Potential Probiotic Lactobacillus Rhamnosus (MTCC-5897) Attenuates Escherichia Coli Induced Inflammatory Response in Intestinal Cells

Taruna Gupta
National Dairy Research Institute
Harpreet Kaur
National Dairy Research Institute
Suman Kapila
National Dairy Research Institute
Rajeev Kapila (rkapila69@rediffmail.com)
ICAR-National Dairy Research Institute https://orcid.org/0000-0002-2795-3720

Research Article

Keywords: Probiotic, Intestinal epithelial cells, Cytokines, Immunomodulation, and NF-κB

Posted Date: May 27th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-533546/v1

Abstract

Probiotics are microbes having tremendous potential to prevent gastrointestinal disorders. In current investigation, immunomodulatory action of probiotic *Lactobacillus rhamnosus* (LR:MTCC-5897) was studied during exclusion, competition and displacement of *Escherichia coli* on intestinal epithelial (Caco-2) cells. The incubation of intestinal cells with *E. coli*, enhanced downstream signalling and activated nuclear factor kappa B (NF-κB). This significantly increased ($p<0.01$) the pro-inflammatory cytokines (IL-8, TNF-α and IFN-Y) expression. While, incubation of epithelial cells with *L. rhamnosus* during exclusion and competition with *E. coli*, counteracted these enhanced expressions. The immunomodulatory feature of *L rhamnosus* was also highlighted with increased ($p<0.05$) transcription of toll like receptor-2 (TLR-2) and single Ig IL-1-related receptor (SIGIRR) along with diminished expression of TLR-4. Likewise, attenuation ($p<0.05$) of *E.coli*-mediated enhanced nuclear translocation of NF-κB p-65 subunit by *L. rhamnosus* during exclusion was confirmed with western blotting. Thus, present finding establishes the prophylactic potential of *L. rhamnosus* against exclusion *of E. coli* in intestinal cells.

1. Introduction

The human gastrointestinal tract (GIT) gets colonized by variety of microbes soon after the birth of neonates. These gut microbes have demonstrated positive impact on human health by modulating metabolic functions including digestion of dietary substances, absorption of nutrients and transformations of xenobiotics. Hence, these commensal organisms are commonly referred as “hidden organ” of the body (Thursby and Juge 2017). Furthermore, intestinal cells, which remain in direct contact with ingested microbes act as the first line of defense against any foreign material entering the gut. The crosstalk between gut microbiota and intestinal epithelial cells cause activation of downstream pathways and play crucial role in the regulation of immune response and the maintenance of barrier integrity (Kawai and Akira 2009). Various factors like diet, stress, unhealthy lifestyle and use of antibiotics adversely disrupt gut microbiota composition (Oriach et al. 2016) to unbalanced state known as dysbiosis, which prompt unregulated induction of local and systemic immune responses (Belkaid and Hand 2014). Unfortunately, these immune-compromised conditions are used by pathobiont or opportunistic pathogens inciting damaging effects in the host through the disruption of epithelial barrier which prompt intestinal disorders like inflammatory bowel diseases (IBD), non-alcoholic fatty liver disease, type I diabetes, obesity and rheumatoid arthritis etc (Lazar et al. 2018). *Escherichia coli*, a common inhabitant of human gastrointestinal tract, behave as a harmless component of normal gut flora. But, commensal *E. coli* strains caused number of human maladies like IBD by prompting a dysregulated immune response when host milieu was modified by specific environmental or genetic conditions (Kittana et al. 2018; Bereswill et al. 2013; Kim et al. 2005). Previous evidences displayed that *E. coli* K12 which is generally referred as “safe” strain, have some virulence genes and regulatory process similar to pathogenic bacteria and could switch to invasive and pathogenic life style without any major change in genetic flux (Koli et al. 2011). These bacteria further translocated to extra intestinal tissue sites like mesenteric lymph nodes, spleen and liver by disruption of gut barrier in immunocompromised aged

mice (Sharma et al. 2014). Similarly, under *in vitro* conditions, *E. coli* K12 enhanced the intestinal permeability by altering the barrier integrity through decreasing the expression of tight junctions (Bhat et al. 2019, a). Furthermore, *E. coli* K12 derived lipopolysaccharides (LPS) also caused enhanced T-helper (Th1/Th17) immune response which resulted in severe intestinal inflammation in mice model (Gronbach et al. 2014).

Thus, balancing the deregulated bacterial ecosystem is only a substitute for the prevention of various gut-related diseases and conferring health benefits. Nowadays, nutritionists and researchers are looking forward to probiotics as a healthier alternative to preserve gut immune homeostasis. Probiotics have been known to have many beneficial effects on metabolism, junctional integrity and regulation of mucosal or systemic immune response (Galdeano et al. 2019; Bron et al. 2017). Therefore, probiotic consumption as a versatile functional food has increased tremendously due to its enormous health effects. Though, the number of indirect evidences depicted the health benefits of probiotics through immune-modulations that displayed changes in expressions of immunoglobulins and pro-inflammatory cytokines in pre-clinical and clinical trials (Oh et al. 2018; Groeger et al. 2013; Milajerdi et al. 2019; Horvath et al. 2016). However, scanty direct evidences are available which can suggest undergoing molecular events to establish microbe's potential individually because probiotic microbes have highly complex and strain specific mode of action (Chiu et al. 2013). Hence, the present study was designed to understand the immunomodulatory signals released from host intestinal epithelial cells, being the first site of interaction after ingestion, in response to probiotic bacteria *L. rhamnosus* in presence of *E. coli.* Earlier, this potential probiotic strain of *L. rhamnosus* (MTCC:5897) restored Th1/Th2 immune homeostasis, anti-oxidative status and antagonize translocation of pathogenic *E. coli* in aging mice (Sharma et al. 2014). Likewise, its feeding in the form of fermented milk in ovalbumin allergen sensitized weaning mice also alleviated symptoms of allergies and depicted its immunomodulatory potential (Saliganti et al. 2015). This potential probiotic also maintained junctional integrity in *E. coli* induced inflammatory response in intestinal cells (Bhat et al. 2019,a) and found safe during feeding to weanling mice (Bhat et al. 2019,b).

2. Materials And Methods

2.1 Cell line and culture conditions

The human epithelial colorectal adenocarcinoma cell line (Caco-2) was purchased from NCCS, Pune, Maharashtra (India) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, Missouri, USA), along with 1% antibiotic solution containing penicillin G (100 µg ml^{-1}), streptomycin (100 µg ml^{-1}) and amphotericin (3 µg ml^{-1}). These cells were routinely grown in 25 cm^2 tissue culture flask with 37°C in an atmosphere of 5% CO_2 with 90% relative humidity. For different experiments, cells were sub-cultured in 6-well plate till confluency. Cells of 20–40 passage numbers were used throughout the experiments.

2.2 Bacterial strains and culture conditions

An indigenous probiotic *L. rhamnosus* (LR:MTCC-5897) used under present investigation was previously isolated from household curd and characterized for probiotic attributes (Sharma et al. 2014). For this study, bacterial culture was activated in sterile de Man, Rogosa and Sharpe (MRS) broth (Hi-Media, Mumbai, Maharashtra, India) for 18 h under aerobic incubation at 37°C before use. Next day, activated culture was used to harvest bacterial pellets by centrifugation at 2000 x *g* for 10 min, followed by washing with phosphate buffered saline (PBS, pH-7.4). For *in vitro* treatments, bacterial pellets were resuspended in antibiotic free DMEM media to obtain 10^9 colony forming unit (CFU) ml^{-1}. The number of bacteria was determined by plate counting on MRS agar plates after aerobic incubation at 37°C for 24–48 h. This particular dose of *L. rhamnosus* was selected on the basis of previous investigation in which this probiotic was found safe in Caco-2 cells upto 24 h and displayed immunomodulatory effects in weanling mice (Bhat et al. 2019,b).

