

Lactobacillus brevis Cell-Free Supernatant Recalibrates TLR-Mediated Inflammatory Signaling While Preserving Immune Competence in HT-29 Intestinal Epithelial Cells

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Abstract

Background

Dysregulated Toll-like receptor (TLR) signaling in intestinal epithelial cells contributes to persistent mucosal inflammation in inflammatory bowel disease (IBD). Although *Lactobacillus brevis* has demonstrated anti-inflammatory potential, the pathway-level mechanisms by which its secreted metabolites influence epithelial innate immune signaling remain insufficiently characterized. This study investigated *L. brevis* cell-free supernatant (CFS) TLR-mediated inflammatory signaling modulation while preserving epithelial immune competence.

Methods

HT-29 human intestinal epithelial cells were treated with 50% *L. brevis* CFS following viability assessment using MTT assay. Transcriptional profiling of 84 key genes involved in the TLR signaling pathway was performed using the RT² Profiler™ PCR Array. Differential gene expression was analyzed using the $\Delta\Delta C_t$ method, and pathway-level mapping was conducted to evaluate coordinated modulation across MyD88- and TRIF-dependent branches.

Results

Treatment with *L. brevis* CFS significantly enhanced HT-29 cell viability and induced a structured transcriptional reprogramming of the TLR signaling network. Core components of the MyD88-dependent pro-inflammatory axis—including *TLR2*, *TLR4*, *MYD88*, *IRAK1/4*, *MAP3K7*, *NFKB1*, and downstream cytokines (*IL1A*, *IL1B*, *IL6*, *IL12A*, *CCL2*)—were significantly downregulated. Concurrent attenuation of TRIF-dependent signaling was observed through modulation of *TLR3*, *TICAM1*, *TBK1*, *IRF3*, and *CXCL10*. Importantly, selective preservation of immune-associated mediators and absence of exaggerated *IL-10* induction indicated maintained basal immune competence rather than global immunosuppression.

Conclusions

L. brevis CFS induces multi-tier recalibration of TLR-mediated inflammatory signaling in intestinal epithelial cells. By attenuating upstream adaptor and kinase modules while preserving essential immune responsiveness, *L. brevis*-derived metabolites promote controlled inflammatory tuning rather than indiscriminate immune suppression. These findings support a mechanistic basis for postbiotic-mediated modulation of epithelial innate immunity relevant to IBD-associated inflammation.

1. Background

Inflammatory bowel disease (IBD), encompassing Crohn's disease and ulcerative colitis, is a group of chronic, relapsing inflammatory disorders of the gastrointestinal tract that impose a substantial global health burden [1]. The pathogenesis of IBD is multifactorial, involving complex interactions between genetic predisposition, environmental triggers, gut microbiota dysbiosis, and dysregulated immune

responses, ultimately resulting in persistent intestinal inflammation [2, 3]. Recent epidemiological data highlight that, although incidence rates in many Western countries have plateaued, the prevalence of IBD continues to rise worldwide, with a marked increase in newly industrialized regions, particularly in Asia, the Middle East, and South America [3]. This epidemiological transition, coupled with the chronic, incurable nature of IBD and the limitations of current therapies, underscores its growing impact on public health and the urgent need for innovative therapeutic approaches [2].

Toll-like receptors (TLRs) are key pattern recognition receptors (PRRs) that sense microbial- and damage-associated molecular patterns, thereby linking the gut microbiota to host innate immunity. Through adaptor proteins such as MyD88 and TRIF, TLR signaling activates downstream cascades including NF- κ B and MAPK, which regulate the expression of pro-inflammatory cytokines and type I interferons [4, 5]. Under physiological conditions, TLR activity helps maintain mucosal homeostasis by balancing immune tolerance and protective responses [6]. However, dysregulated TLR signaling has been implicated in epithelial barrier dysfunction, exaggerated cytokine release, and chronic intestinal inflammation characteristic of IBD [7]. Clinical studies further confirm this association, reporting increased expression of TLR2, TLR4, and TLR9 in the colonic mucosa of ulcerative colitis patients, correlating with disease severity and microbial dysbiosis [8].

