12) and Gram-negative probiotic (*E. coli* Nissle1917), and (ii) investigate the mechanisms by which *E. coli* Nissle modulates rotavirus infections in vitro. Numerous cell lines have been used to investigate the in vitro mechanisms of probiotics. The distinct features of human colonic adenocarcinoma (HT-29) cells closely mimicking the in vivo functional intestinal epithelium [30–34] make the HT-29 cells an ideal model. These differentiated cells possess apical brush border proteins, Cl- channels, Cl- secretion, mucus production, disaccharidases and peptidases, domes on impermeable substrates, trans-epithelial resistance (TER), and intracellular tight junction proteins similar to intestinal epithelium. In this study, we have established a polarized HT-29 cell monolayer model to investigate anti-RV properties of the above-mentioned probiotics. We tested three different probiotic treatment regimens to determine their effects on RV infection. (1) Pre-inoculation: This approach mimics the in vivo effects of probiotics administered prior to RV infection as a prophylactic measure in humans. (2) Co-inoculation: This regimen was used to model probiotic administration during ongoing RV infections in infants. (3) Pre-incubation and co-inoculation: Our rationale was that incubating RV with probiotics would provide sufficient time to induce structural and functional alterations that would not be possible in the direct co-inoculation experiment. Of the four probiotics tested in this study, *E. coli* Nissle 1917 (Dr. Ulrich Sonnenborn) pre-treatment exhibited the most prominent anti-RV properties, while *L. acidophilus* (ATCC 700396) and L. *rhamnosus* GG (ATCC-53103) induced intermediate and the least effects, respectively. *E. coli* Nissle's superior anti-RV properties were attributed to its ability to bind RV, modulate expression of TJ, innate immune response and PRR signaling genes, via specific upstream regulators.

Materials and Methods

Bacteria and Virus Culturing

Probiotic bacteria *E. coli* Nissle 1917 (Dr. Ulrich Sonnenborn, Department of Biological Research, Ardeypharm GmbH, Germany) was cultured in Luria Bertani (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) while *Lacticaseibacillus rhamnosus* GG, ATCC-53103, *Lactobacillus acidophilus* NCFM™ (ATCC 700396), and *Bifidobacterium animalis* subsp. *lactis* Bb12 (Chr. Hansen Inc. Milwaukee, WI, USA) were cultured in De Man, Rogosa, and Sharpe (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) media and enumerated as described previously [35, 36]. The virulent human RV Wa G1P [8] strain at pig passages 25–26 was used in this study [37].

Culturing of Polarized HT-29 Cells

A unique feature of HT-29 cells observed in the absence of glucose and presence of galactose is that the cells closely mimic the in vivo enterocyte architecture. Therefore, in the present study, we adapted previously established protocols to induce HT-29 polarization [31–33]. Briefly, HT-29 cells (ATCC HTB-38™) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY, USA) containing 4.5 g/L D-glucose (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Amarillo, TX, USA), 2 mM glutamine, 1% non-essential amino acids (Gibco, Amarillo, TX), and streptomycin-penicillin antibiotic (Sigma-Aldrich, St. Louis, MO) mix for 2 days. Subsequently, cells were cultured in DMEM with gradually decreasing concentrations of D-glucose (4 mM, 3

mM, 2 mM) with other cell culture ingredients added daily as mentioned above. After that, the cells were cultured in the presence of 1 mM glucose and 1 mM galactose (Sigma-Aldrich, St. Louis, MO, USA) for 24 h before replacing glucose source from medium. In subsequent days, galactose source was gradually increased in 1 mM increments to reach the final concentration of 5 mM with other ingredients. Finally, the cells were sub-cultured to from monolayer and freezer stocks of polarized HT-29 cells were prepared using routine cell culture techniques.Transmission electron microscopy (TEM) was carried as described previously [38] at the Molecular and Cellular Imaging Center (http://www.oardc.ohio-state.edu/mcic) to confirm the polarization of the HT-29 cells. Here and onward, all in vitro experiments (unless specified otherwise) were performed using the polarized HT-29 monolayers in 96- or 48-wells plates with passage number ranging from P1 to P13. Each treatment including controls were performed in triplicate wells in 2 to 3 independent experiments. Average mean and standard deviation were used to express the results.

Probiotics-HT-29 Cell Adhesion Assay

E. coli Nissle, *L. rhamnosus* GG, *L. acidophilus*, and *B. lactis* Bb12 were tested to assess their cell adhesion properties after 30 and 60 min of incubation using multiplicity of infections (MOI) 0.01 as described previously [39]. HT-29 cells were washed with Dulbecco's phosphate-buffered saline (DPBS) without $CaCl_2$ and $MgCl_2$ (Invitrogen, Grand Island, NY, USA) 2-times and maintained in the cell culture medium without FBS for 2 h. Bacterial cultures were pelleted at $10{,}000 \times g$ for 10 min and washed with antibiotic-free cell culture medium. Desired $OD600_{nm}$ for different probiotic bacteria were adjusted in antibiotic-free cell culture medium according to the established standard curve ($OD600_{nm}$ *E. coli* Nissle ~ 0.10, *L. rhamnosus* GG ~ 0.16, *L. acidophilus* ~ 0.23, and *B. lactis* Bb12 ~ 0.78) that corresponds to MOI of 0.01. HT-29 cells were inoculated with bacterial cells resuspended in antibiotic free cell culture media in triplicate wells. *E. coli* K-12 strain ($OD600_{nm}$ ~ 0.10) and the cell culture medium were included as controls. At the end of the assay, media were carefully removed, and the cell monolayers were washed 2 times using the cell culture medium with antibiotics. The HT-29 cells were then lysed with 0.1% Triton-100X, and suspension was used for colony forming unit (CFU) enumeration.

RV Infection of Polarized HT-29 Cells

Earlier studies have demonstrated that RV can effectively infect HT-29 cells [40]. However, the efficacy of RV replication could be affected by HT-29 polarization status and RV strains used, which prompted us to determine the optimal conditions for RV infection in HT-29 cells. The infectivity of RV Wa strain to polarized HT-29 cells was evaluated using tenfold dilutions [1×10^8 to 1×10^3 focus forming units (FFU/mL)] of RV Wa. RV infection and quantification were measured as described previously [41].

Probiotic Treatment Regimens

We tested three different probiotic treatment regimens (pre-inoculation, co-inoculation, and pre-incubation and co-inoculation) to determine their effects on RV infection:

1. Pre-inoculation: This approach mimics the in vivo effects of probiotics administered prior to RV infection as a prophylactic measure in humans. Our rationale was since probiotic treatment induces beneficial changes in the host cells by up-regulating innate immune and tight junction genes that would either prevent or inhibit RV replication.
2. The co-inoculation: This regimen was used to model probiotic administration during ongoing RV infections in infants. Our hypothesis was that probiotic and RV may share similar binding sites on HT-29 cells, and thus probiotics could interfere with RV binding when probiotics were mixed with the RV particles and then allowed to infect HT-29 cells simultaneously.
3. Pre-incubation and co-inoculation: Our rationale was that incubating RV with probiotics would provide sufficient time to induce structural and functional alterations that would not be possible in direct co-inoculation experiment. We expected that direct interactions between probiotics and RV may alter ligand/receptor expression needed for productive infection of HT-29 cells.

(a) Pre-inoculation: probiotic bacteria to HT-29 cell ratio 100:1 was used to pretreat the HT-29 cell monolayers for 1, 2, and 4 h prior to RV Wa infection.
(b) Co-inoculation: RV and probiotic bacteria were mixed at the ratio of 1:100 and were used to inoculate the HT-29 cells.
(c) Pre-incubation/co-inoculation: a 1:100 mixture of RV and probiotic bacteria was prepared and incubated at room temperature for 1 and 2 h. At respective time interval, suspension was used to infect HT-29 cells.

Optimizing Probiotic Cell Killing

The cyclic freezing–thawing was used to kill the probiotic cells to better preserve the outer membrane structure compared to dry heat killing. For this, 5 mL of OD adjusted bacterial cultures were frozen (− 80 °C) for 24-, 48-, 72-h, and 1-week time interval and followed by thawing at 37 °C and an aliquot of the culture was used to enumerate the number of live bacterial cells on the respective

solid agar. Increasing freeze–thaw cycles (7 freeze cycles for 1-week incubation) decreased the number of live cells but it did not result in 100% loss of viability. Thus, the dry heat killing method was used to conduct the experiment. The dry heat-killing protocol was optimized for these probiotics by incubating at different temperatures and for variable durations. The lowest temperature and the shortest duration that resulted in 100% loss of viability were selected to ensure that the bacterial outer membrane surface architecture was preserved as much as possible. The optimal condition was achieved for *E. coli* Nissle using 65 °C for 5 min [42] and for *L. acidophilus*, *L. rhamnosus* GG, and *B. lactis* Bb12 using 65 °C for 10 min. These conditions were used throughout the study. Killed bacteria were pre-incubated with the HT-29 cells for 4 h to determine the anti-RV effects.

Probiotic-RV Binding Using Flow Cytometry

Binding of probiotics to RV was determined by flow cytometry as described previously [13]. Briefly, probiotic bacteria stained with 5 µM SYTO 9 (Life Technologies, Carlsbad, CA, USA), followed by incubation with semipurified RV or Alexa Fluor 647 (Life Technologies, Carlsbad, CA)–conjugated rotavirus like particles (VLP) at 37 °C for 1.5 h. The unbound virus particle on the probiotic bacteria were removed by washing 3 times with sterile PBS and bacteria were incubated with Alexa Fluor 647–conjugated anti-RV mAb (clone RG23B9C5H11) or isotype control at 4 °C for 45 min. The samples were washed and bacterial-RV complexes were acquired using BD Accuri C6 flow cytometer (Ann Arbor, MI, USA).

Selection of Probiotics and In vitro Strategy to Investigate Anti-RV effects

Unlike co-incubation strategies, pre-incubation of probiotic has a number of advantages to demonstrate the probiotics anti-RV properties like the following: (i) probiotics can induce host innate and adaptive responses like defensins, anti-microbial peptides, etc. that might prevent or inhibit RV infection; (ii) probiotics can up-regulate tight junction proteins like ZO1 and occludin, thereby improve gut barrier function resulting in prevention/inhibition of RV infection; and (iii) probiotics that are strongly adhered to host cells might even compete or interfere with RV binding activity. Because of these advantages and as expected, outcome of different probiotic treatment strategies led us to investigate the mechanisms regulating probiotic effects using the pre-incubation regimen. We have focused on evaluating two probiotics (*E. coli* Nissle and *L. acidophilus*) that showed significant anti-RV properties compared to other two probiotics when incubated for 4 h.

Effect of *E. coli* Nissle and *L. acidophilus* Pretreatment on HT-29 Cells

Prior to conducting the experiments**,** we assessed the cytotoxicity of *E. coli* Nissle and *L. acidophilus* pre-treatment. The total number of cells and % dead cells were compared to no probiotic treatment at 0 and 4 h using conventional trypan blue staining technique.