E. coli strain (ATCC:14948) was obtained from National Collection of Dairy Cultures (NCDC), NDRI, Karnal, Haryana (India). The culture was activated in nutrient broth under aerobic conditions for 18 h at 37°C and preceded as above for further work. For various experiments, the desired bacterial count ($1x10^8$ CFU ml^{-1}) was obtained by plate counting on eosin methylene blue (EMB) agar. Thereafter, the bacteria were re-suspended in antibiotic free DMEM media to attain 100:1/well multiplicity of infection. This specific dose of *E. coli* was selected through preliminary study performed on barrier integrity in intestinal cells (Bhat et al. 2019, a).

2.3 Stimulation of intestinal cells with bacteria

Caco-2 cells with $1x10^5$ cells ml^{-1} density were seeded in 6-well plate and after obtaining confluency, cells were treated with *L. rhamnosus* ($1x10^9$ CFU ml^{-1}) or *E. coli* ($1x10^8$ CFU ml^{-1}) for 3 h at 37°C in 5% CO_2. For further experiments, Caco-2 cells were incubated with probiotic/*E. coli* under three different challenge modes known as exclusion (Ex: pre-treatment), competition (Com) and displacement (Dis: post-treatment) respectively. In exclusion assay, Caco-2 cells were incubated with probiotic *L. rhamnosus* for 3 h, then media was removed and cells were washed with PBS followed by 3 h incubation with *E. coli* containing DMEM medium for inflammatory stimulation. In competition assay, Caco-2 cells were simultaneously incubated with *L. rhamnosus* and *E. coli* for 3 h. While, during displacement assay, Caco-2 cells were initially treated with *E. coli* containing medium which was then removed after 3 h of incubation and cells were washed with PBS. Later, these intestinal cells were incubated with *L. rhamnosus* for 3 h. In all these experimental assays, Caco-2 cells grown in DMEM media acted as a negative control. All sets of experiments were carried out in triplicate. The treated cells were washed with ice-cold PBS twice and used for RNA extraction and western blotting.

2.4 RNA isolation and relative expression of genes associated with immune response

Total RNA was isolated from Caco-2 cells following Trizol method as described in manufacturer protocol and further used for relative quantification of genes associated with immune response. Purity of the RNA

was confirmed by determining O.D. at 260/280 ratio using microplate spectrophotometer (BioTek Instruments, Winooski, Vermont, USA). RNA integrity was confirmed on 1.5% agarose gel through electrophoresis. Total RNA (1 µg) was reverse transcribed to cDNA using a reverse transcription kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) following user manual. The prepared cDNA was stored in -20°C until used further. Quantitative real-time PCR (qRT) analysis, reactions were conducted to determine the relative gene expression by using ABI-fast 7500 thermocycler system (Applied Biosystems, California, USA). For mRNA expressions, qRT-PCR reactions were performed in 10 µl reaction volume containing 1 µl of test sample, 5 µL of syber (Thermo scientific, USA), 0.5 µl of each primer and 3 µl nuclease free water. Sequences of primers are shown in Table 1. GAPDH was used as a reference gene throughout the experiments. The thermal profile for reaction was: initial denaturation of 5 min at 94°C, 35 cycles of denaturation (94°C for 30 sec), annealing (60°C for 30 sec) and extension (60°C for 45 sec) and final extension cycle at 60°C for 5 min. After amplification, threshold (Ct) values of both control and treatment groups with reference genes (GAPDH) were used for calculating fold changes in respective target genes expression (Livak and Schmittgen 2001).

Table 1
Sequence of primers along with their corresponding amplicon size for mRNA quantification immunity related genes using qRT-PCR

Gene	Sequence of primer	Amplicon Length (bp)	Accession number
Genes related to Interleukins			
IL-8	F 5'-GGTGCAGTTTTGCCAAGGAG-3'	183	NM_001354840.2
	R 5'-TTCCTTGGGGTCCAGACAGA-3'		
IL-6	F 5'-GGCACTGGCAGAAAACAACC-3'	85	NM_001371096.1
	R 5'-GCAAGTCTCCTCATTGAATCC-3'		
TNF-α	F 5'-GGGACCTCTCTCTAATCAGC-3'	103	NM_000594.4
	R 5'-TCAGCTTGAGGGTTTGCTAC-3'		
IL-23	F 5'-GTGGGACACATGGATCTAAGAGA-3'	135	NM_016584.3
	R 5'- CTGGTGGATCCTTTGCAAGC-3'		
Genes related to pathogen recognition receptor (PRR)			
TLR-2	F 5'-AGCACTGGACAATGCCACAT-3'	113	NM_001318796.2
	R 5'-ACCATTGCGGTCACAAGACA-3'		
TLR-4	F 5'-CAAGAACCTGGACCTGAGCTT-3'	200	NM_138554.5
	R 5'-AAAAGGCTCCCAGGGCTAAA-3'		
MyD-88	F 5'-CAGCGACATCCAGTTTGTGC-3'	146	NM_002468.5
	R 5'-GGCGGCACCTCTTTTCGAT-3'		
Genes related to NF-κB pathway			
NF-κB	F 5'-ATGTGGGACCAGCAAAGGTT-3'	134	NM_001319226.2
	R 5'-CACCATGTCCTTGGGTCCAG-3'		
SIGIRR	F 5'-GGTATGTCAAGTGCCGTCTCAAC-3'	120	NM_001135054.2
	R 5'- AGCTGCGGCTTTAGGATGAAGT-3'		

2.5 Enzyme linked immunosorbent assay

Cytokines secretions (TNF-α, IL-10, TGF-β and IFN-Y) were evaluated by enzyme linked immunosorbent assay (ELISA) (Biolegend Inc., San Diego, California, USA) in supernatants of Caco-2 cells according to manufacturer protocol. In brief, 96-well immune plates (Hi-media, Mumbai, Maharashtra, India) were coated with 100 µl of capture antibody and incubated overnight at 4°C. Next day after PBS/T washing,

200 µl of blocking buffer was added to each well to avoid non-specific binding. Afterward, serially diluted respective standard cytokines or undiluted culture supernatant was used to determine the concentrations of interleukins. Then, 100 µl well^{-1} of detection antibody of respective cytokines were added. Later, 100 µl well^{-1} HRP-conjugated avidin was added as detection enzyme. In final step, TMB substrate (3,3,5,5-tetramethyl diamine benzidine containing 0.03% H_2O_2) was added for the development of colour and 2 N H_2SO_4 was used to stop the reaction. After that plate was read at 450 nm by using a microplate reader (BioTek Instruments, Winooski, Vermont, USA).

2.6 Extraction of protein and determination of NF-κB nuclear translocation by western blotting

Cytoplasmic and nuclear extracts of experimentally treated Caco-2 cells were collected by cell lysis using extraction reagents (Cytoplasmic nuclear extraction reagents Kit, Infobio, India) and protein concentrations were determined using Lowry method (Lowry 1951). After that, protein samples (30 µg well^{-1}) were separated on SDS-PAGE (Laemmli 1970) followed by blotting on PVDF membrane for detection of a specific protein. This membrane was blocked with 5% BSA (overnight) and incubated with primary antibody for 4 h at room temperature against NF-κB p-65 (1:100 dilution, Santa Cruz Biotech Inc, Dallas, Texas, USA), β-actin (1:1000 dilution, Sigma-Aldrich, St Louis, Missouri, USA) and Lamin (1:500 dilution, Santa Cruz Biotech Inc, Dallas, Texas, USA). The membrane was washed and incubated with horseradish peroxidase conjugated secondary antibody (Sigma-Aldrich, St Louis, Missouri, USA) for 2 h at room temperature. Signals were detected by using Dab Membrane Peroxidase tablets (Genetix, San Jose, California, USA).