Probiotics, defined as live microorganisms that confer health benefits when administered in adequate amounts, have been increasingly recognized as promising adjunctive strategies for IBD management. By modulating gut microbiota composition, enhancing epithelial barrier integrity, and regulating host immune responses, probiotics exert significant anti-inflammatory effects [9, 10]. Among them, *Lactobacillus* species are the most widely studied, with evidence showing that various strains can attenuate colitis severity, restore immune balance, and reduce mucosal inflammation in both preclinical models and clinical trials [11]. Specifically, *Lactobacillus brevis* (*L. brevis*) has demonstrated potent immunomodulatory properties, including the suppression of pro-inflammatory cytokines (e.g., TNF- α , IL-6, IL-1 β) and the induction of anti-inflammatory mediators such as IL-10, while simultaneously strengthening epithelial tight junction proteins (e.g., ZO-1, occludin, and E-cadherin) [12]. These findings highlight the therapeutic potential of *L. brevis* as a strain-specific probiotic capable of modulating key pathways implicated in IBD pathogenesis, particularly through the regulation of TLR-mediated signaling and cytokine networks.

Despite growing evidence supporting the beneficial effects of probiotics in IBD, the precise molecular mechanisms through which *L. brevis* exerts its immunomodulatory functions remain poorly understood. In particular, limited data are available on how *L. brevis*-derived metabolites influence Toll-like receptor-mediated signaling in intestinal epithelial cells, a pathway central to the initiation and perpetuation of mucosal inflammation. To address this gap, the present study employed an in vitro HT-29 cell model to investigate the transcriptional alterations induced by *L. brevis* cell-free supernatant (CFS). By profiling the expression of 84 key genes within the TLR signaling pathway, we aimed to elucidate the potential regulatory effects of *L. brevis* on innate immune responses relevant to IBD pathogenesis.

2. Methods

2.1. *L. brevis* culture and Preparation of CFS

The probiotic strain *L. brevis* (ATCC-14869) was obtained from the National Genetic and Biological Resources Center (Tehran, Iran). The bacterium was cultivated on de Man, Rogosa, and Sharpe (MRS) broth (Merck, Germany) (37°C, anaerobic conditions, 24h). After confirmation of bacterial identity using Gram staining, a 0.5 McFarland standard (OD ≈ 0.6) of *L. brevis* was centrifuged (4,000 × g for 15 min at 4°C) using a refrigerated centrifuge (Eppendorf 5810R, Germany). The resulting supernatant was collected and passed through a 0.22 µm sterile membrane filter (Sartorius, Germany) to ensure removal of bacterial cells. The sterile filtrate, designated as CFS, was aliquoted and stored at – 20°C until further use in cell treatment experiments. Serial dilutions of the CFS (100%, 50%, 25%, and 12.5%) were prepared in culture medium for subsequent assays.

2.2. HT-29 cell culture

The human colorectal adenocarcinoma epithelial cell line HT-29 (ATCC HTB-38) was obtained from the Pasteur Institute (Tehran, Iran). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco™, USA) supplemented with 10% fetal bovine serum (FBS, Gibco™, USA) and 1% penicillin–streptomycin (Gibco™, USA) and incubated (37°C, humidified atmosphere with 5% CO₂). Cells were subcultured upon reaching ~ 80% confluency using 0.25% trypsin–EDTA solution (Gibco™, USA).

2.3. Cell Viability (MTT) and Treatment Design

HT-29 cells (2×10⁴/well) were cultured in 96-well plates for 24 h. After medium replacement with serial dilutions of the CFS (100%, 50%, 25%, 12.5%), cells were incubated for an additional 24 and 48 h. Cell viability was then assessed using MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, Germany) solution (5 mg/mL, 3 h incubation), followed by DMSO solubilization. Absorbance was measured at 570 nm, and viability was calculated relative to untreated controls.