Effects of *E. coli* Nissle and *L. acidophilus* Supernatant on RV Infection

Bacterial culture supernatants were harvested to determine their contribution to the observed anti-RV properties of *E. coli* Nissle and *L. acidophilus*. Briefly, overnight grown probiotic culture was centrifuged for 10,000 × *g* for 10 min to collect the supernatants. Collected supernatants were filtered through 0.22-micron filter to generate cell-free extracts. Further, portions of the original filtrates were used to prepare 10 × filtrates using vacuum concentrator (Thermo Fischer Scientific, Waltham, MA, USA). Instrument was run on low vacuum mode to preserve any labile molecule until one tenth of volume remained. In vitro pre-incubation experiment was performed at 4 h of treatment with 1 × and 10 × cell-free extract as described above.

RT² Profiler™ PCR Arrays Analysis

The expressions of sets of 84 genes involved in each PCR arrays, i.e., Human Tight Junction (TJ), Innate Immune Response were determined using RT^2 Profiler™ PCR Array (Qiagen, Array # PAHS-143Z and PAHS-148Z, Germantown, MD, USA). The list of genes, 96 well format, protocol used to perform these arrays can be found on Qiagen RT^2 Profiler PCR array [42, 43]. The genes list for TJ array included critical genes encoding proteins that form impermeable barriers between epithelia cells to regulate polarity, proliferation and differentiation (https://www.qiagen.com/us/shop/pcr/primer-sets/rt2-profiler-pcr-arrays?catno=PAHS-143Z#geneglobe) while for, innate immune response and PRR signaling included cellular growth and development, proliferation and maintenance, and anti-inflammatory and pro-inflammatory responses and antimicrobial responses and cell apoptosis- associated genes. HT-29 cells in 48 well tissue culture plates were treated with *E. coli* Nissle and *L. acidophilus* for 4 h and then infected with RV as described previously [41]. Controls were included in the experiment: *E. coli* Nissle, *E. coli* Nissle+RV, *L. acidophilus*, *L. acidophilus*+RV, and RV. Treated HT-29 cells were washed and suspended in the TRIzol reagent (Life technologies, Carlsbad, CA USA). Total RNA was extracted from the wells using the miRNeasy Mini

Kit (Qiagen, Germantown, MD),and traces of DNA were removed using the Qiagen RT2 First Strand Kit (Qiagen, Germantown, MD, USA). The cDNA was synthesized using the Qiagen RT2 First Strand Kit and analyzed using RT² Profiler™ PCR Arrays. The Ct-values for each gene were normalized using house-keeping genes that were included in the TJ and innate response arrays. Subsequently, fold-changes in genes expression were determined using the ΔΔCt method. To examine the potential functions and cellular pathways that were modulated in HT-29 cells in response to different treatments, ingenuity pathway analysis (IPA; Qiagen, Redwood City, CA, USA) was performed as described previously [44]. Each treatment was performed in four replicate wells and was repeated in three independent experiments.

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons test was used for the probiotic adhesion assay, while unpaired *t*-test was used to analyze the results of the RV infection of HT-29 cells. The data generated in all other experiments were analyzed using two-way ANOVA followed by Tukey's multiple comparisons. ANOVA was also used to analyze the qPCR data. A fold change of $\pm 1.5 \geqslant$ or $\leqslant 1.5$ and a p value ≤ 0.05 was used to determine significant differences in the expression of the genes. All the statistical analysis were performed using a GraphPad Prism software (San Diego, CA). Significance level of the data analyzed in IPA was determined by default Fisher's Exact test. In all statistical analysis a p value of ≤ 0.05 was used to determine level of significance.

Results

TEM Confirmed Polarization of HT-29 Cells

HT-29 cells possessed both differentiated and undifferentiated epithelial cells of different types including goblet cells, M cells, and enterocytes, more closely mimicking in vivo conditions when grown in the presence of galactose [34, 45]. Thus, we adapted the above protocol to induce HT-29 cells and confirmed the polarized state of HT-29 cells. Transmission electron microscopy (TEM) imaging indicated that polarized HT-29 cells were characterized by the presence of dense granules, large vacuoles, tight junctions, and microvilli (Fig. 1A, B), closely resembling the in vivo structure of intestinal epithelial cells.

Probiotic Bacteria Adhere to HT-29 Cells

Probiotic bacteria like *B. lactis* Bb12 and *E. coli* Nissle were shown to adhere to epithelial cell lines like Caco2; however, many factors are also known to influence the host cell adhesion ability [46]. Thus, in the present study, we confirmed the probiotic ability to adhere to the HT-29 cells before investigating their anti-RV properties. Compared to 30 min incubation (data not shown), higher numbers of bacterial cells adhered to HT-29 cells at 60 min. Gram-negative bacteria *E. coli* Nissle and *E. coli* K-12 adhered more efficiently to HT-29 cells compared to Gram-positive probiotic bacteria (Fig. 2A).

There were no significant differences in the binding ability of the Gram-positive probiotic bacteria where 10% of bacterial cells were bound to HT-29 cells.

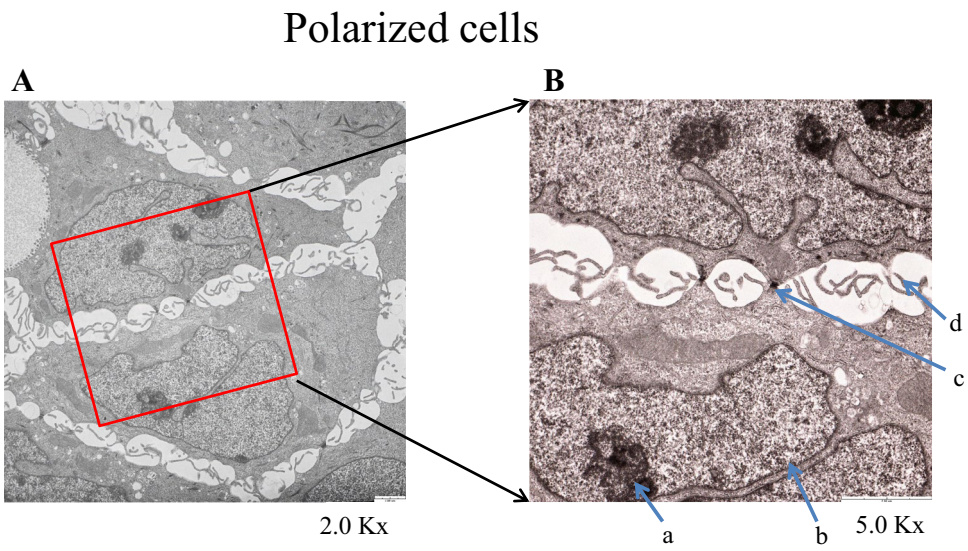


Fig. 1 TEM cross section images of induced HT-29 cells in the presence of galactose and in the absence of glucose in cell culture medium. (**A**) 2K magnification and (**B**) 5K magnification of selected image A, where arrows respectively indicate (a) dense granule (b) large vacuole (c) tight junction and (d) microvillus presence

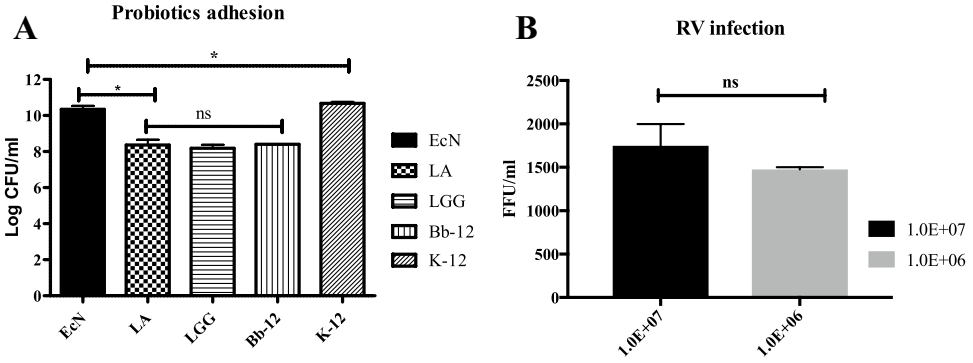


Fig. 2 **A** Probiotic adhesion assay confirms tested probiotics efficiently adhere to polarized HT-29 cells after 60 min of incubation. EcN: *E.coli Nissle*, LA: *L. acidophilus*, LGG: *L. rhamnosus* GG, and Bb12: *B.lactis* Bb12. One-way ANOVA, Sidak's multiple comparisons test where * refers to $p \leq 0.05$. **B** RV Wa strain infects polarized HT-29 cells at MOIs 1 and 0.1. Unpaired *t*-test. ns, non-significant

RV Infects Polarized HT-29 Cells

Rhesus monkey kidney (MA104) cells are more commonly used for the growth and characterization of both animal and human culture-adapted RVs. In addition to MA104 cells and depending upon the strain, RVs were shown to infect other types of continuous cell lines including HT-29 cells [40, 47]. Our immediate question to answer was whether polarized HT-29 cells are equally efficiently infected by RV Wa strain used in our present study. Concurrent with previous findings, RV Wa strain infected polarized HT-29 cells, but to some degree less efficiently compared to MA104 cells. MOI of RV Wa strain for MA104 cells varied from 0.1 to 0.5 while HT-29 cells required 1 to 0.1 MOI [47]. Infection with either 1.0E+07 and 1.0E+06 FFU of RV resulted in comparable number of FFU (Fig. 2B); therefore, we used 1.0E+07 FFU for our subsequent infections. Interestingly, no RV was detected when infected with lower than 1.0E+06 FFU.

Pre- and Co-inoculation with *E. coli* Nissle, *L. rhamnosus* GG, and *L. acidophilus* Inhibited RV Replication in HT-29 Cells

Except *B. lactis* Bb-12, other probiotic pretreatments significantly ($p < 0.05$) inhibited RV replication in HT-29 cells (Fig. 3A). Irrespective of *E. coli* Nissle pre-treatment duration, RV Wa replication was inhibited, although most inhibition was observed at 4 h of pre-treatment. While comparable trends were observed for *L. acidophilus* and *L. rhamnosus* GG, the magnitude of the inhibition was lower, and reached significance only at 4 h of pre-treatment (Fig. 3A). In the co-inoculation treatment, an overall similar trend was observed with pre-treatment, wherein co-incubation with *E. coli* Nissle, *L. acidophilus* and *L. rhamnosus* GG for 4 h resulted in significant RV inhibition in HT-29 cells (Fig. 3B). As observed with pre-treatment above, *B. lactis* Bb12 co-inoculation did not affect RV replication (Fig. 3B). In the pre-incubation and co-inoculation regimen, like pre- and co-inoculation experiments, *E. coli* Nissle induced the most significant inhibition of RV infection, followed by *L. acidophilus* and *L. rhamnosus* GG that resulted in a slight numeric, but not significant inhibition. In contrast, *B. lactis* Bb12 incubation and co-inoculation resulted in significantly increased RV replication compared to RV alone and other probiotic treatments (Fig. 3C). Interestingly, RV alone infectivity in HT-29 cells decreased progressively during incubation at room temperature, which precluded us from performing the 4 h incubation and co-inoculation experiment.