2.7 Statistical analysis

Data were analysed using GraphPad Prism (Version 5.01) software. Experimental results are presented as means ± S.E.M. Data were subjected to analysis of variance (ANOVA) and the Tukey test was used to separate the means ($p < 0.05$) which were considered statistically significant.

3. Results

3.1 Modulation in intestinal cytokines

In initial set of experiments, the immunomodulatory effect of probiotic *L. rhamnosus* on *E. coli* evoked inflammatory responses were analysed through measuring change in gene expressions of pro-inflammatory cytokines by qRT-PCR in Caco-2 cells. Stimulation of Caco-2 cells with *E. coli* for 3 h tremendously enhanced ($p \leq 0.01$) the mRNA expressions of pro-inflammatory cytokines IL-8, TNF-α, and IL-23 by 8.12 ± 1.74, 8.75 ± 2.18 and 2.81 ± 0.49 folds as well as pleotropic cytokine IL-6 by 3.81 ± 0.44 folds as compared to control cells (Fig. 1A-D). Interestingly, probiotic LR treatment individually for equivalent time did not bring much change in expressions of pro-inflammatory cytokines than control. Incubation of Caco-2 cells with *L. rhamnosus* during exclusion and completion of *E. coli* significantly ($p \leq 0.05$) reduced the expression of pro-inflammatory chemo-attractant IL-8 to 3.38 ± 1.18 and 4.19 ± 0.24

folds than *E. coli* (8.12 ± 1.74 folds) stimulated cells (Fig. 1A). Likewise, mRNA levels of pro-inflammatory marker TNF-α, decreased considerably ($p \leq 0.01$) during *L.rhamnosus* incubation in presence of *E.coli* irrespective of the mode of challenge (Fig. 1B). Pro-inflammatory cytokine IL-23 merely showed significantly ($p \leq 0.05$) diminished mRNA transcription from intestinal cells during exclusion by probiotic LR than other modes of challenges with *E. coli* (Fig. 1C). On the other hand, no major alterations occurred in expression of IL-6 irrespective of the type of challenge between *E. coli* and probiotic bacteria during incubation with intestinal cells.

The results of actually released pro-inflammatory (TNF-α, IFN-Y) and regulatory cytokines (IL-10 and TGF-β) measured by ELISA are shown in Fig. 2. *E. coli* challenged Caco-2 cells showed significantly ($p \leq 0.05$) higher release of inflammatory cytokines (TNF-α and IFN-Y) along with diminished secretions of anti-inflammatory cytokine (IL-10) as compared to negative control cells. It was also observed that incubation of probiotic *L. rhamnosus* individually or during challenge with *E. coli* during exclusion, competition and displacement assays prevented inflammatory response by significantly ($p \leq 0.05$) reducing the secretions of inflammatory cytokines (TNF-α, IFN-Y) than *E. coli* inflamed cells (Fig. 2A and B). On the other hand, probiotic *L. rhamnosus* treated intestinal cells showed much higher ($p \leq 0.05$) production of TGF-β and IL-10 in comparison to *E. coli* inflamed cells (Fig. 2C and D). Besides, *E. coli* exclusion by *L. rhamnosus* significantly ($p \leq 0.05$) enhanced the release of IL-10 and TGF-β from intestinal cells, though assays based variations were observed during competition and displacement. Thus, it is clearly depicted that probiotic *L. rhamnosus* has an inhibitory effect on inflammatory milieu induced by *E. coli* in intestinal cells by suppressing pro-inflammatory cytokines and modulating anti-inflammatory cytokines more effectively during exclusion assay.

3.2 Modulation in expression of Toll like receptors (TLRs)

To get more insight in molecular events related to immunomodulation brought by *L. rhamnosus*, mRNA expressions of key pathogen recognition receptors (PRR's) were assessed in Caco-2 cells (Fig. 3). Stimulation of Caco-2 cells with *E. coli* or probiotic *L. rhamnosus* induced differential expression of these genes. mRNA transcription of TLR-4 was enhanced ($p \leq 0.05$) to 2.83 ± 0.13 folds after exposure of *E. coli* than control cells (Fig. 3A) while it remained near to control levels on incubation with probiotic *L. rhamnosus* individually or suppressed it significantly ($p \leq 0.05$) during respective exclusion and competition assays as compared to *E. coli* inflamed cells. On the other hand, exposure of probiotic *L. rhamnosus* noticeably ($p \leq 0.05$) enhanced the TLR-2 expression by 2.42 ± 0.42 folds than *E. coli* infected cells. Similarly, probiotic *L. rhamnosus* caused significantly higher ($p \leq 0.05$) TLR-2 mRNA expression in intestinal cells than *E. coli* treated epithelial cells during exclusion as well as competition with inflammatory agent *E. coli*, (Fig. 3B). Though, displacement assays showed its transcriptional expression almost same to control or *E. coli* treated cells. mRNA expression of adaptor protein MyD-88, which regulates downstream signalling of all PRR's in intestinal cells showed statistically higher ($p < 0.05$) transcriptional activity in *E. coli* inflamed cells (Fig. 3C) without any major modulations in treatment groups.

3.3 Inhibition of NF-κB signalling in intestinal epithelial cells

In present investigation, infection of Caco-2 cells with *E. coli* resulted in increased mRNA expression of NF-κB to 4.73 ± 0.34 folds ($p \leq 0.05$) than control. In contrast, probiotic *L. rhamnosus* exposure to intestinal cells kept mRNA transcription (1.84 ± 0.14 folds) close to control and significantly less ($p \leq 0.05$) than *E. coli* treated cells. Exclusion and competition of *E. coli* with *L. rhamnosus* caused 3.05 ± 0.62 and 2.44 ± 0.64 folds of mRNA expression, respectively which differed insignificantly from control or *E. coli* treated intestinal epithelial cells (Fig. 4A). The inflammatory challenge of *E. coli* to epithelial cells suppressed ($p \leq 0.05$) transcription of single immunoglobulin IL-1R-related receptor (SIGIRR), a negative regulator of NF-κB, than control (Fig. 4B). While, *L. rhamnosus* incubation to intestinal epithelial cells individually or during various modes of challenge with *E. coli* resulted into significantly higher ($p \leq 0.05$) transcription of SIGIRR than *E. coli* inflamed cells (Fig. 4B).

Figure 4C shows western blot analysis of nuclear translocation of NF-κB, p-65 subunit from cytoplasm which was essentially required for activation of inflammatory responses by secretion of various inflammatory cytokines. The nuclear translocation of p-65 subunit increased significantly ($p < 0.05$) in the cells infected with *E. coli* as compared to unstimulated negative control cells (Fig. 4d & e). In opposition, cells treated with *L. rhamnosus* showed reduced nuclear translocation of p-65. Likewise, pre-incubation of Caco-2 cells with *L. rhamnosus* before *E. coli* during exclusion assay also resulted in much less ($p \leq 0.05$) translocation of this factor than *E. coli* inflamed cells (Fig. 4d & e), which was contrary to the observations made during competition or displacement assays.