For treatment experiments, HT-29 cells were seeded into six-well plates at a density of 1×10⁶ cells/well and allowed to adhere for 24 h. Subsequently, the cells were exposed to the 50% CFS of *L. brevis* (48 h), which have been identified in preliminary MTT assays as the concentration and time providing the highest cell viability (> 90%). Untreated cells cultured under identical conditions served as the control group.

2.4. RNA Extraction, cDNA Synthesis, and Gene Expression Analysis of TLR Signaling Pathway by Real-Time PCR

Total RNA was extracted from cultured cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany, Cat. No. 74106) according to the manufacturer's protocol. Briefly, approximately 1 × 10⁶ cells were harvested, pelleted, and lysed in 350 µL of RLT lysis buffer. The lysate was homogenized by pipetting and mixed thoroughly with 350 µL of 70% ethanol. The mixture was then applied to a RNeasy Mini spin column and

centrifuged (8000× g, 15 s). The column was washed sequentially with 350 µL of RW1 buffer and twice with 500 µL of RPE buffer (for each centrifuged at 8000× g, 15 s and a final centrifugation at 8000 × g, 2 min). Finally, RNA was eluted in 30 µL of RNase-free water (8000× g, 1 min). RNA concentration and purity were determined using a NanoDrop spectrophotometer, and integrity was confirmed by 1% agarose gel electrophoresis. RNA samples were stored at – 80°C until further use.

cDNA was synthesized using the QuantiNova Reverse Transcription Kit (Qiagen, Cat. No. 205411, Germany) following the manufacturer's protocol. Briefly, up to 5 µg of total RNA was combined with 2 µL of gDNA Removal Mix in a 15 µL reaction volume and incubated at 45°C for 2 min. Subsequently, 5 µL of the Reverse-transcription Master Mix (containing 4 µL of Reverse Transcription Mix and 1 µL of Reverse Transcription Enzyme) was added. The complete reaction was incubated in a thermal cycler for annealing (25°C for 3 min), Reverse-transcription (45°C for 10 min), and final Inactivation of reaction (85°C for 5 min) steps. Concentration of synthesized cDNA was measured using a NanoDrop spectrophotometer and were normalized to a uniform concentration with RNase-free water. The synthesized cDNA was stored at -20°C.

Gene expression profiling of the Toll-like receptor (TLR) signaling pathway was performed using the RT² Profiler™ PCR Array Human Toll-Like Receptor Signaling Pathway (Qiagen, Germany, Cat. No. PAHS-018Z). A PCR master mix was prepared by combining 1350 µL of 2× RT² SYBR Green Mastermix, 102 µL of synthesized cDNA, and 1248 µL of RNase-free water (total volume: 2700 µL). Then, 25 µL of the master mix was loaded into each well of the pre-designed 96-well array plate (Fig. 2.1). The plate contains primers for 84 TLR pathway-related genes, 5 housekeeping genes, 1 genomic DNA control, 3 reverse transcription controls (RTC), and 3 positive PCR controls (PPC). Quantitative real-time PCR was run on a Roche LightCycler 480 with the following cycling protocol: initial activation (95°C for 10 min), followed by 40 cycles of denaturation (95°C for 15 s) and annealing/extension (60°C for 1 min) (with fluorescence acquisition).

Data quality was validated by requiring the genomic DNA control to have a Cq > 38 and the positive PCR controls to have a Cq < 20. Relative gene expression was analyzed using the GeneGlobe Data Analysis Center (<https://geneglobe.qiagen.com/us/analyze>), which calculates the fold change/regulation of the investigated genes using the delta Ct (ΔCt) method. Briefly, ΔCt was calculated between each gene and an average of the housekeeping genes. Then, $\Delta\Delta Ct$ was extrapolated as the difference between ΔCt of genes in the tested group and ΔCt of the same genes in the control group. Finally, fold change was calculated using $2^{(-\Delta\Delta Ct)}$ formula.