Observed Anti-RV Effects of Probiotics Were Induced by Live Bacteria

To investigate the mechanisms of the probiotic effects, we first sought to understand whether live probiotics are essential to induce the observed anti-RV effects. Except for *B. lactis* Bb-12, pre-treatment with other live probiotics resulted in inhibition of RV as observed previously. However, this effect was not observed when heat-killed bacteria were used. Neither live nor dead *B. lactis* Bb-12 pre-treatment resulted in RV inhibition (Fig. 4A).

Only *E. coli* Nissle Binds to RV But Not *L. acidophilus*, *L. rhamnosus* GG, and *B. lactis* Bb12

The ability of probiotics to directly bind RVs is one of the mechanisms that contributes to the anti-RV properties as

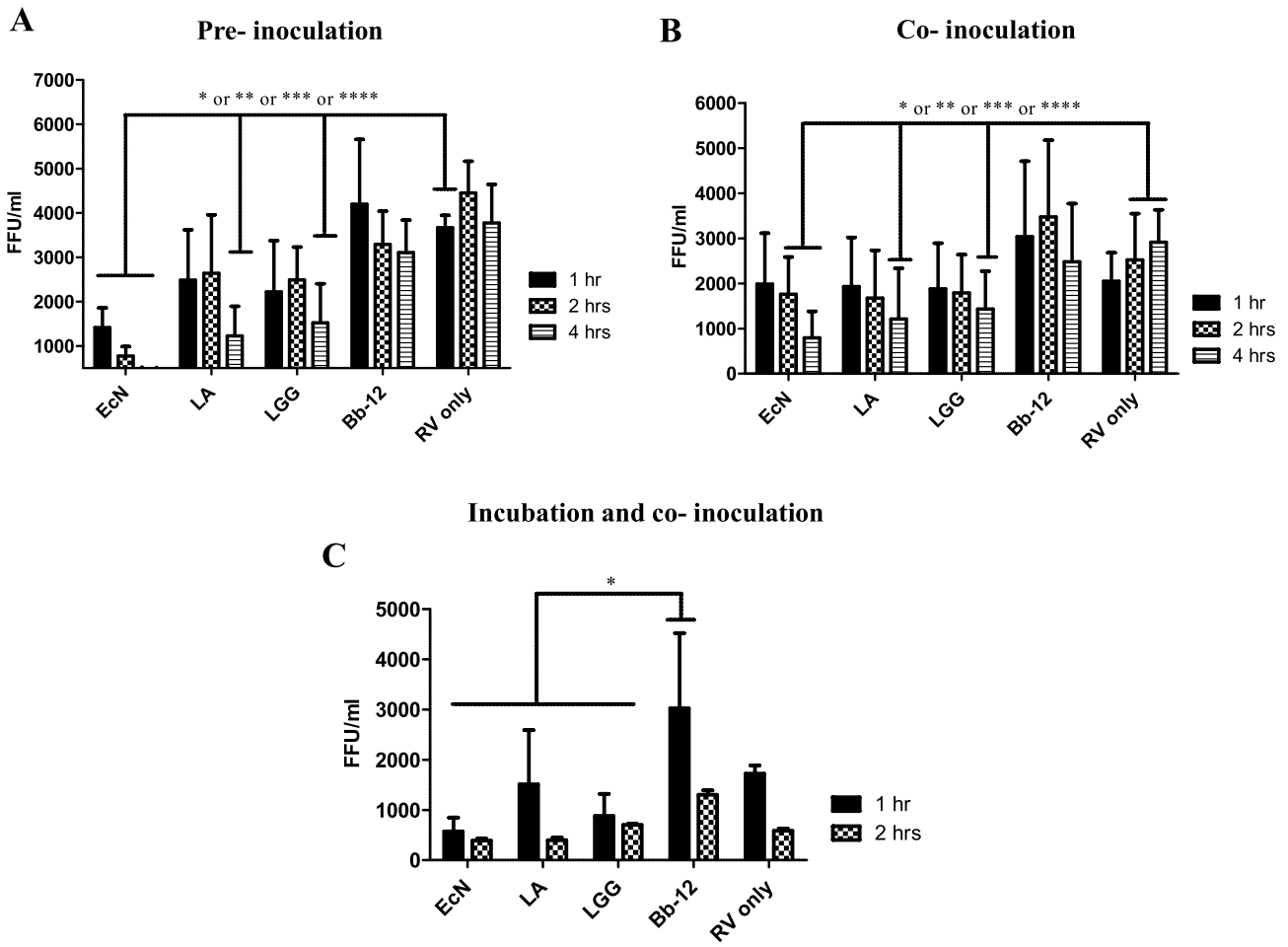


Fig. 3 Probiotics treatment strategies to demonstrate the anti-RV properties. **A** pre-inoculation, **B** co-inoculation, and **C** incubation and inoculation. EcN: *E. coli Nissle*, LA: *L. acidophilus*, LGG: *L. rhamnosus* GG, and Bb12: *B.lactis* Bb12. Two-way ANOVA, Tukey's multiple comparisons test where * refers to $p \leq 0.02$, ** refers to $p \leq 0.002$, *** refers to $p \leq 0.0005$, **** refers to $p \leq 0.0001$, and ns refers to non-significant

we reported previously [13]. Therefore, we investigated the probiotics-RV binding properties. Less than 2% of *L. acidophilus* and *B. lactis* Bb12 bound to RV (Fig. 4B), while significantly higher percentages (15%) of *E. coli* Nissle bind to RV and no RV binding by *L. rhamnosus* GG was evident.

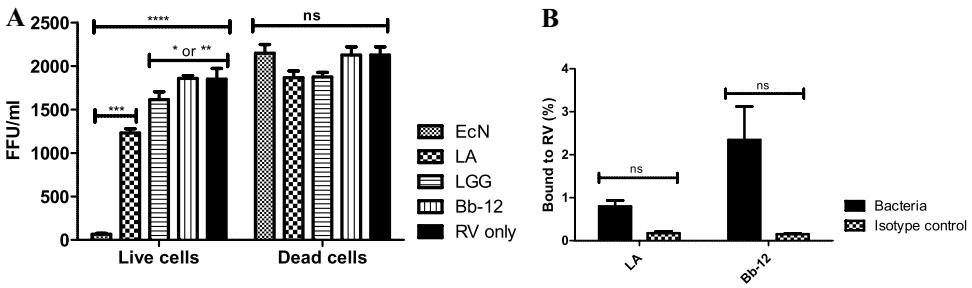


Fig. 4 **A** Assessment of dead bacterial cell effects on RV infection to HT-29 cells. EcN: *E.coli Nissle*, LA: *L. acidophilus*, LGG: *L. rhamnosus* GG, and Bb12: *B.lactis* Bb12. Two-way ANOVA, Tukey's multiple comparisons test where * refers to $p \leq 0.02$, ** refers to $p \leq 0.002$, *** refers to $p \leq 0.0005$, **** refers to $p \leq 0.0001$, and ns refers to non-significant. **B** Probiotics RV binding ability assessed by flow cytometry. Two-way ANOVA, Tukey's multiple comparisons test where ns refers to non-significant

E. coli Nissle and *L. acidophilus* Pre-treatments Did Not Result in Cytotoxic Effects in HT-29 Cells

Increasing duration of probiotic treatment is generally not recommended due to rapid multiplication of bacteria that ultimately damage the host cell monolayer. In our present study, except for *E. coli* Nissle, probiotic treatments did not induce any monolayer alterations. After, 4 h of treatment, *E. coli* Nissle induced up to 50% HT-29 cells monolayer clumping, which raised a question whether the observed superior anti-RV effects of *E. coli* Nissle were due to the loss of host cells viability resulting in reduced numbers of HT-29 cells available for RV infection. To address this concern, we determined the total number of cells and % dead cells. There was no difference in the total numbers and viability of HT-29 cells compared to no probiotic treatment controls. This suggests that the observed anti-RV effects were due to probiotic induced alterations of the cell physiology and/or structure (Fig. 5A).

Both *E. coli* Nissle and *L. acidophilus* Culture Supernatants Possess Anti-RV Properties

To establish if probiotic secreted factors present in culture supernatant induce beneficial host responses to prevent or interfere with RV replication in HT-29 cells, we assessed the effects of pretreatment with 1× and 10× cell free culture supernatants prepared from probiotics grown in their respective media [35, 36]. Both 1× and 10× *E. coli* Nissle and *L. acidophilus* supernatants possessed RV inhibitory properties; however, therewere no differences between pretreatment with 1× and 10× *E. coli* Nissle and *L. acidophilus* supernatants. This suggests that *E. coli* Nissle and *L. acidophilus* secrete biologically active molecules essential for the observed anti-RV effects (Fig. 5B).

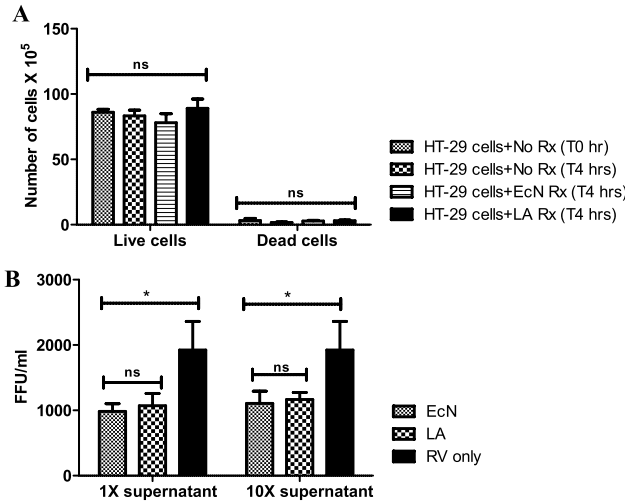


Fig. 5 **A** Cytotoxic effect of HT-29 cells evaluated by percentage of number of live and dead cells at 4 h of *E. coli* Nissle and *L. acidophilus* treatment. Two-way ANOVA, Tukey's multiple comparisons test where ns refers to non-significant. **B** Effect of *E. coli* Nissle and *L. acidophilus* culture supernatants (1× and 10×) on RV replication. Two-way ANOVA, Tukey's multiple comparisons test * refers to $p \leq 0.04$

IPA

To further study the mechanisms of the *E. coli* Nissle and *L. acidophilus* anti-RV properties, we performed targeted PCR arrays and compared the fold-change expression of genes in HT-29 cells in probiotic (*E. coli* Nissle, *L. acidophilus*, *E. coli* Nissle + RV, and *L. acidophilus* + RV) and RV infection alone treated groups (Tables S1 and S2 and Figs. S1 and S4). A comprehensive summary of gene regulations was depicted as radar diagrams (Figs. 6 and 11). The IPA tool was used to predict the biological functions by analyzing networks and canonical pathways. The top two gene regulatory networks associated with *E. coli* Nissle, *L. acidophilus*, RV, *L. acidophilus* + RV, and *E. coli* Nissle + RV based on focus molecules and scores are shown in Table 1.