4. Discussion

Intestinal epithelial cells, besides acting as a physical barrier, also play crucial role as immune modulator because epithelium is the site of interaction between microbes and host. Upon microbial pattern recognition by PRR's present at intestinal surface, epithelial cells initiate immune response through secretions of cytokines or chemokines. Probiotic stimulation modulates levels of cytokines through innate immune response mediated by pathogen recognition receptors (Brito et al. 2013), therefore evaluation of TLRs expression were utmost helpful in understanding the modulations of immune responses in intestinal cells. The mRNA expression of TLR-2 enhanced in the presence of *L. rhamnosus* was quite justifiable since Gram-positive bacteria have specific surface signature such as peptidoglycans, lipothechoic acid and lipopeptides which use TLR-2 signalling (Pinto et al. 2009). Probiotic utilized TLR-2 signalling for the release of IL-10 from regulatory T-cells to induce immune tolerance response (Konieczna et al. 2012). TLR-2 also played important role in induction of anti-microbial peptides which acted against the invasion of pathogens causing epithelial damage (Jia et al. 2019). Moreover, present study also confirmed that probiotic *L. rhmanosus* maintains immunotolerance through decreasing the expression of TLR-4 even in the presence of *E. coli*. Earlier probiotic *L. jensenii* TL2937 inhibited the increased TLR-4 levels induced by pathogenic *E. coli* or LPS infection in porcine epithelial cells (Shimazu et al. 2012). Likewise, treatment of intestinal cell with *L. amylovorous* or its supernatant reduced enterotoxigenic *E. coli* (ETEC) induced inflammatory response by decreasing TLR-4 regulated signalling

(Finamore et al. 2014). Thus, these data clearly suggested that probiotic bacteria are exerting protective effect by differentially modulating the TLR signalling cascade and cytokines production that regulate immune response. Under current investigation, significant up-regulation of genes encoding pro-inflammatory markers IL-8, TNF-α, IFN-Y and IL-23 were observed in *E. coli* treated intestinal cells. These results are consistence with previous finding where increased expression of potent inflammatory markers (TNF-α and IL-8) were observed in response to *E. coli* (Dhanani and Bagchi 2013). Similarly, Ghadimi et al. (2012) reported enhanced expression of pro-inflammatory cytokine IL-23 in LPS stimulated intestinal cells. These increased syntheses of cytokines, induced inflammatory conditions, which could often be linked to the development of chronic inflammation and auto immune diseases causing serious health complications (Chen et al. 2018). Moreover, incubation of intestinal cells with probiotic *L. rhamnosus*, under present investigation, showed levels of pro-inflammatory cytokines similar to control which were quite lower than cells treated with *E. coli*. These observations are consistent with the earlier reports where no major change in the levels of pro-inflammatory cytokines with probiotic bifidobacteria or lactobacilli were observed (Bahrami et al. 2011). Probiotic strains are known to display their protective role against inflammatory stimulus either through down-regulation of pro-inflammatory cytokines expressions and up-regulation of regulatory cytokines (Duary et al. 2014). This was also evident in the present investigation depending upon the mode of inflammatory challenge with *E. coli* wherein *L. rhamnosus* suppressed inflammatory responses by reducing pro-inflammatory (IL-8, TNF-α, IFN-Y) cytokines and simultaneously increased the release of anti-inflammatory cytokines (IL-10 and TGF-β). Similarly, previous findings with probiotic *L. jensenii* treatment before exposure with heat-killed enterotoxigenic *E. coli* reduced IL-8 expressions at transcriptional levels (Takanashi et al. 2013). Likewise, probiotic *Bifidobacterium animalis MB5* and *L. rhamnosus* GG defended intestinal cells from pathogenic *E. coli* K88 induced inflammatory response by diminishing the production of inflammatory markers such as IL-8 and TNF-α (Roselli et al. 2006). On the same lines, probiotic *L. acidophilus* was able to counteract *Salmonella typhimurium* inflammatory responses by enhancing TGF-β production in intestinal cells (Caco-2) (Haung et al. 2015). Although, under present investigation, post-treatment of probiotic *L. rhamnosus* during displacement assay was not found much effective in controlling inflammatory response but it clearly mitigated inflammatory responses and maintained intestinal homeostasis by achieving balance between pro and anti-inflammatory cytokines (Shadnoush et al. 2013) during exclusion and competition assays. For further insight into plausible mechanism of immunomodulation by probiotic *L. rhamnosus* in presence of *E. coli* induced inflammatory response, mRNA expression of NF-κB and its translocation from cytoplasm to nucleus were also explored. It was well established that regulated NF-κB dependent signalling is critical for efficient immune response, but prolonged activation contributes to generation of inflammatory diseases (Yan and Polk 2010). During present study, *E. coli* stimulation induced significant increase in mRNA expression of NF-κB in intestinal cells. On the other hand, *L rhamnosus* suppressed expression of NF-κB at transcriptional level than *E. coli* treated cells. Likewise, probiotic *L. acidophilus* diminished *Salmonella* induced NF-κB transcriptional activity in intestinal epithelial cells (Huang et al. 2015). Similarly, co-stimulation of intestinal cells with probiotic *Bifidobacterium lactis* and inflammatory stimulus such as IL-1β and LPS also suppressed NF-κB activation (Kim et al. 2010). Inhibition of NF-κB signalling can be controlled through action of negative regulator such Tollip, SIGIRR, SOCS1 and IRAK-M

which in turn help in maintaining homeostasis in intestinal cells (Finamore et al. 2014). These membrane-bound regulators modulate TLR signalling pathways by disrupting TLR and ligand interactions. Likewise, SIGIRR control NF-κB activation through interaction with IRAK and TRAF6 by inhibiting TLR signalling (Villena and Kitazawa 2014). Thus, reduced mRNA expression of SIGIRR in Caco-2 cells during *E. coli* infection in contrast to much enhanced expression with *L. rhamnosus* stimulation individually or during respective challenge assays, under present investigation, indicated inhibition of NF-κB signalling. These results are in consonance with previous finding which displayed better expression of SIGIRR in intestinal cells on stimulation with probiotic bifidobacteria (Tomosada et al. 2013). Similarly, in another study, probiotic *L. delbrueckii* inhibited *E. coli* 987P induced NF-κB pathways through enhancing TLR negative regulators in porcine intestinal cells (Wachi et al. 2014). Higher translocation of NF-κB p-65 subunit from cytosol to nucleus in intestinal cells by *E. coli*, observed under present investigation, also supported previous findings of Finamore et al. (2014). Impediment of *E. coli* induced inflammatory response by diminished translocation of p-65 subunit from cytosol to nucleus by *L. rhamnosus* also supported previous results where pre-treatment of Caco-2BBe cells with *L. rhamnosus* GG diminished NF-κB activation induced by TNF-α (Donato et al. 2010). Thus, inhibition of NF-κB translocation during exclusion of *E.coli* with probiotic *L. rhamnosus* emerged as crucial factor for anti-inflammatory activity which in turn decreased the expression and release of pro-inflammatory cytokines and simultaneously substantiated the release of regulatory cytokines.

5. Conclusion

In summary, this study clearly provided direct insight into the mode of action of probiotic *L. rhamnosus* (LR:MTCC-5897) under inflammatory milieu induced by *E. coli* in intestinal cells. This potential probiotic strain displayed immunomodulatory and anti-inflammatory functions to varying extent in intestinal cells depending upon the type of *E. coli* challenge. However, differential expression of TLRs caused effective reduction in NF-κB p-65 nuclear translocation during exclusion of *E.coli* with *L.rhamnosus*. Thus immune homeostasis was achieved by reducing the expression of pro-inflammatory and enhancing the release of regulatory cytokines. Thus, it can be concluded that *L. rhamnosus* (LR:MTCC-5897) may be a potential candidate to produce nutraceuticals products for prevention of *E. coli* induced intestinal inflammation.

Declarations

Acknowledgments

The authors acknowledge ICAR-National Dairy Research Institute (NDRI) Karnal, for providing the laboratory facilities for execution of this work. We are also thankful to Department of Biotechnology, Ministry of Science and Technology, New Delhi for providing required funds.

Funding

The authors are highly indebted to the Department of Biotechnology, Ministry of Science and Technology, New Delhi for providing necessary funds. (BT/PR15109/PFN/20/1174/2015) required for the present work. The funding agency has no role in the design, analysis or writing of this article.