Data analyses were also performed using PathVisio software, KEGG String, and WikiPathways online websites (<https://www.wikipathways.org/index.php/WikiPathways>).

2.5. Statistical Analysis

All experiments were performed in triplicate, and data are presented as mean ± standard deviation (SD). Statistical analyses were carried out using GraphPad Prism version 8.0 (GraphPad Software, USA).

Differences between treated and control groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. 50% *L. brevis* CFS Enhances HT-29 Cell Proliferation in MTT Assay

The MTT assay demonstrated both concentration- and time-dependent effects of *L. brevis* CFS on HT-29 cell viability (each treatment group normalized to the unstimulated HT-29 control) (Fig. 3.1). Treatment with 50% *L. brevis* CFS significantly enhanced HT-29 cell proliferation, with no significant difference between 24 h and 48 h exposure. Since 24 h exposure was sufficient to induce significant proliferation, we chose 50% CFS at 48 hours with the aim of subsequent gene expression and molecular pathway analysis, as the extended exposure was expected to provide a more robust model for examining signaling pathways relevant to IBD pathogenesis.

3.2. *L. brevis* Induced Significant Alterations in Gene Expression of HT-29 Cells

The qPCR array analysis of 84 key genes involved in the Toll-like receptor (TLR) signaling pathway revealed significant transcriptional changes following treatment of HT-29 cells with 50% *L. brevis* CFS (Fig. 3.2). While a concise group of seven genes was significantly upregulated—including *BTK* ($p = 0.016$), *FOS* ($p < 0.001$), *HSPA1A* ($p < 0.001$), *JUN* ($p < 0.001$), *TICAM2* ($p < 0.001$), *TLR1* ($p = 0.016$), and *TLR10* ($p = 0.030$) (Fig. 3.2.A)—the overall response was predominantly characterized by a broad and coordinated downregulation across three major innate immune signaling modules. First, the TRIF-dependent antiviral and interferon response axis was markedly inhibited, evidenced by downregulation of *TLR3* ($p < 0.001$), its adaptor *TICAM1* ($p < 0.001$), the kinase *TBK1* ($p < 0.001$), the transcription factor *IRF3* ($p < 0.001$), and the chemokine *CXCL10* ($p < 0.001$) (Fig. 3.2.B1). Second, the core components of the canonical TLR/MyD88-dependent pro-inflammatory pathway were suppressed, encompassing receptors (*TLR2* $p < 0.001$, *TLR4* $p < 0.001$), adaptors and kinases (*MYD88* $p < 0.001$, *TIRAP* $p < 0.001$, *IRAK1* $p < 0.001$, *IRAK4* $p < 0.001$, *MAP3K7* $p < 0.001$), transcription factors (*NFkB1* $p < 0.001$, *REL* $p < 0.001$), and effector cytokines (*IL1A* $p < 0.001$, *IL1B* $p < 0.001$, *IL6* $p < 0.001$, *IL12A* $p < 0.001$, *CCL2* $p < 0.001$, *PTGS2* $p < 0.001$) (Fig. 3.2.B2). Third, a network of integrated modulatory pathways was attenuated, including mediators of apoptosis (*CASP8* $p < 0.001$, *FADD* $p < 0.001$), key elements of the MAPK/JNK stress-signaling cascade (*MAP3K1* $p < 0.001$, *MAP2K3* $p < 0.001$, *MAP2K4* $p < 0.001$, *MAPK8* $p < 0.001$), the immunomodulatory nuclear receptor *PPARA* ($p < 0.001$), and pleiotropic cellular regulators (*HRAS* $p < 0.001$, *HMGB1* $p < 0.001$) (Fig. 3.2.B3). Collectively, these data demonstrate that *L. brevis* CFS induces a potent immunomodulatory transcriptomic signature in intestinal epithelial cells, primarily through the

simultaneous dampening of multiple TLR-driven inflammatory and antiviral programs, suggesting a mechanistic basis for promoting an anti-inflammatory milieu.