RV Has Limited Influence on TJ Gene Functions and Pathways Compared to Either of the Probiotic Treatments

RV influenced a limited number of pathways compared to probiotic and probiotic + RV treatments. For example, RV did not influence the electric resistance, transmigration of granulocytes (lymphocyte and neutrophil) functions (Fig. 7A). Correspondingly, only leukocyte excavation and PTEN signaling were affected by RV treatment (Fig. 7B). Paxillin signaling, which is involved in cell adhesion, was activated only by *E. coli* Nissle or *E. coli* Nissle + RV but not in other treatments. *E. coli* Nissle or *E. coli* Nissle + RV affected the most functions and pathways compared to others. Interestingly, *L. acidophilus* + RV treatment activated functions and pathways that were not affected by RV treatment alone. Though RV treatment influenced the function and pathways the least, it affected the greatest number of upstream regulators compared with that of probiotic or probiotic + RV treatments. The one upstream regulator CDX2, was only influenced by probiotic + RV treatment (Fig. 7C). CDX2 is a nuclear transcriptional factor involved in intestinal cell proliferation, differentiation, adhesion, and apoptosis. Overall, *E. coli* Nissle or *E. coli* Nissle + RV treatment affected several biological functions and cellular pathways while RV alone influenced the least. The important TJ gene clusters that were differentially regulated on RV and probiotics alone (*E. coli* Nissle and *L. acidophilus*) or probiotic + RV (*E. coli* Nissle + RV, and *L. acidophilus* + RV) treatments are indicated in Table 2.

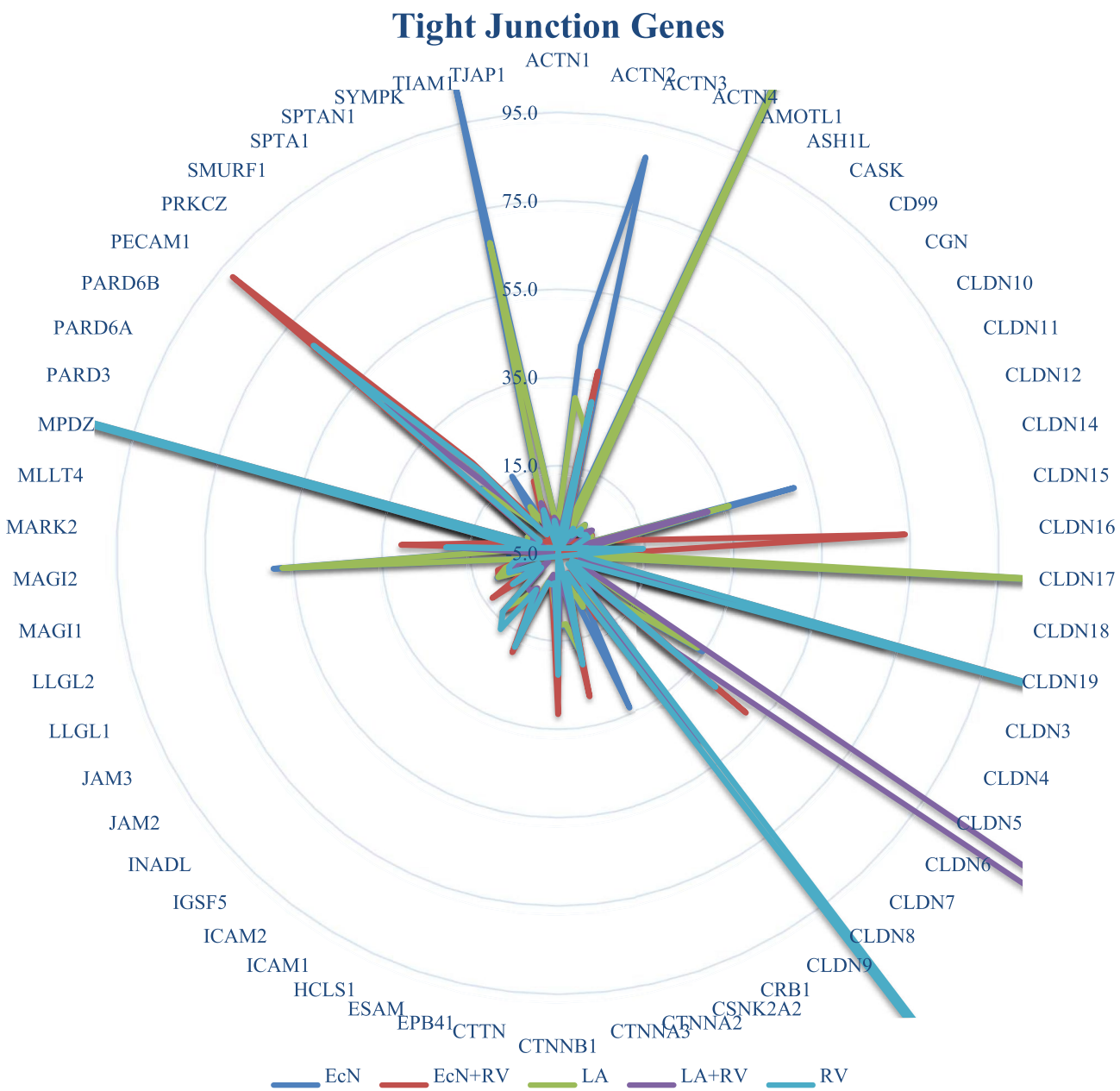


Fig. 6 Fold change expression of TJ genes for different treatment groups were depicted in the form of radar diagram by normalizing HT-29 cells basal level expressions. RV alone infection resulted in increased expression of CLDN 8 and 19 genes that were some extents reduced on *L. acidophilus* pre-treatment. Interestingly, MAGI2 gene expression was down regulated on RV infection but not rescued by probiotics pre-treatment. EcN, *E.coli Nissle*; LA, *L. acidophilus*

E. coli Nissle Treatment Improves Cell–Cell Adhesion and Cell Contact

The five major predicted TJ cellular functions affected by the *E. coli* Nissle treatment alone included: cell polarity formation, cell–cell contact, formation of adherens junctions, cell movement, and cell–cell adhesion, wherein more specifically, cell–cell adhesion and cell contact biological functions were highly activated while, cell polarity was least activated (Fig. S2). The genes like ACTN1&4, AFDN, alpha actinin, CTNNA3, ERK1/2, JAM3, and MAGI1 lead to activation of cell–cell contact, while AFDN, alpha catenin, CTNNA3, and MAGI1 genes are responsible for the activated biological function of cell–cell adhesion. A complete list of genes involved in the respective biological functions is highlighted in Fig. S2. Similar cellular pathways (cell polarity formation, cell–cell contact, cell–cell adhesion, polarity of cells, formation of adherens junctions, and cell movement) were affected

Table 1 Top gene regulatory networks associated with treatment of the HT-29 cells with *E. coli* Nissle, *L. acidophilus*, RV, *L. acidophilus* + RV, and *E. coli* Nissle + RV based on focus molecules and scores

Tight junction			Innate immune response		
Top network involved	Score	Focus molecules	Top network involved	Score	Focus molecules
E. coli Nissle					
(1) Cell morphology, cell-to-cell signaling and interaction, cellular assembly, and organization	58	23	(1) Cell morphology, cellular function and maintenance, protein synthesis	16	8
(2) Cell-to-cell signaling and interaction, cellular assembly and organization, cellular function, and maintenance	48	20	(2) Cell death and survival, infectious diseases, organismal survival	12	6
RV					
(1) Cell-to-cell signaling and interaction, cellular assembly and organization, cellular function, and maintenance	67	25	(1) Cell-to-cell signaling and interaction, infectious diseases, protein synthesis	23	10
(2) Cellular assembly and organization, cellular function and maintenance, tissue development	21	25	(2) Cell-to-cell signaling and interaction, cellular movement, inflammatory response	15	7
E.coli Nissle + RV					
(1) Cell morphology, cellular assembly and organization, cellular function, and maintenance	65	25	(1) Cell-to-cell signaling and interaction, cellular movement, hematological System Development and Function	24	11
(2) Cell-to-cell signaling and interaction, cellular assembly and organization, cellular function, and maintenance	37	16	(2) Antimicrobial response, cell death and survival, inflammatory response	21	10
L. acidophilus			NA		
(1) Cell morphology, cellular assembly and organization, cellular development	55	22			
(2) Cell-to-cell signaling and interaction, cellular assembly and organization, cellular function, and maintenance	40	17			
L. acidophilus + RV			NA		
(1) Cell-to-cell signaling and interaction, cellular assembly and organization, cellular function, and maintenance	75	27			
(2) Cardiovascular system development and function, cell morphology, cell-to-cell signaling, and interaction	18	9			

Only top networks are shown, where "score" reflects number of network eligible molecules; the higher scores indicate that the given network is more likely modulated by different treatment. Focus molecules are the affected genes in different treatments and were considered for generating networks. Since only *E. coli* Nissle showed significantly higher RV binding characteristic, innate response PCR array was not analyzed for *L. acidophilus* or *L. acidophilus* + RV group

NA not applicable

in *E. coli* Nissle + RV-treated cells; however, one additional pathway was also influenced, i.e., polarity of cells. Though all cellular pathways were activated, cell–cell contact, and polarity of cells were highly triggered (Fig. 8). The polarity of cells was activated due to ACTN4, F-actin, JAM3, and PRKCZ genes while the cell–cell contact was activated via ACTN1, ACTN4, AFDN, alpha catenin, ERK1/2, MAGI1, and Par6 genes.

Cell–Cell Contact Activated While Cell Movement Inhibited upon *L. acidophilus* Treatment

Affected cellular functions by the LA treatment alone included: cell polarity formation, cell–cell contact, organization of cytoskeleton, formation of cellular protrusions, formation of adherens junctions, and cell–cell adhesion. All the cellular functions were activated of which, cell–cell contact, and cell–cell adhesion were highly stimulated by the LA treatment (Fig. S3). Genes involved in the respective cellular functions are illustrated in Fig. S3. The genes ACTN1, AFDN, alpha catenin, CTNNA3, and MAGI1 simultaneously activated the functions of cell–cell contact and cell–cell adhesion (Fig. S3). While after LA + RV treatment, formation of tight junctions, cell polarity formation, cell–cell contact, formation of adherens junctions, cell movement, formation of intercellular junctions, and polarity of cells were affected (Fig. 9).