Declarations

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Author's Contribution

Conceptualization : **[Taruna Gupta], [Rajeev Kapila]**; Methodology : [**Taruna Gupta], [Harpreet Kaur]** ; Formal analysis : [**Taruna Gupta], [Rajeev Kapila**],**[Suman Kapila]**; Investigation : [**Taruna Gupta**] ; Writing-original draft : [**Taruna Gupta**]; Writing - Review & Editing **[Harpreet Kaur], [Suman Kapila];** Software : **[Taruna Gupta], [Harpreet Kaur];** Validation : [**Taruna Gupta], [Rajeev Kapila];** Funding acquisition : **[Rajeev Kapila];** Resources: **[Rajeev Kapila],** Supervision : **[Rajeev Kapila]**

References

1. Thursby E, Juge N (2017) Introduction to the human gut microbiota. Biochem J 474(11):1823-1836. https://doi.org/10.1042/BCJ20160510
2. Kawai T, Akira S (2009) The roles of TLRs, RLRs and NLRs in pathogen recognition. Int Immunol 21(4):317-337. https://doi.org/10.1093/intimm/dxp017
3. Oriach CS, Robertson RC, Stanton C, Cryan JF, Dinan TG (2016) Food for thought: The role of nutrition in the microbiota-gut–brain axis. Clin Nutr Exp 6:25-38. https://doi.org/10.1016/j.yclnex.2016.01.003
4. Belkaid Y, Hand TW (2014) Role of the microbiota in immunity and inflammation. Cell, 157(1), 121-141
5. Lazar V, Ditu L, Pircalabioru G, Gheorghe I, Curutiu C, Holban AM, Chifiriuc CM (2018) Aspects of gut microbiota and immune system interactions in infectious diseases, immunopathology and cancer. Front. Immunol 9:1830. https://doi.org/10.3389/fimmu.2018.01830
6. Kittana H, Gomes-Neto JC, Heck K, Geis AL, Segura Muñoz RR, Cody LA, Schmaltz RJ, Bindels LB, Sinha R, Hostetter JM, Benson AK (2018) Commensal *Escherichia coli* strains can promote intestinal inflammation via differential interleukin-6 production. Front Immunol 9:2318
7. Bereswill S, Fischer A, Dunay IR, Kühl AA, Göbel UB, Liesenfeld O, Heimesaat MM (2013) Pro-inflammatory potential of *Escherichia coli* strains K12 and Nissle 1917 in a murine model of acute ileitis. Eur J Microbiol Immunol 3(2):126-34
8. Kim SC, Tonkonogy SL, Albright CA, Tsang J, Balish EJ, Braun J, Huycke MM, Sartor RB (2005) Variable phenotypes of enterocolitis in interleukin 10-deficient mice mono-associated with two different commensal bacteria. Gastroenterology 1;128(4):891-906

9. Koli P, Sudan S, Fitzgerald D, Adhya S, Kar S (2011) Conversion of commensal *Escherichiacoli* K-12 to an invasive form via expression of a mutant histone-like protein. MBio 2(5)
10. Sharma R, Kapila R, Dass G, Kapila S (2014) Improvement in Th1/Th2 immune homeostasis, antioxidative status and resistance to pathogenic *coli* on consumption of probiotic *Lactobacillus rhamnosus* fermented milk in aging mice. Age 36(4):9686. https://doi.org/10.1007/s11357-014-9686-4
11. Bhat MI, Sowmya K, Kapila S, Kapila R. Potential probiotic *Lactobacillus rhamnosus* (MTCC-5897) inhibits *Escherichia coli* impaired intestinal barrier function by modulating the host tight junction gene response (2019,a) Probiotics Antimicro 15:1-2. https://doi.org/10.1007/s12602-019-09608-8
12. Gronbach K, Flade I, Holst O, Lindner B, Ruscheweyh HJ, Wittmann A, Menz S, Schwiertz A, Adam P, Stecher B, Josenhans C (2014) Endotoxicity of lipopolysaccharide as a determinant of T-cell mediated colitis induction in mice. Gastroenterology 146(3):765-75
13. Galdeano CM, Cazorla SI, Dumit JML, Velez E, Perdigon G (2019) Beneficial effects of probiotic consumption on the immune system. Ann Nutr Metab 74(2):115-124. https://doi.org/10.1159/000496426
14. Bron PA, Kleerebezem M, Brummer RJ, Cani PD, Mercenier A, MacDonald TT, Garcia-Rodenas CL, Wells JM (2017) Can probiotics modulate human disease by impacting intestinal barrier function? Br J Nutr 117(1):93-107. https://doi.org/10.1017/S0007114516004037
15. Oh NS, Joung JY, Lee JY, Kim Y (2018) Probiotic and anti-inflammatory potential of *Lactobacillusrhamnosus* 4B15 and *Lactobacillus gasseri* 4M13 isolated from infant feces. PloS One 13. https://doi.org/10.1371/journal.pone.0192021
16. Groeger D, O'Mahony L, Murphy EF, Bourke JF, Dinan TG, Kiely B, Shanahan F, Quigley EM (2013) *Bifidobacterium infantis* 35624 modulates host inflammatory processes beyond the gut. Gut Microbes 4, 325-339. https://doi.org/10.4161/gmic.25487
17. Milajerdi A, Mousavi SM, Sadeghi A, Salari-Moghaddam A, Parohan M, Larijani B, Esmaillzadeh A (2019) The effect of probiotics on inflammatory biomarkers: A meta-analysis of randomized clinical trials. Eur J Nutr 1-17. https://doi.org/10.1007/s00394-019-01931-8
18. Horvath A, Leber B, Schmerboeck B, Tawdrous M, Zettel G, Hartl A, Madl T, Stryeck S, Fuchs D, Lemesch S, Douschan P (2016) Randomised clinical trial: the effects of a multispecies probiotic vs. placebo on innate immune function, bacterial translocation and gut permeability in patients with cirrhosis. Aliment Pharmacol Ther 44, 926-935. https://doi.org/10.1111/apt.13788
19. Chiu YH, Lu YC, Ou CC, Lin SL, Tsai CC, Huang CT, Lin MY (2013) *Lactobacillusplantarum* MYL26 induces endotoxin tolerance phenotype in Caco-2 cells. BMC Micro 13, doi.org/10.1186/1471-2180-13-190
20. Saliganti V, Kapila R, Sharma R, Kapila S (2015) Feeding probiotic *Lactobacillus rhamnosus* (MTCC 5897) fermented milk to suckling mothers alleviates ovalbumin-induced allergic sensitisation in mice offspring. Br J Nutr 114(8):1168-117. https://doi.org/10.1017/S000711451500286X