3.3. Pathway-Level Impacts of *L. brevis* CFS on TLR Signaling

To obtain a systems-level understanding of the transcriptional alterations induced by *L. brevis* 50% CFS, differentially expressed genes were integrated into a curated TLR signaling pathway adapted for HT-29 intestinal epithelial cells (Fig. 3.3.). Pathway mapping revealed coordinated modulation of both the MyD88-dependent and MyD88-independent (TRIF-dependent) signaling branches.

As illustrated in Fig. 3.3., multiple components of the MyD88-dependent cascade—including adaptor molecules and downstream kinase modules—displayed altered expression profiles. These changes collectively suggest attenuation of NF- κ B and MAPK activation, which is consistent with the observed modulation of downstream inflammatory mediators and chemokines. The pathway visualization demonstrates that transcriptional regulation was not confined to a single node but extended across adaptor proteins, signal transducers, and effector genes.

To further delineate the effects within the canonical inflammatory axis, the MyD88-dependent arm was separately highlighted (Fig. 3.4.). This focused representation emphasizes the modulation of IRAK-associated signaling, TRAF6-mediated ubiquitination events, MAPK cascades, and NF- κ B family transcription factors, ultimately impacting the expression of pro-inflammatory genes. The collective pathway-level analysis indicates that *L. brevis* CFS exerts broad regulatory effects on TLR-mediated signaling networks in HT-29 cells rather than selectively targeting an isolated downstream effector.

The Toll-like receptor (TLR) signaling pathway was adapted for intestinal epithelial cells (HT-29). Gene expression changes are color-coded as follows: gray indicates no statistically significant change; dark blue indicates significantly downregulated genes ($p < 0.05$ and fold regulation > 2); light blue indicates significantly downregulated genes ($p < 0.05$ and fold regulation < 2); dark red indicates significantly upregulated genes ($p < 0.05$ and fold regulation > 2); light red indicates significantly upregulated genes ($p < 0.05$ and fold regulation < 2). Signaling cascades include MyD88-dependent and MyD88-independent pathways leading to NF- κ B, MAPK, and IRF activation and subsequent inflammatory gene transcription.

4. Discussion

The intestinal epithelium maintains mucosal homeostasis through tightly regulated TLR signaling [13]. In inflammatory bowel disease (IBD), dysregulation of this system leads to chronic inflammation [5]. Although probiotics have been reported to modulate epithelial immune responses, the pathway-level mechanisms by which bacterial metabolites influence TLR signaling remain incompletely defined. In the present study, exposure of HT-29 cells to *L. brevis* cell-free supernatant (CFS) induced a coordinated

transcriptional reprogramming across the TLR signaling network, indicating broad dampening of TLR activation rather than isolated regulation of individual cytokines.