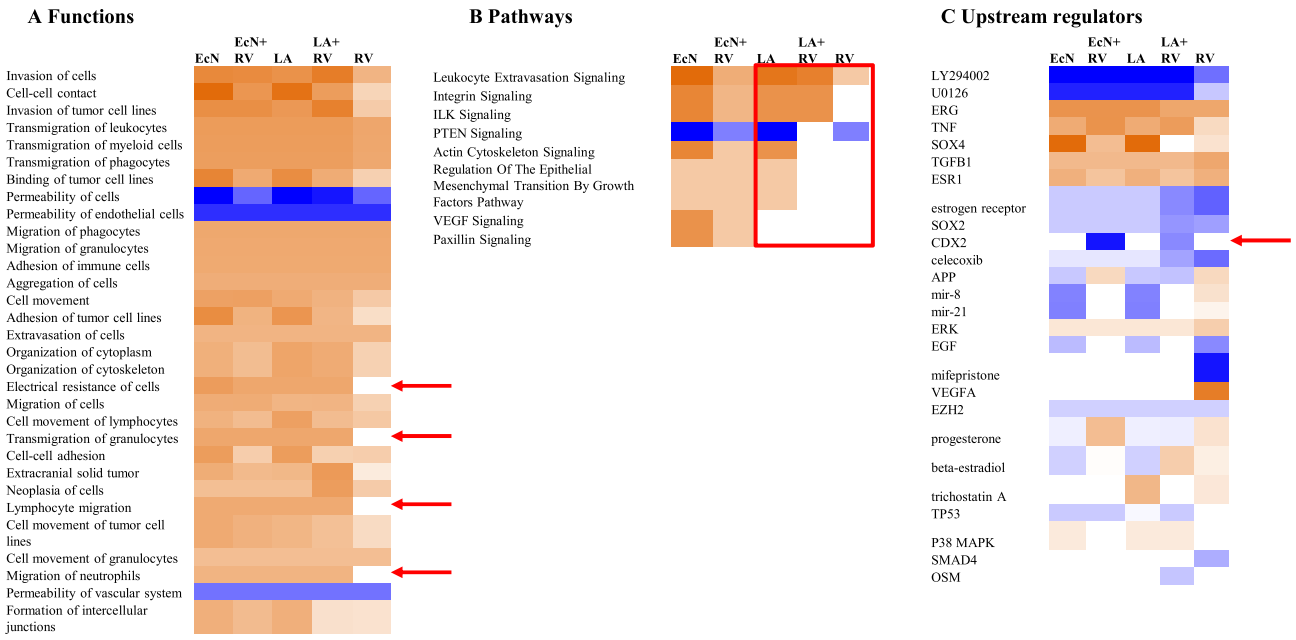


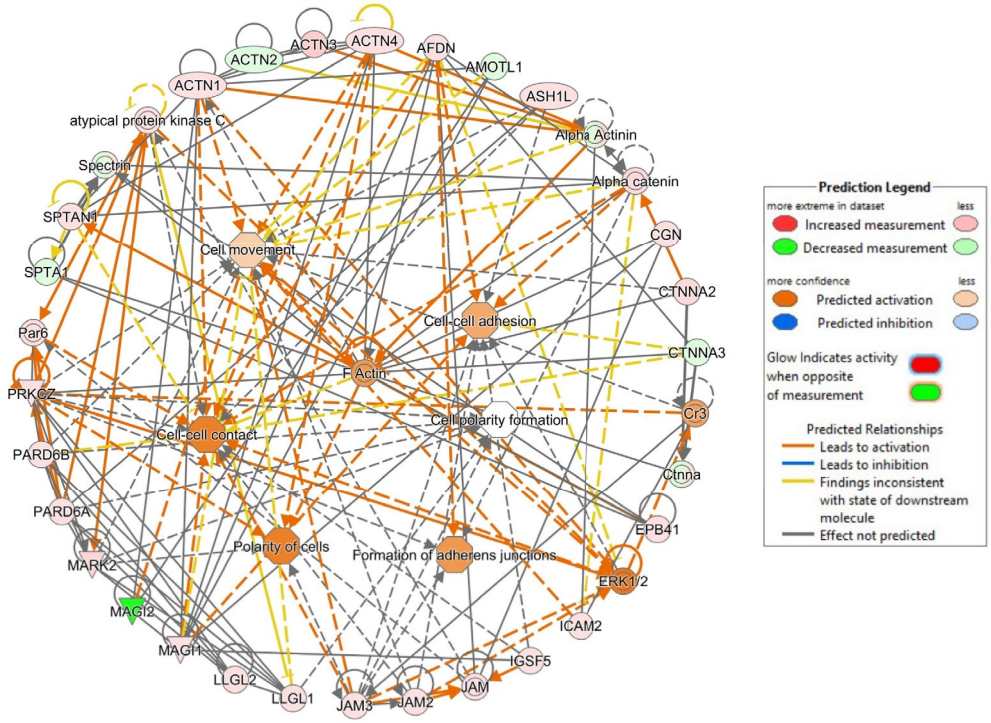
Fig. 7 An overall summary of TJ genes' IPA predicted **A** cellular functions, **B** their corresponding pathways that are in turn regulated by **C** upstream regulators. The heatmap shows the predicted activation (blue color) or inhibition (brown) of several selected cellular functions for each of the treatment groups. The intensity of color correspondence to expression status. The functions, pathways, and upstream regulators are organized in descending order of –log(*p*) values that represents the extent to which the gene set for a particular group overlaps with the given cellular function/pathways. The red color arrows and a box highlight unique signatures at each level. EcN, *E.coli* Nissle; LA, *L. acidophilus*

Compared to other cellular functions, cell–cell contact, and cell movements were highly activated or inhibited, respectively. The activated function of cell–cell contact was due to AFDN, ERK1/2, JAM3, and MPDZ genes while inhibited cell movement function was due to AFDN, CLDN19, CLDN5, LLGL1, MAGI2, PARD3, and PATJ genes (Fig. 9).

Table 2 The fold change expressions of selected tight junction (TJ) genes in signaling pathways

Gene symbol	Name	*E. coli* Nissle	*E. coli* Nissle + RV	*L. acidophilus*	*L. acidophilus* + RV	RV
CLDN14	Claudin-14	50.56		35.26	30.27	
CLDN19	Claudin-19	30.62	140.17	29.71	71.68	142.09
CLDN8	Claudin-8	33.81		31.45	48.42	326.40
CTNNB1	Catenin Beta 1	11.66	31.51	11.35	15.55	22.61
ICAM2	Intercellular adhesion molecule 2	8.16	10.00	7.92	5.62	16.61
JAM2	Junctional adhesion 2	2.66	13.04	2.96	5.55	7.48
JAM3	Junctional adhesion 3	2.84	2.76	2.80	1.95	
MAGI2	Membrane Associated Guanylate Kinase, WW And PDZ Domain Containing 2	59.71	− 83.87	57.68	− 3.66	− 24.25
PARD6A	Par-6 Family Cell Polarity Regulator Alpha	2.89	1.82	2.85	1.51	
PARD6B	Par-6 Family Cell Polarity Regulator Beta	1.99	2.25	2.00	1.87	
TIAM1	T-lymphoma invasion and metastasis-inducing protein 1	103.97		67.18		− 3.81

The fold change expressions were calculated using HT-29 cells basal level genes expressions, where a cutoff value of ± 1.5-fold change was considered to tabulate

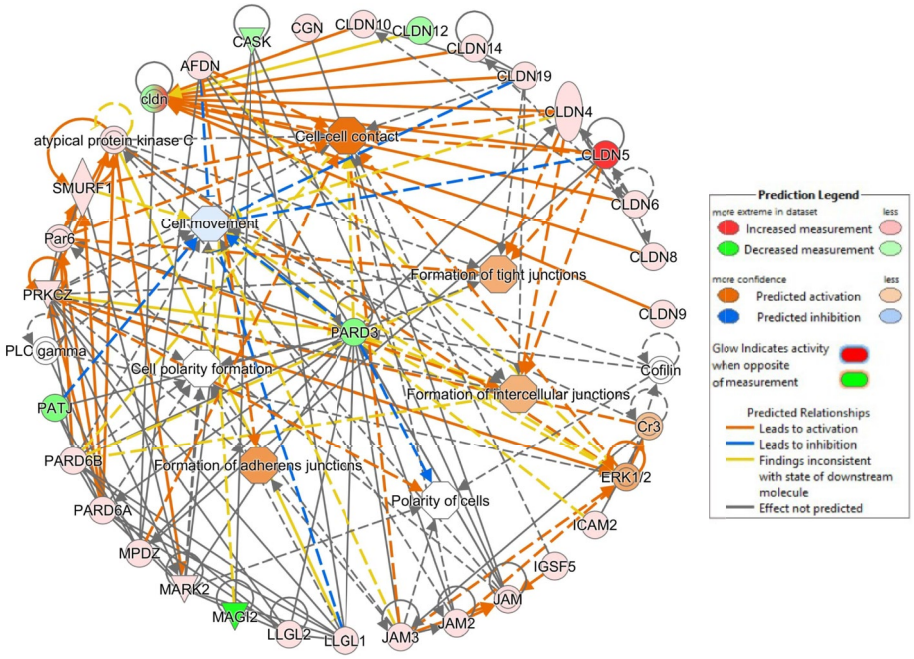


Functions Annotation	p-Value	Molecules	# Molecules
Cell polarity formation	6.09E-09	JAM3,LLGL1,MARK2,PARD6A,PARD6B, PRKCZ	6
Cell-cell contact	9.78E-08	ACTN1,ACTN4,AFDN,Alpha catenin,ASH1L,atypical protein kinase C, CTNNA3,ERK1/2,F Actin,IGSF5,JAM2, JAM3,MAGI1,Par6,PARD6B,PRKCZ,SPTAN1	17
Cell-cell adhesion	0.00000931	AFDN,Alpha catenin,ASH1L,CTNNA3, ERK1/2,IGSF5,JAM2,JAM3,MAGI1	9
Polarity of cells	0.0000225	ACTN4,F Actin,JAM3,MARK2,PRKCZ	5
Formation of adherens junctions	0.0000352	AFDN,Alpha catenin,JAM3,PRKCZ	4
Cell movement	0.000198	ACTN1,ACTN4,AFDN,Alpha Actinin,Alpha catenin,AMOTL1,ASH1L,Cr3,CTNNA2,ERK1/2,F Actin,ICAM2,JAM2,JAM3,LLGL1, MAGI1,MAGI2,MARK2,PARD6B,PRKCZ	20

Fig. 8 Ingenuity pathway analysis (IPA) predicted TJ cellular functions that are consistently activated and inhibited following *E. coli* Nissle + RV treatment. The networks of differentially expressed genes were algorithmically generated based on their connectivity such that the highly interconnected networks likely represent significant biological function. The Fischer's exact test was used to calculate a *p* value for each biological function assigned to a particular network. The name and number of molecules involved are described in the table

The Migration of Cells Is Inhibited upon RV Infection

Six TJ biological functions like formation of tight junctions, cell–cell contact, cell–cell adhesion, formation of intercellular junctions, morphology of tight junctions, and migration of cells were modulated on RV infection of HT-29 cells, wherein most of the functions were activated except migration of cells (Fig. 10). The inhibited function of migration of cells was associated with AFDN, AMOTL1, CLDN19, Cr3, LLGL1, and MAGI1 genes (Fig. 10).