21. Bhat MI, Singh VK, Sharma D, Kapila S, Kapila R (2019,b) Adherence capability and safety assessment of an indigenous probiotic strain *Lactobacillus rhamnosus* MTCC-5897. Microb pathogen 130:120-130. https://doi.org/10.1016/j.micpath.2019.03.009
22. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262
23. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193, 265-275. http://www.jbc.org/content/193/1/265
24. Laemmli UK (1970) SDS-page Laemmli method. Nature 227, 680-5
25. Bermudez-Brito M, Munoz-Quezada S, Gomez-Llorente C, Matencio E, Bernal MJ, Romero F, Gil A (2013) Cell-free culture supernatant of *Bifidobacterium breve* CNCM I-4035 decreases proinflammatory cytokines in human dendritic cells challenged with *Salmonella typhi* through TLR activation. PLoS One 8(3):59370. https://doi.org/10.1371/journal.pone.0059370.
26. Pinto MGV, Gomez MR, Seifert S, Watzl B, Holzapfel WH, Franz CM (2009) Lactobacilli stimulate the innate immune response and modulate the TLR expression of HT-29 intestinal epithelial cells *in vitro*. Int J Food Micro 133, 86-93. https://doi.org/10.1016/j.ijfoodmicro.2009.05.013
27. Konieczna P, Groeger D, Ziegler M, Frei R, Ferstl R, Shanahan F, Quigley EM, Kiely B, Akdis CA, O'Mahony L (2012) *Bifidobacterium infantis* 35624 administration induces Foxp3 T regulatory cells in human peripheral blood: potential role for myeloid and plasmacytoid dendritic cells. Gut 61:354–366. https://doi.org/10.1136/gutjnl-2011-300936
28. Jia Y, Si W, Hong Z, Qu M, Zhu N, Liu S, Li G (2019) Toll-like receptor 2-mediated induction of avian β-defensin 9 by *Lactobacillus rhamnosus* and its cellular components in chicken intestinal epithelial cells. Food Agr Immuno 30(1):398-417. https://doi.org/10.1080/09540105.2019.1593325
29. Shimazu T, Villena J, Tohno M, Fujie H, Hosoya S, Shimosato T, Aso H, Suda Y, Kawai Y, Saito T, Makino S (2012) Immunobiotic *Lactobacillus jensenii* elicits anti-inflammatory activity in porcine intestinal epithelial cells by modulating negative regulators of the Toll-like receptor signaling pathway. Infect Immune 80(1):276-288. https://doi.org/10.1128/IAI.05729-11
30. Finamore A, Roselli M, Imbinto A, Seeboth J, Oswald IP, Mengheri E (2014) *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 9(4):94891. https://doi.org/10.1371/journal.pone.0094891
31. Dhanani AS, Bagchi T (2013) *Lactobacillus plantarum* 2 prevents *Escherichia coli* adhesion to HT-29 cells and also down- regulates enteropathogen induced tumor necrosis factor-α and interleukin-8 expression. Microbiol Immunol 57(4):309-315. https://doi.org/10.1111/1348-0421.12038
32. Ghadimi D, Helwig U, Schrezenmeir J, Heller KJ, de Vrese M (2012) Epigenetic imprinting by commensal probiotics inhibits the IL- 23/IL- 17 axis in an in vitro model of the intestinal mucosal immune system. J Leukoc Biol 92(4):895-911. https://doi.org/10.1189/jlb.0611286

33. Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, Li Y, Wang X, Zhao L (2018) Inflammatory responses and inflammation-associated diseases in organs. Oncotarget 9, 7204
34. Bahrami B, Macfarlane S, Macfarlane GT (2011) Induction of cytokine formation by human intestinal bacteria in gut epithelial cell lines. J Appl Microbiol 110(1):353-363. https://doi.org/10.1111/j.1365-2672.2010.04889.x
35. Duary RK, Batish VK, Grover S (2014) Immunomodulatory activity of two potential probiotic strains in LPS-stimulated HT-29 cells. Genes & Nutr 9(3):398. https://doi.org/10.1007/s12263-014-0398-2
36. Takanashi N, Tomosada Y, Villena J, Murata K, Takahashi T, Chiba E, Tohno M, Shimazu T, Aso H, Suda Y, Ikegami S (2013) Advanced application of bovine intestinal epithelial cell line for evaluating regulatory effect of lactobacilli against heat-killed enterotoxigenic *Escherichia coli*-mediated inflammation. BMC Microbiol 13(1):54. https://doi.org/10.1186/1471-2180-13-54
37. Roselli M, Finamore A, Britti MS, Mengheri E (2006) Probiotic bacteria *Bifidobacterium animalis* MB5 and *Lactobacillus rhamnosus* GG protect intestinal Caco-2 cells from the inflammation associated response induced by enterotoxigenic *Escherichia coli* Br J Nutr 95(6):1177-1184. https://doi.org/10.1079/bjn20051681
38. Huang IF, Lin IC, Liu PF, Cheng MF, Liu YC, Hsieh YD, Chen JJ, Chen CL, Chang HW, Shu CW (2015) *Lactobacillus acidophilus* attenuates *Salmonella*-induced intestinal inflammation via TGF-β signaling. BMC Microbiol 15(1):203. https://doi.org/10.1186/s12866-015-0546-x
39. Shadnoush M, Hosseini RS, Mehrabi Y, Delpisheh A, Alipoor E, Faghfoori Z, Mohammadpour N, Moghadam JZ (2013) Probiotic yogurt affects pro-and anti-inflammatory factors in patients with inflammatory bowel disease. IJPR 12, 929
40. Yan F, Polk DB (2010) Disruption of NF-κB signalling by ancient microbial molecules: novel therapies of the future. BMJ 421-426. https://doi.org/10.1136/gut.2009.179614
41. Kim SW, Kim HM, Yang KM, Kim SA, Kim SK, An MJ, Park JJ, Lee SK, Kim TI, Kim WH, Cheon JH (2010) *Bifidobacterium lactis* inhibits NF-κB in intestinal epithelial cells and prevents acute colitis and colitis-associated colon cancer in mice. Inflamm Bowel Dis 16(9),1514-1525
42. Villena J, Kitazawa H (2014) Modulation of intestinal TLR4-inflammatory signaling pathways by probiotic microorganisms: lessons learned from *Lactobacillus jensenii* Front Immunol 4:512. https://doi.org/10.3389/fimmu.2013.00512
43. Tomosada Y, Villena J, Murata K, Chiba E, Shimazu T, Aso H, Iwabuchi N, Xiao JZ, Saito T, Kitazawa H (2013) Immunoregulatory effect of bifidobacteria strains in porcine intestinal epithelial cells through modulation of ubiquitin-editing enzyme A20 expression. PLoS One 8(3):59259. https://doi.org/10.1371/journal.pone.0059259
44. Wachi S, Kanmani P, Tomosada Y, Kobayashi H, Yuri T, Egusa S, Shimazu T, Suda Y, Aso H, Sugawara M, Saito T (2014) *Lactobacillus delbrueckii* TUA 4408 L and its extracellular polysaccharides attenuate enterotoxigenic *Escherichia coli*- induced inflammatory response in porcine intestinal epitheliocytes via Toll- like receptor- 2 and 4. Mol Nutr Food Res 58 (10):2080-2093. https://doi.org/10.1002/mnfr.201400218

45. Donato KA, Gareau MG, Wang YJJ, Sherman PM (2010) *Lactobacillus rhamnosus* GG attenuates interferon-γ and tumour necrosis factor-α-induced barrier dysfunction and pro-inflammatory signalling. Microbiol 156(11):3288-3297. https://doi.org/10.1099/mic.0.040139