At the receptor level, *L. brevis* CFS induced structured remodeling rather than uniform suppression. TLR2 was markedly downregulated, whereas TLR1 and TLR10 were upregulated. TLR2 is known to drive MyD88-NF- κ B inflammatory activation in intestinal inflammation [14], and its overexpression has been associated with inflammatory amplification in colitis models [15]. Thus, its reduction aligns with attenuation of classical pro-inflammatory signaling. In contrast, TLR10 has been recognized as a modulatory receptor capable of suppressing NF- κ B activation [16, 17], and probiotic-induced TLR2/TLR10-dependent signaling has been linked to epithelial immune tolerance [18]. The asymmetric regulation of the TLR2–TLR6 pair (TLR2 down; TLR6 unchanged) further supports disruption of canonical lipoteichoic acid-driven inflammatory signaling [14]. The most coordinated suppression occurred within the TLR4 sensing module. TLR4 downregulation paralleled reduced CD14 and HMGB1 expression, alongside modulation of the CD180–LY86 regulatory complex. Because CD14 facilitates LPS delivery to the TLR4–MD-2 complex and HMGB1 amplifies TLR2/4-mediated NF- κ B activation, their concurrent reduction suggests attenuation of a high-gain inflammatory amplification loop rather than isolated transcriptional fluctuation. Probiotic-mediated suppression of TLR4/MyD88/NF- κ B signaling has similarly been reported in DSS colitis models [19], and decreased TLR4 expression with enhanced tolerogenic cytokine production has been observed in immune cells from IBD patients treated with probiotics [20]. Our data extend these observations by demonstrating coordinated modulation across multiple components of the LPS recognition complex at the epithelial level. In contrast to surface bacterial sensors, endosomal TLRs exhibited selective modulation. TLR3 and TLR9 were reduced, whereas TLR7 and TLR8 remained unchanged. Given that TLR3 and TLR9 overactivation contributes to mucosal inflammatory amplification in IBD and colitis-associated pathology [21, 22], their attenuation may reflect restoration of epithelial activation thresholds rather than compromised antiviral defense. Preservation of TLR7/8 is notable, as TLR7–MyD88 signaling is critical for antiviral competence [23]. Consistent with this receptor pattern, adaptor redistribution (MYD88 and TIRAP decreased; TICAM1 decreased while TICAM2 increased) suggests rebalancing between inflammatory MyD88-dependent signaling and TRIF-associated interferon routing rather than global innate suppression.

Within the MYD88-dependent arm, transcriptional modulation extended into the core Myddosome architecture. IRAK1/2, MAP3K7 (TAK1), TAB1, TRAF6, and TIRAP were coordinately regulated, indicating attenuation at the level of signalosome assembly and TRAF6–TAK1 complex formation rather than isolated receptor dampening. Because this complex controls activation of both the IKK–NF- κ B axis and MAPK cascades, modulation at this tier constrains inflammatory signal propagation upstream of cytokine transcription [24]. Consistently, components governing IKK activation and NF- κ B nuclear translocation—including CHUK, IKBKB, UBE2N, NFKB1/2, REL, and RELA—were transcriptionally modulated. Similar adaptor- and kinase-level suppression has been reported in HT-29 cells treated with *Lactobacillus/Bifidobacterium* mixtures, where TIRAP, IRAK4, and NEMO were downregulated alongside reduced NF- κ B activity [25]. Likewise, *L. acidophilus* and *B. animalis* suppressed phosphorylated p65 and p38 MAPK in stimulated HT-29 models [26]. Notably, *L. brevis* itself reduced RELA expression and shifted

the RELA–IKB balance in HT-29 cells [27], supporting restrained NF-κB transcriptional activity. Parallel modulation of MAP3K7–TAB1 and downstream MAP2K3/MAP2K4/MAPK8 modules further indicates dampening of JNK and p38 signaling, pathways that cooperate with NF-κB to drive AP-1-dependent transcription. In vivo, *L. brevis* G-101 inhibited IRAK1 phosphorylation and suppressed NF-κB/MAPK activation in TNBS colitis while reducing TNF-α, IL-1β, and IL-6 production [24], aligning with our kinase-tier findings. Importantly, MYD88 modulation should be interpreted as recalibration rather than abrogation. Complete MyD88 loss disrupts microbial homeostasis and can worsen colitis through compensatory inflammatory pathways [28, 29]. Thus, the coordinated attenuation observed here most likely reflects controlled signal gating, preserving basal innate responsiveness while limiting excessive amplification relevant to epithelial homeostasis in IBD.