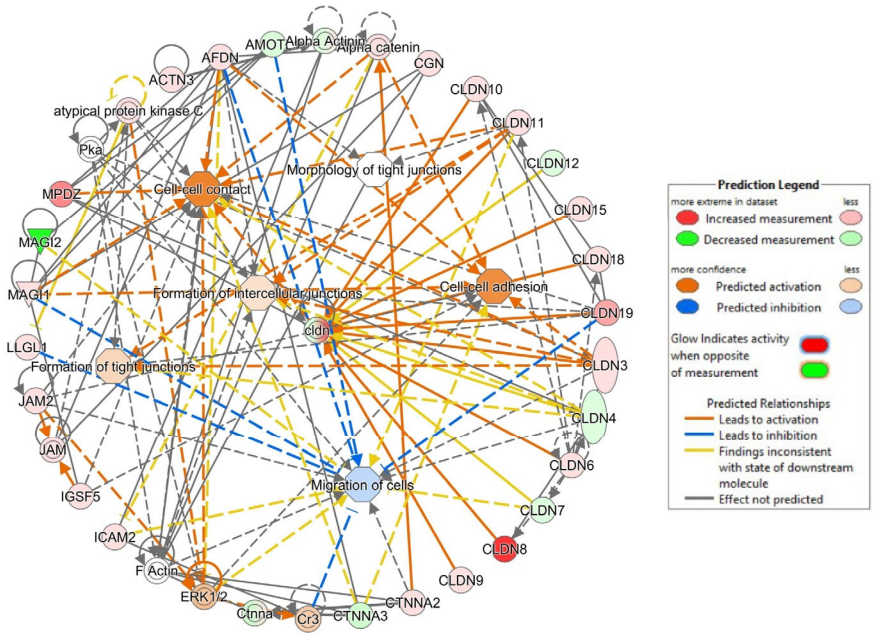


Functions Annotation	p-Value	Molecules	# Molecules
Formation of tight junctions	1.02E-10	atypical protein kinase C,CLDN19, CLDN4,CLDN5, Par6,PARD3,PARD6B,PRKCZ	8
Cell polarity formation	1.74E-10	JAM3,LLGL1,MARK2,PARD3,PARD6A,PARD6B, PRKCZ	7
Cell-cell contact	2.82E-07	AFDN,atypical protein kinase C,cldn,CLDN19,CLDN4, CLDN5,ERK1/2,IGSF5,JAM2,JAM3,MPDZ,Par6,PAR D3,PARD6B,PRKCZ,SMURF1	16
Formation of adherens junctions	0.0000446	AFDN,JAM3,PRKCZ	3
Cell movement	0.000624	AFDN,CLDN19,CLDN4,CLDN5,Cofilin,Cr3,ERK1/2,I CAM2,JAM2,JAM3,LLGL1,MAGI2,MARK2,PARD3,P ARD6B,PATJ,PLC gamma,PRKCZ,SMURF1	19
Formation of intercellular junctions	0.00000401	AFDN,atypical protein kinase C,CLDN19,CLDN4, CLDN5,Par6,PARD3,PARD6B,PRKCZ	9
Polarity of cells	0.0000309	Cofilin,JAM3,MARK2,PARD3,PRKCZ	5

Fig. 9 Ingenuity pathway analysis (IPA) predicted TJ cellular functions that are consistently activated and inhibited following LA + RV treatment. The networks of differentially expressed genes were algorithmically generated based on their connectivity such that the highly interconnected networks likely represent significant biological function. The Fischer's exact test was used to calculate a *p* value for each biological function assigned to a particular network. The name and number of molecules involved are described in the table

Innate Functions and Pathways Not Affected by RV Were Influenced in the Presence of *E. coli* Nissle Treatment

Since only *E. coli* Nissle showed significantly higher RV binding characteristics, innate response PCR array was analyzed for *E. coli* Nissle and control groups but not for LA group (Figs. 11 and S4, and Table S2). One function of innate genes not affected by RV, i.e., differentiation of blood cells, was influenced on *E. coli* Nissle or *E. coli* Nissle + RV treatments (Fig. 12A). Similarly, LPS stimulated MAPK, B cell receptor, PI3K in B lymphocytes, PI3K/AKT, role of NFAT, Nf-kB, LXR/RXR, Ga12/13 signaling were only influenced in the presence of *E. coli* Nissle treatments suggesting *E. coli* Nissle activates robust innate immune responses (Fig. 12B). For upstream regulators, RV influenced all the regulators but with lesser intensity compared with *E. coli* Nissle or *E. coli* Nissle + RV treatments (Fig. 12C). The important innate gene clusters that were differentially regulated on RV and *E. coli* Nissle alone or *E. coli* Nissle + RV treatments are indicated in Table 3.



Functions Annotation	p-Value	Molecules	# Molecules
Formation of tight junctions	6.23E-07	atypical protein kinase C,CLDN11,CLDN19,CLDN3,CLDN4	5
Cell-cell contact	0.0000089 3	AFDN,Alpha catenin,atypical protein kinase C,cldn,CLDN11, CLDN19,CLDN3,CLDN4,CTNNA3,ERK1/2,F Actin,IGSF5,JAM2,MAGI1,MPDZ	15
Cell-cell adhesion	0.000107	AFDN,Alpha catenin,CLDN3,CTNNA3,ERK1/2,IGSF5, JAM2,MAGI1	8
Formation of intercellular junctions	0.000377	AFDN,atypical protein kinase C,CLDN11,CLDN19, CLDN3,CLDN4,F Actin	7
Morphology of tight junctions	0.000421	AFDN,CLDN11	2
Migration of cells	0.00121	AFDN,AMOTL1,CLDN11,CLDN19,CLDN3,CLDN4,CLDN7 ,Cr3,CTNNA2,ERK1/2,F Actin,ICAM2,JAM2, LLGL1,MAGI1,MAGI2,Pka	17

Fig. 10 Ingenuity pathway analysis (IPA) predicted TJ cellular functions that are consistently activated and inhibited RV infection. The networks of differentially expressed genes were algorithmically generated based on their connectivity such that the highly interconnected networks likely represent significant biological function. The Fischer's exact test was used to calculate a *p* value for each biological function assigned to a particular network. The name and number of molecules involved are described in the table

E. coli Nissle Activates Innate Immunity and Inflammatory Response via TNF Production

The top 5 biological functions that were affected on *E. coli* Nissle treatment alone included: production of cytokines, production of proteins, innate immune responses, function of dendritic cells, and function of phagocytes (Fig. S5). All the functions were activated, and the genes involved in activating these functions in turn activated the robust production of TNF. On *E. coli* Nissle + RV treatment, six biological functions that were affected included, inflammatory response, cytokine and chemokine mediated signaling pathway, cell movement of granulocytes, immune response of cells, activation of phagocytes, and activation of macrophages (Fig. 13). Interestingly, activation of phagocytes and inflammatory responses were highly activated. The genes involved in the activation of inflammatory response include CXCL8, Cpla2, Eotaxin, Ifn, Ifn gamma, LBP, pro-inflammatory, and Tnf (family) genes. The detailed list of genes involved and their status in respective biological pathways can be found in Fig. 13.

NK Cells and Inflammatory Response Are Strongly Activated on RV Infection

Eight biological functions were affected on RV infection alone, i.e., activation of cells, inflammatory response, innate immune responses, production of cytokines, function of antigen

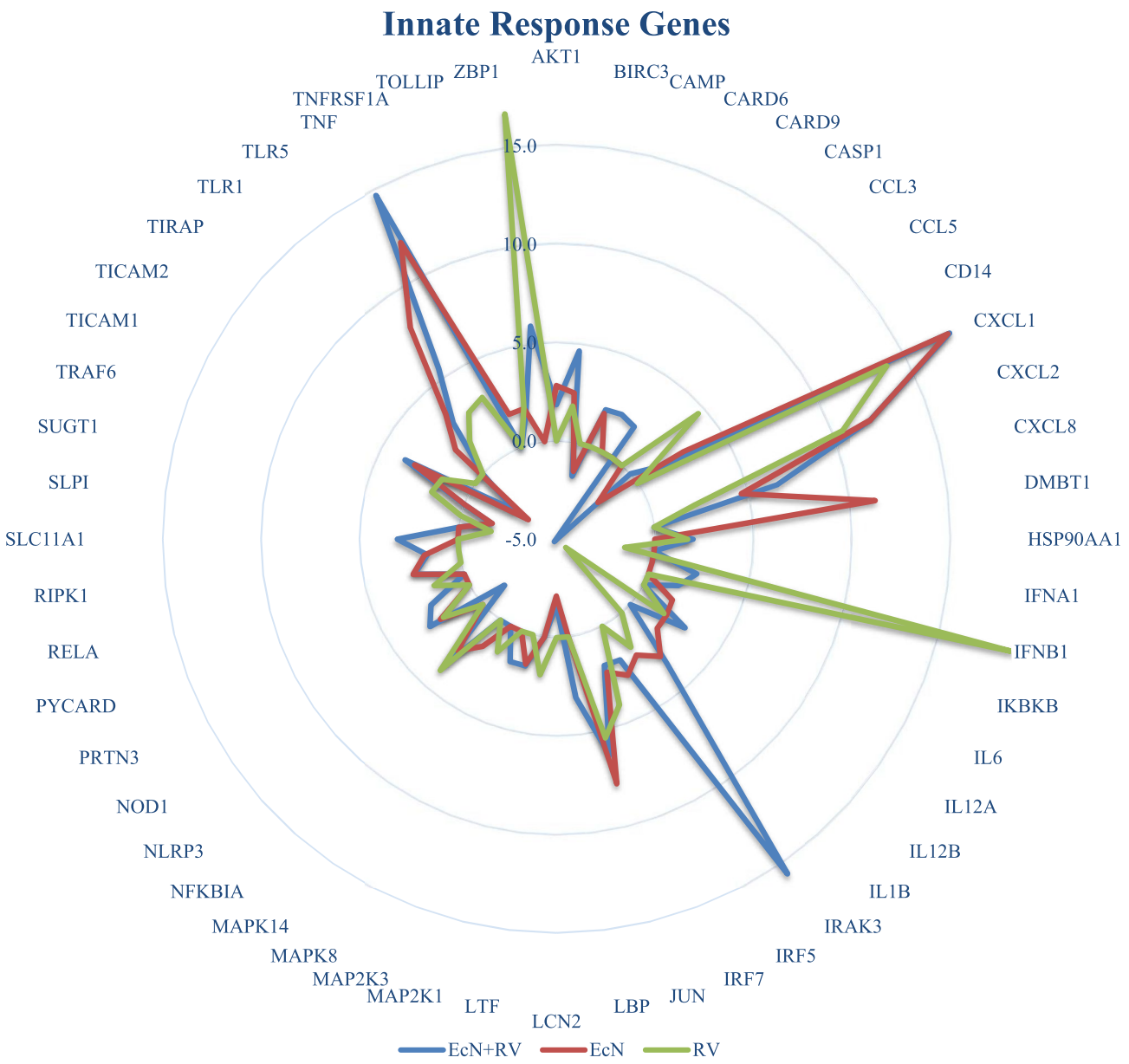


Fig. 11 Fold change expression of innate response genes for different treatment groups were depicted in the form of radar diagram by normalizing HT-29 cells basal level expressions. RV alone infection resulted in significant increase in expression of IFNB1 and ZBP1 that were reduced on *E. coli* Nissle treatment. EcN, *E.coli Nissle*; LA, *L. acidophilus*

presenting cells, activation of natural killer (NK) cells, replication of virus, and antiviral response (Fig. 14). Of these, activation of NK cells and inflammatory responses were strongly activated, and the genes involved in both these functions are shown in Fig. 14.