Figures

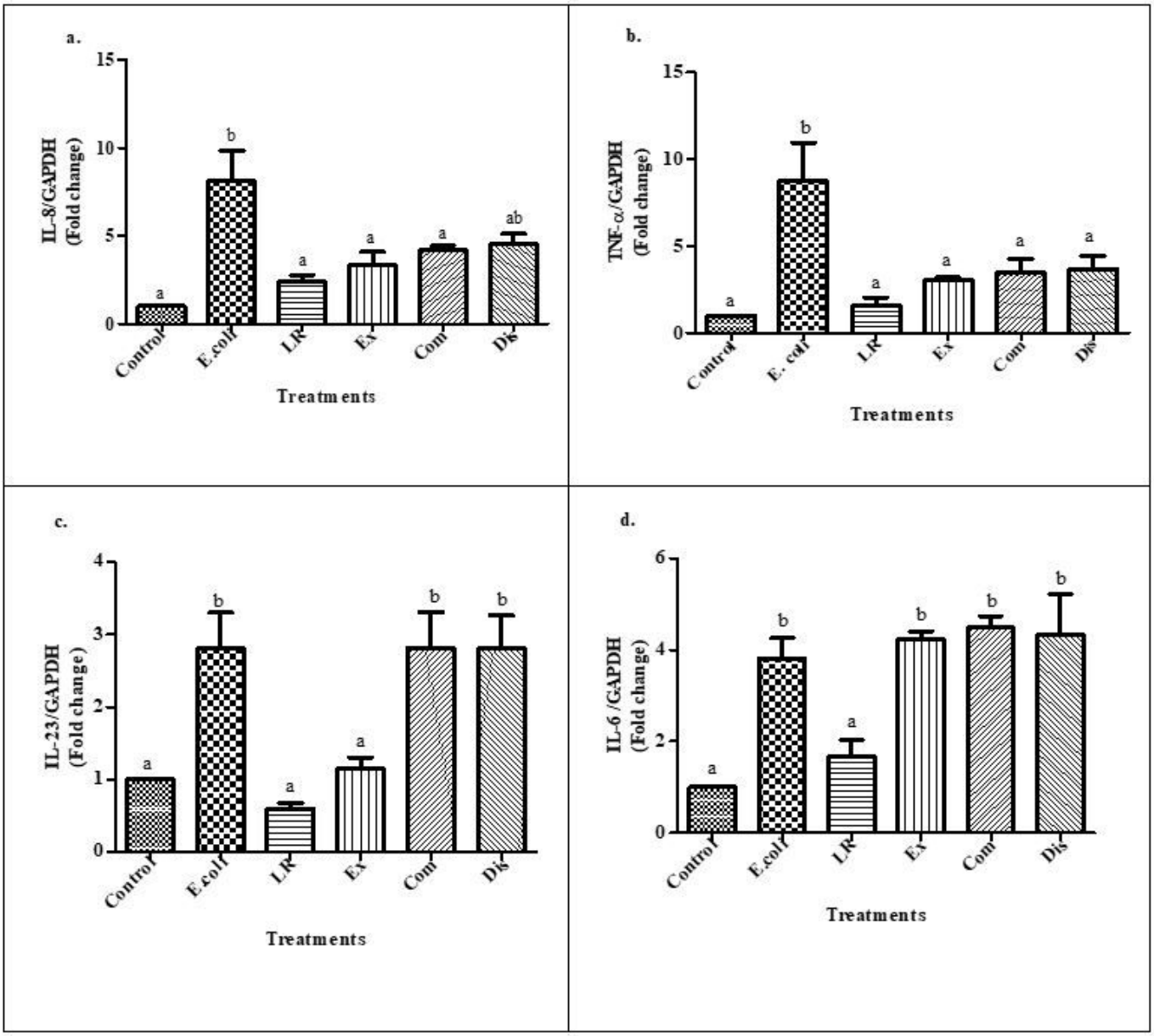

Figure 1

Transcriptional modulations of genes associated with immune signals (A: IL-8; B: TNF-α; C: IL-23; D: IL-6) during challenge with E. coli in the presence of probiotic L. rhamnosus. Values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$)

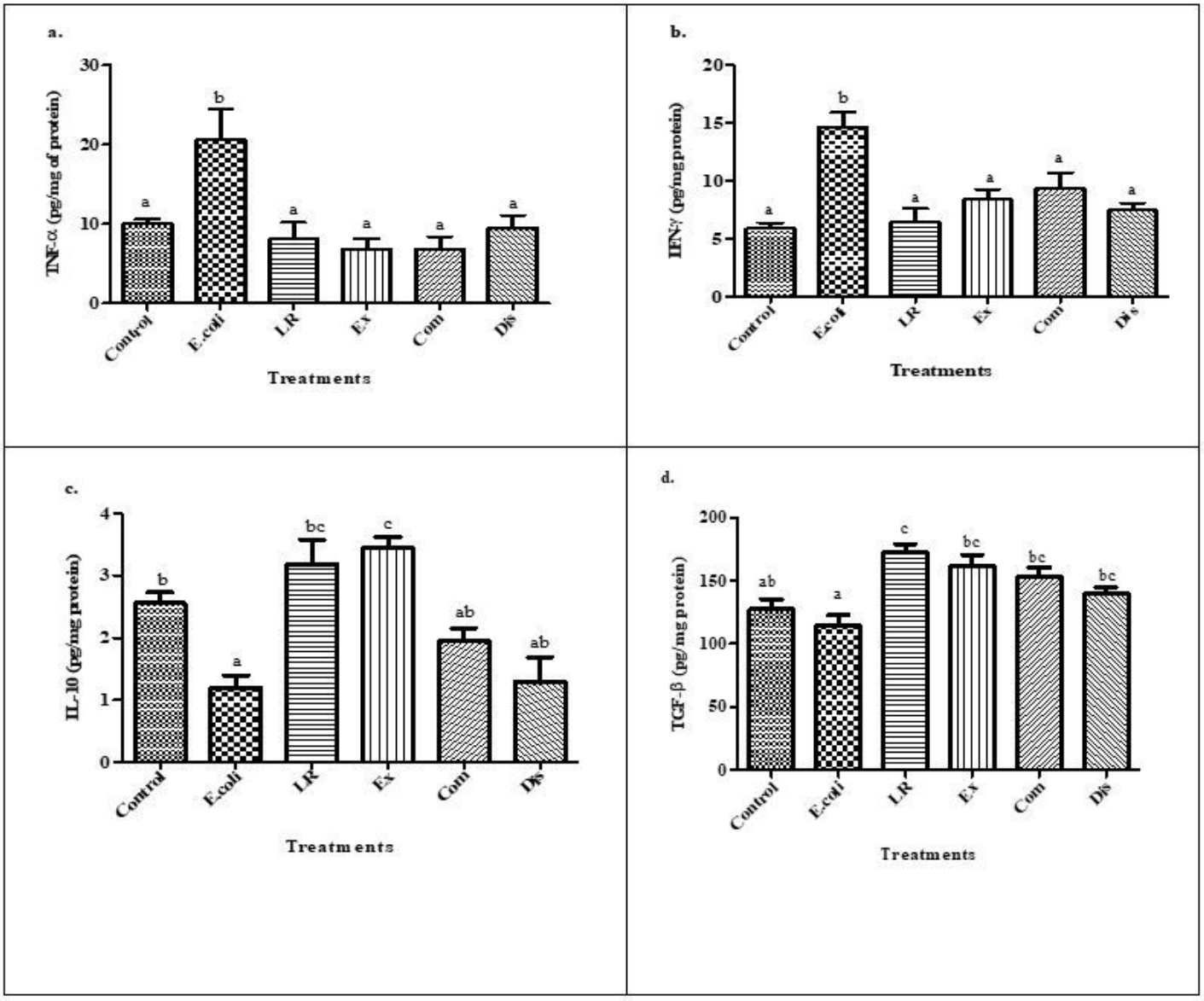

Figure 2

Release of interleukins (A: TNF-α; B: IFN-γ; C: IL-10; D: TGF-β) as immune signals by intestinal epithelial cells during challenge with E. coli in the presence of probiotic L. rhamnosus. Values are expressed as means ±SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$)