In parallel with MyD88-dependent attenuation, components of the TICAM1 (TRIF)-dependent pathway were also selectively regulated, indicating broader recalibration of innate signaling topology. Core TRIF-axis elements—including TICAM1, TICAM2, TBK1, TRAF6, MAP3K7, and PELI1—were transcriptionally modulated. Because TRIF signaling downstream of TLR3/4 governs TBK1–IRF-mediated interferon responses while intersecting with NF-κB via TRAF6–TAK1 [25], regulation at this tier reflects control of signal branching rather than restriction of a single inflammatory output. Consistently, downstream IRF1/IRF3 and interferon-related genes (*IFNA1*, *IFNB1*, *IFNG*, *CXCL10*) were selectively modulated. In pathogenic contexts, TRIF-linked signaling can drive high-amplitude interferon activation. For example, Caco-2 cells exposed to *E. coli* OMVs exhibited marked upregulation of *IFNA1/IFNB1* alongside TLR3/7/8 activation [30], illustrating the amplification capacity of this branch. Compared with such hyperactivation, the pattern observed here suggests controlled attenuation rather than antiviral shutdown. Similarly, native *Lactobacillus* spp. have been reported to modulate MYD88-independent transcripts without abolishing immunoregulatory effects, supporting a model of pathway recalibration rather than collapse [31].

Given that several differentially expressed cytokines in our dataset—including *CCL2*, *CSF2*, *IFNG*, *IL12A*, *IL2*, and *IL6*—signal through canonical JAK/STAT pathways, the observed transcriptional shifts extend beyond primary TLR activation into secondary cytokine-driven amplification loops. IL-6 primarily activates JAK1/JAK2–STAT3, whereas IFNγ and IL-12 preferentially engage STAT1 and STAT4, respectively [25]. Since STAT phosphorylation sustains inflammatory gene transcription, modulation at this level directly influences the persistence of mucosal inflammation. In HT-29 IBD models, probiotic cocktails have been shown to downregulate JAK genes alongside NF-κB components, with concomitant reductions in IL-6 and IL-1β production [25, 32], supporting functional linkage between pathway modulation and cytokine suppression. Similarly, *Lactobacillus* spp. have been reported to coordinately downregulate JAK genes together with TIRAP and IRAK4 in HT-29 cells, disrupting the TLR–cytokine–STAT feed-forward loop [33]. Because JAK/STAT signaling intersects with NF-κB transcriptional programs in epithelial cells [34], simultaneous modulation of both axes suggests coordinated restriction of inflammatory reinforcement circuits. Consistent with this, prior work on *L. brevis* demonstrated regulation of RELA and IKB expression in HT-29 cells [27], indicating that probiotic-derived factors can influence transcriptional regulators upstream of cytokine amplification.

Modulation of downstream effector molecules—including *CASP8*, *FADD*, *EIF2AK2* (PKR), *PRKRA*, *PPARA*, *ECSIT*, *UBE2N*, and *TRAF6*—further underscores that *L. brevis* CFS influences multiple tiers of intracellular signaling architecture beyond proximal adaptor complexes. At the level of ubiquitin-dependent NF-κB regulation, coordinated modulation of TRAF6 and UBE2N is particularly notable. TRAF6 functions as a central E3 ubiquitin ligase within TLR pathways, mediating K63-linked ubiquitination events required for IKK activation and NF-κB nuclear translocation. Recent evidence demonstrates that alterations in TRAF6 protein stability critically influence IBD severity; ASB3-mediated K48-linked polyubiquitination of TRAF6 promotes dysregulated NF-κB activation and intestinal microbiota imbalance [35]. Therefore, transcriptional regulation of TRAF6 and its associated ubiquitination machinery in our dataset suggests upstream tuning of NF-κB activation thresholds. Consistent with this, modulation of ECSIT further implicates control at the level of ubiquitin-dependent NF-κB activation. ECSIT serves as a TRAF6-interacting scaffold within TLR4 signaling and requires ubiquitination at lysine 372 for effective interaction with p65/p50 NF-κB and nuclear colocalization [36]. Because ubiquitinated ECSIT facilitates NF-κB DNA-binding activity and pro-inflammatory cytokine production, its regulation implies potential attenuation of nuclear NF-κB transcriptional competence rather than simple cytoplasmic signal restriction. The observed shifts in CASP8 and FADD indicate additional cross-talk between inflammatory and apoptotic signaling nodes. In the context of IBD, epithelial apoptosis and barrier dysfunction contribute directly to mucosal inflammation. Therefore, coordinated regulation of *CASP8/FADD* suggests impact on the epithelial survival–inflammation balance. Modulation of *EIF2AK2* (PKR) and its activator *PRKRA* extends this regulatory influence into antiviral and stress-response pathways. Finally, transcriptional regulation of *PPARA* introduces a nuclear receptor-mediated anti-inflammatory layer. PPARα exerts inhibitory effects on NF-κB-dependent gene expression and represses inflammatory mediators such as IL-6 and COX-2 [37]. Collectively, the coordinated modulation of ubiquitination regulators, scaffold proteins, apoptotic mediators, antiviral kinases, and nuclear receptors indicates structured regulation across cytoplasmic, nuclear, and post-translational signaling layers.