Discussion

Understanding interactions among pathogens, probiotics and host epithelial cells are of utmost importance to maintain enteric health. In this study we utilized polarized HT-29 cells as an in vitro model to investigate the anti-RV properties of the selected Gram-positive and Gram-negative probiotics and their underlying mechanisms. The Gram-positive and Gram-negative probiotics were tested for their ability to inhibit RV replication using established three-way treatment strategies mainly pre-inoculation, co-inoculation, and pre-incubation/co-inoculation. The *E. coli* Nissle and *L. acidophilus* in a pre-inoculation strategy showed significantly higher ability to prevent RV replication in HT-29 cells. In agreement with our findings, recent studies also highlighted the RV inhibitory effects of

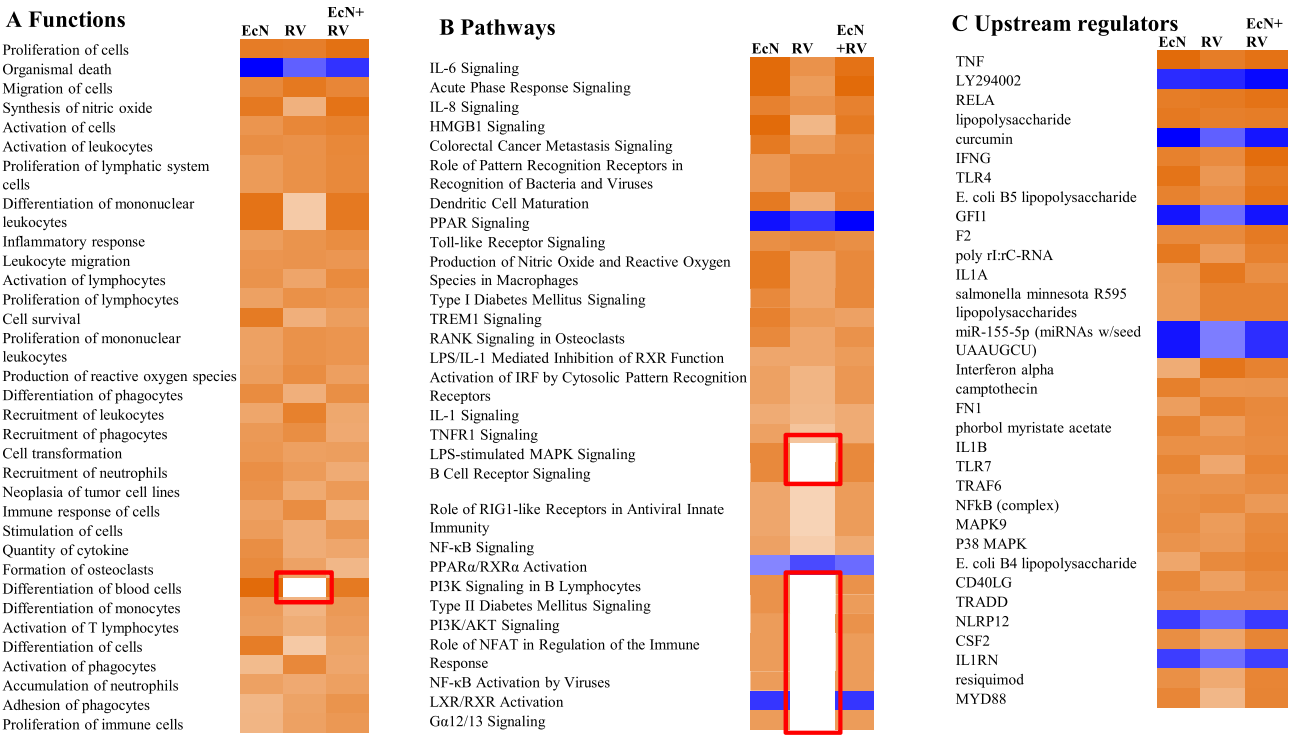


Fig. 12 An overall summary of innate response genes' IPA predicted **A** cellular functions, **B** their corresponding pathways that are in turn regulated by **C** upstream regulators. The heatmap shows the predicted activation (blue color) or inhibition (brown) of several selected cellular functions for each of the treatment groups. The intensity of color corresponds to expression status. The functions, pathways and upstream regulators are organized in descending order of –log(*p*) values that represents the extent to which the gene set for a particular group overlaps with the given cellular function/pathways. The red color box highlights the unique signatures at each level

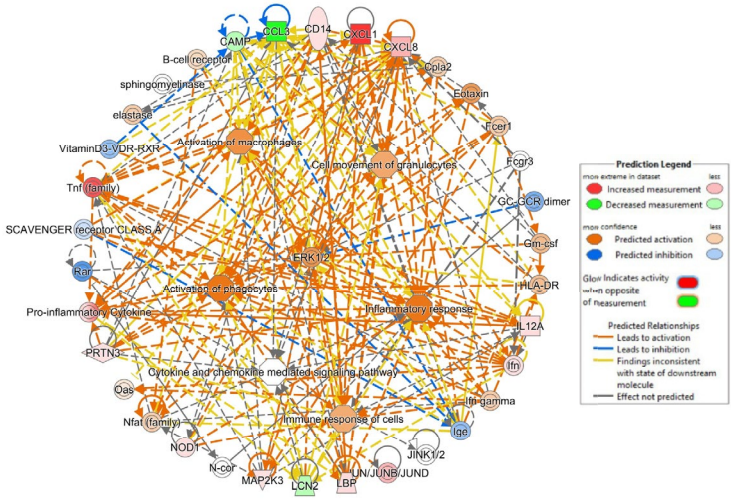
probiotics like *Lactobacillus* and *Bifidobacterium* species using in vitro cell culture system [20, 48, 49], wherein up to 50% reduction in plaque forming units (PFUs) was observed. Yet, in another study, six different *Bifidobacterium*

Table 3 The fold change expressions of selected innate genes in signaling pathways

Gene symbol	Name	*E.coli* Nissle	*E.coli* Nissle + RV	RV
AKT1	V-akt murine thymoma viral oncogene homolog 1	2.81	1.84	
CXCL8	Interleukin 8	4.68	6.54	2.22
CARD6	Caspase recruitment domain family, member 6	1.83	2.03	
CD14	CD14 molecule	2.84	1.55	
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	17.44	17.55	13.94
IFNA1	Interferon, alpha 1			-1.51
IFNB1	Interferon, beta1, fibroblast		2.34	18.75
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	1.80	1.63	
IRF5	Interferon regulatory factor 5	2.80	1.93	
IRF7	Interferon regulatory factor 7	2.23	1.86	4.00
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	2.56	3.52	3.91
NOD1	Nucleotide-binding oligomerization domain-containing protein 1	2.16	2.81	1.98
RELA	V-rel reticulendotheliosis viral oncogene homolog A (avian)	2.49	2.42	
RIPK1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	1.74	1.61	
TLR1	Toll like receptor 1	3.47	2.94	1.66
TLR5	Toll like receptor 5	8.07	5.53	2.83
TNF	Tumor necrosis factor	12.02	14.72	3.14

The fold change expressions were calculated using HT-29 cells basal level genes expressions, where a cutoff value of ± 1.5-fold change was considered to tabulate

C

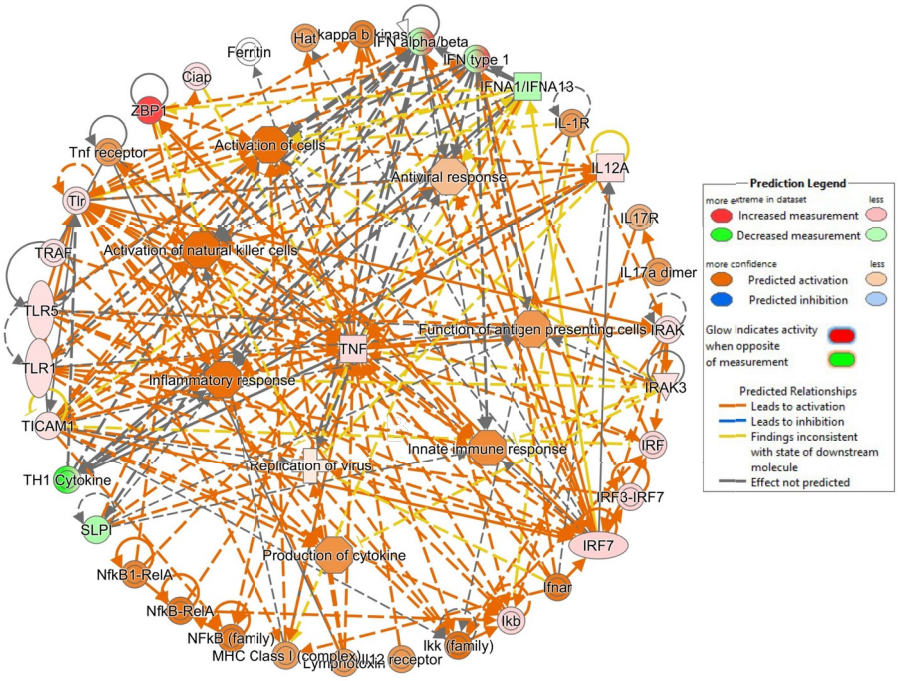


Functions Annotation	p-Value	Molecules	# Molecules
Inflammatory response	2.9E-12	B-cell receptor,CAMP,CCL3,CD14,Cpla2,CXCL1,CXCL8,Eotaxin,ERK1/2,Ifn,Ifn gamma,Ige,IL12A,LBP,LCN2,MAP2K3,Nfat (family),NOD1,Pro-inflammatory Cytokine,PRTN3,SCAVENGER receptor CLASS A,Tnf (family)	22
Cytokine and chemokine mediated signaling pathway	8.34E-10	CCL3,CXCL1,CXCL8,IL12A,LBP,LCN2,PRTN3	7
Cell movement of granulocytes	1.65E-09	CAMP,CCL3,CD14,CXCL1,CXCL8,Eotaxin,ERK1/2,LBP,LCN2,PRTN3	10
Immune response of cells	1.76E-09	CAMP,CCL3,CD14,CXCL1,CXCL8,ERK1/2,Fcgr3,Ifn,Ifn gamma,IL12A,LCN2,NOD1,PRTN3,Tnf (family)	14
Activation of phagocytes	1.41E-11	CAMP,CCL3,CD14,CXCL1,CXCL8,ERK1/2,Fcer1,HLA-DR,Ifn,Ifn gamma,Ige,LBP,LCN2,MAP2K3,Pro-inflammatory Cytokine,PRTN3,SCAVENGER receptor CLASS A,Tnf (family)	18
Activation of macrophages	2.02E-09	CAMP,CCL3,CD14,CXCL8,ERK1/2,Ifn,Ifn gamma,Ige,LBP,LCN2,MAP2K3,Pro-inflammatory Cytokine,SCAVENGER receptor CLASS A,Tnf (family)	14