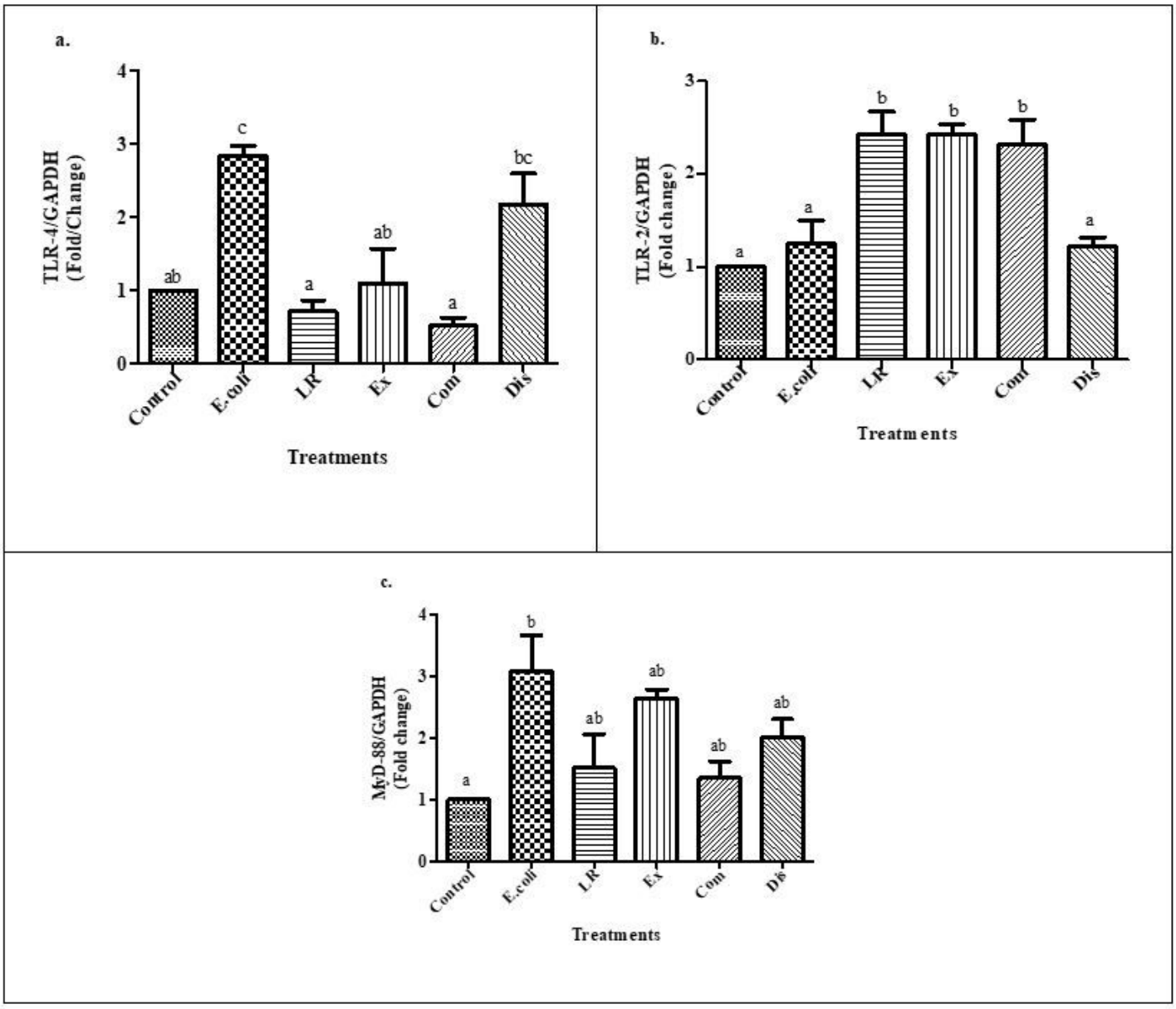

Figure 3

Transcriptional modulations of pathogen recognition receptor genes (A: TLR-2; B: TLR-4) and adaptor protein (C: MyD-88) during challenge with E. coli in the presence of probiotic L. rhamnosus. Values are expressed as means ± SEM (n=3). Means with different alphabets indicate significant difference ($p < 0.05$)

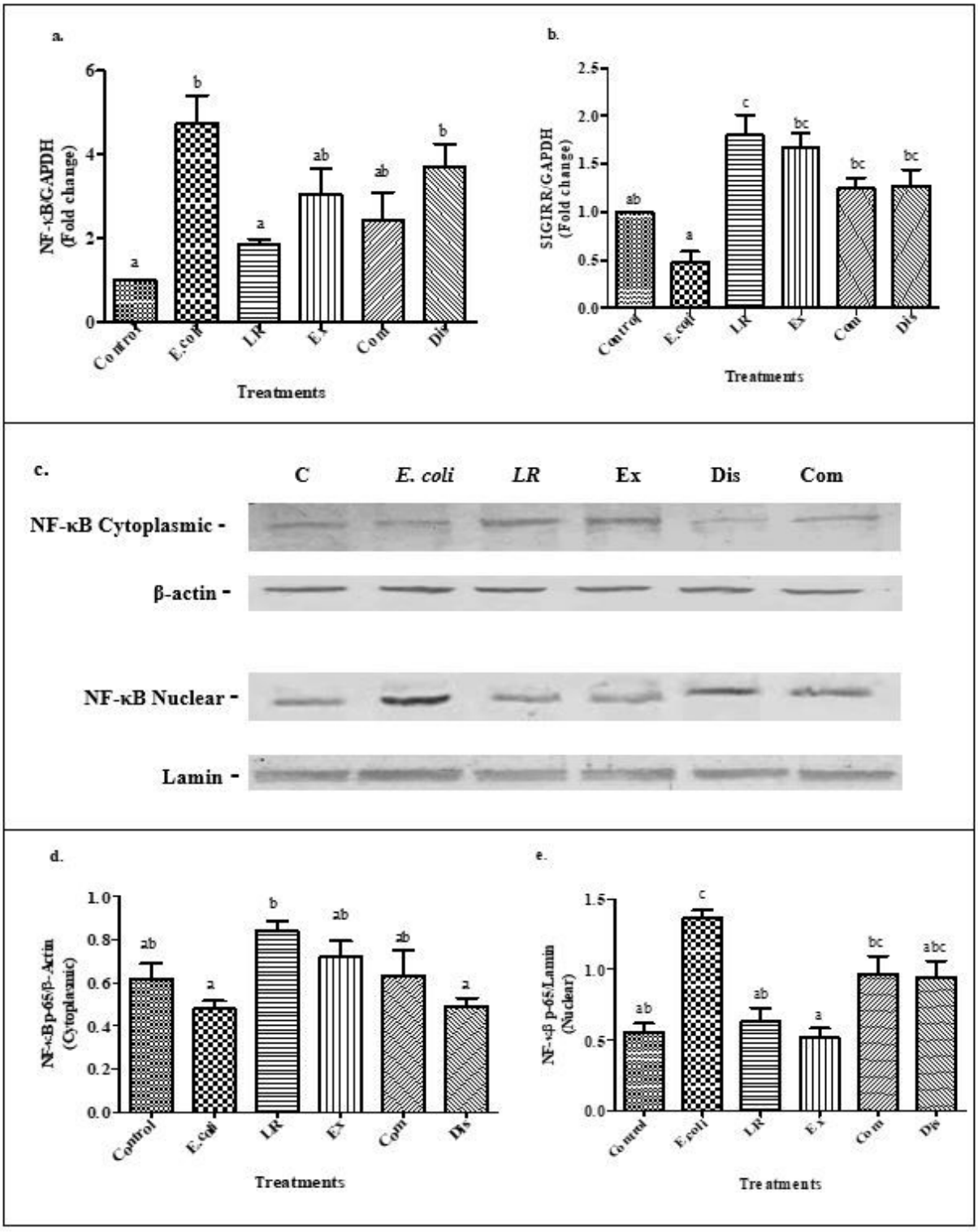

Figure 4

Effect of E. coli challenge on NF-κB master regulator of inflammation in the presence of probiotic L. rhamnosus. A: Modulation in transcriptional expression of NF-κB; B: SIGIRR genes; C: Translocation of NF-κB (p-65) from cytosol to nucleus by western blotting; D: Relative density of cytoplasmic NF-κB (p-65) bands; E: Relative density of nuclear NF-κB (p-65 bands). Values are expressed as means ± SEM (n=3).

Means with different alphabets indicate significant difference ($p < 0.05$) LR: Lactobacillus rhamnosus, Ex: Exclusion, Com: Competition, Dis: Displacement