Fig. 13 Ingenuity pathway analysis (IPA) predicted innate response cellular functions that are consistently activated and inhibited following *E. coli* Nissle + RV treatment. The networks of differentially expressed genes were algorithmically generated based on their connectivity such that the highly interconnected networks likely represent significant biological function. The Fischer's exact test was used to calculate a *p* value for each biological function assigned to a particular network. The name and number of molecules involved are described in the table

strains were tested for anti-RV activity using both HT-29 and MA-104 cell lines and showed up to 50% reduction in PFU, and further higher RV reduction was evident in HT-29 cells compared to MA-104 cells, more specifically in a pre-inoculation strategy [50]. Like previous studies, our findings confirmed the ability of the *Lactobacillus* probiotics (*L. acidophilus* and *L. rhamnosus* GG) to reduce RV replication and showed that the pre-inoculation strategy was more efficient in reducing RV load in the HT-29 cells. Although the exact reasons for the lack of effects of Bb12 treatment is unknown, factors like, probiotic strain and/or species specific effects, RV strain used, dose and duration of probiotic treatment, etc., can undoubtedly influence the experimental outcome.

Pre-inoculation of HT-29 cells with probiotics was more efficient in inhibiting RV in our present study and from others' studies [48, 49, 51–54]. This strategy has numerous advantages including the following: (i) limiting the cell adsorption and internalization of RV due to the direct trapping of the virus by the probiotic bacteria [13, 53, 56], (ii) "cross-talk" with the host epithelial cells in establishing antiviral protection [44, 52, 53, 55], (iii) enhancing gut mucosal barrier thereby reducing permeability [14, 54], (iv) production of metabolites with direct antiviral properties [52, 55], and (v) interfering with intracellular RV replication by regulating NSP4 and Ca2+ [49, 51]. Interestingly, anti-RV effects were not observed when dead probiotic cells were used (Fig. 4A). However, RV replication inhibition was still evident when cell culture supernatants were used (Fig. 5B) in the pre-inoculation regimen, suggesting that live probiotics cells and their secreted products are necessary to provide the RV inhibition effects. In this regards, recent studies have demonstrated that *E. coli* Nissle supernatant contain both soluble proteins and outer membrane vesicles that positively modulate the epithelial barrier through upregulation and redistribution of TJ proteins mainly, ZO-1, ZO-2, and CLDN14 [56, 57]. Similarly, in our study, both *E. coli* Nissle and *L. acidophilus* pretreatment upregulated the expression of CLDN 14 (Table 2). The probiotic's ability to bind RV particles was also shown to contribute to the anti-RV properties. In the present and in our previous study [13], none of the tested probiotics other than *E. coli* Nissle



Functions Annotation	p-Value	Molecules	# Molecules
Activation of cells	3.73E-10	IFN alpha/beta,IFN type 1,IFNA1/IFNA13,Ifnar,IL-1R,IL12A,IRAK3,IRF7,Lymphotoxin,MHC Class I (complex),SLPI,TH1 Cytokine,TICAM1,Tlr,TLR1,TLR5, TNF,Tnf receptor,ZBP1	19
Inflammatory response	3.35E-09	IFN alpha/beta,IFN type 1,IFNA1/IFNA13,IL12A,IRAK3, Lymphotoxin,SLPI,TH1 Cytokine,TICAM1,Tlr,TLR1,TLR5, TNF,ZBP1	14
Innate immune response	1.27E-08	IFN alpha/beta,IFN type 1,IRAK3,IRF7,SLPI,Tlr,TLR1,TNF, Tnf receptor,ZBP1	10
Production of cytokine	1.73E-08	IRAK3,TICAM1,Tlr,TLR1,TLR5	5
Function of antigen presenting cells	3.63E-08	IFN type 1,IRAK3,SLPI,TICAM1,TLR1,TLR5,TNF	7
Activation of natural killer cells	6.28E-08	IFN alpha/beta,IFN type 1,IFNA1/IFNA13,IL12A,MHC Class I (complex),TICAM1,Tlr,TLR1,TNF	9
Replication of virus	2.63E-07	IFN alpha/beta,IFN type 1,IFNA1/IFNA13,Ifnar,IL12A,IRF7, Lymphotoxin,SLPI,TICAM1,TNF,ZBP1	11
Antiviral response	0.0000188	IFN alpha/beta,IFN type 1,IFNA1/IFNA13,IRF7,TH1 Cytokine,TICAM1,Tlr,TNF	8

Fig. 14 Ingenuity pathway analysis (IPA) predicted innate response cellular functions that are consistently activated and inhibited following RV infection. The networks of differentially expressed genes were algorithmically generated based on their connectivity such that the highly interconnected networks likely represent significant biological function. The Fischer's exact test was used to calculate a *p* value for each biological function assigned to a particular network. The name and number of molecules involved are described in the table

possessed any significant RV binding properties (Fig. 4B). This further suggests that the direct binding of RV particles by *E. coli* Nissle is an important mechanism by which the reduction in RV replication is achieved following *E. coli* Nissle treatment. Additionally, *E. coli* Nissle decreased cell movements and increased cell-to-cell contact and TJ formation (Figs. 8 and S2), the critical cellular functions that are important for RV proliferation in the intestinal cells. Previous study has shown that in polarized MDCK cells RV infection activates RhoA/ROCK/MLC signaling, which alters TJ protein distribution and disrupts TJ integrity thereby facilitating RV access to coreceptors and entry into the cells [58]. Though no specific activation of MLC signaling was observed, we found that RVinduced inhibition of morphology of TJ and formation of TJ/intercellular junctions (Fig. 9). Inhibition of these TJ formation events help RV to

readily access the TJ and adherence junction's proteins that acts as receptors or coreceptors for RV infection [59, 60]. Specifically, JAM2, occludin, and ZO-1 of TJ proteins play important receptor or coreceptor roles during RV entry into the cells [59]. Our study reports that upregulation of JAM2 gene by RV infection, which to a certain extent was repressed on probiotic treatments (Table 2). Another gene CLDN14 critical for TJ formation is highly expressed after E. coli Nissle and LA treatments (Table 2) suggesting roles for these probiotics in improving TJ formation [61]. Previously, *E. coli* Nissle was shown to induce protein kinase C-ζ (PKCζ) and extracellular-signal-regulated kinase 1/2 (ERK1/2) phosphorylation mediated events to upregulate the TJ protein claudin-14 [61]. Further, we found that transcription factor 2 (CDX2), which is critical in early intestinal differentiation and has been implicated as a master regulator of intestinal homeostasis and permeability is only regulated and inhibited by probiotic+RV infection (Fig. 7) suggesting the tested probiotics likely negate the RV-induced intestinal inflammation and perturbed homeostasis [62].

As predicted, *E. coli* Nissle modulated the expression of key genes involved in the innate immune and inflammatory responses. TLR genes like TLR1, TLR5, and NOD1 were upregulated on *E. coli* Nissle treatment (Fig. 9 and Table 3). It is likely that *E. coli* Nissle may antagonize the RV induced inhibition of NF-κB pathway, as it was previously reported that RV employs several strategies to inhibit immune responses in cells, specifically the prevention of the nuclear accumulation of NF-κB [63]. Another protein, encoded by the RELA gene, is also uniquely upregulated by *E. coli* Nissle treatment (Table 3). This protein forms a dimer with NF-κB in the nucleus and acts as the transcription factor for many of the genes that are regulated by NF-κB [64]. The upregulation of RELA by *E. coli* Nissle treatment supports the idea that *E. coli* Nissle can boost the transcription of genes regulated by NF-κB, which are inhibited by RV. Interestingly, TNF was highly upregulated by *E. coli* Nissle treatment. Besides the implications of higher levels of TNF for the inflammatory response, TNF is known to recruit proteins that interact with RIPK1, causing it to promote cell survival via the activation of the NF-κB pathway [65]. Other mechanisms that *E. coli* Nissle exploits to antagonize RV infection include, the enhancement of antiviral sensing in HT-29. The protein encoded by the CARD6 gene (also uniquely upregulated by *E. coli* Nissle; Table 3 and Fig. 9) has been shown to play a key role in recognition of intracellular viral dsRNA [66]. Since RV is a dsRNA virus, and if *E. coli* Nissle treated cells have an enhanced ability to detect the presence of RV, then host cells may have an enhanced ability to mount a virus specific attack and/or signal other cells about the presence of RV.

Conclusion

Taken together, our in vitro study, which represents a simplified model for probiotic-RV-epithelial cell interactions, demonstrated that the probiotic *E. coli* Nissle acts via multiple mechanisms: (i) RV binding, (ii) up-regulation of critical TJ genes via specific upstream regulator (e.g., CDX2) to maintain gut barrier homeostasis, and (iii) activation of innate immune and inflammatory responses to neutralize RV infection. Our in vitro findings further suggest that *E. coli* Nissle supplementation, especially prior to enteric infections in infants, can confer more benefits in reducing enteric infectious diseases.

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Author Contribution AK and GR conceptualize the study. AK, YH, and ZF performed the experiments and analyze the data. AK, YH, and ZF prepared the original draft of the manuscript. AK, YH, AV, LS, and GR reviewed and edited the manuscript. All authors have acknowledged the final version of the manuscript.

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Data Availability All data generated or analyzed during this study are included in this published article (and in supplementary information files).

Declarations

Conflict of Interest The authors declare no competing interests.

Consent for Publication Not applicable.

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