The coordinated modulation of *SIGIRR*, *SARM1*, and *TOLLIP* indicates reinforcement of intrinsic inhibitory checkpoints within the TLR network rather than indiscriminate pathway suppression. These regulators operate at distinct tiers—TOLLIP at the IRAK level, SIGIRR at receptor complex assembly, and SARM1 at adaptor-routing control—collectively constraining excessive MYD88-driven amplification. TOLLIP inhibits IRAK activation within the TLR4 cascade. In Caco-2 cells, *Lactobacillus plantarum* MYL26 induced LPS tolerance via TOLLIP upregulation, impairing TLR4–NF-κB signaling [38], and *Bifidobacterium adolescentis* increased intestinal TOLLIP while reducing TLR4 expression in NEC models [39]. This aligns with our observation that TOLLIP modulation accompanies upstream TLR attenuation, suggesting active negative-feedback reinforcement. SIGIRR (TIR8) functions as a decoy receptor limiting IL-1R/TLR signaling. SIGIRR deficiency enhances NF-κB activation and susceptibility to colitis [40]. Probiotic strains—including *L. brevis*—have been shown to upregulate SIGIRR in HT-29 cells while suppressing pathogen-induced cytokines [41], and *Bifidobacterium breve* similarly enhanced SIGIRR alongside A20 and Tollip to restrain NF-κB/MAPK activation [42]. SARM1 further modulates MYD88–TRAF signaling dynamics, with recent data indicating that SARM1 regulates MYD88-mediated inflammatory routing [43] and influences

intestinal inflammatory responses in IBD models [44]. Together, the regulation of TOLLIP, SIGIRR, and SARM1 supports a model in which *L. brevis* CFS strengthens endogenous braking mechanisms—limiting NF- κ B/MAPK amplification while preserving basal antimicrobial competence.

Functional stratification of the differentially expressed genes into pathogen-associated response modules revealed structured and non-uniform tuning across microbial sensing axes. Genes linked to bacterial sensing (*TLR2*, *TLR4*, *CD14*, *LY96*, *RIPK2*), viral recognition (*TLR3*, *TLR7*, *TLR8*, *TBK1*, *IRF3*, *EIF2AK2*), and fungal/parasitic pathways (*CLEC4E*, *TIRAP*) were differentially regulated rather than globally suppressed. Importantly, antiviral competence appears preserved. In contrast to pharmacologic suppression of the TLR–TBK1–IRF3/7 axis, which reduces IFN- α/β transcription [45], our dataset did not demonstrate collapse of interferon-associated mediators. Preservation of TNF expression in our system similarly argues against impaired antibacterial defense. Equally important, no disproportionate IL-10 induction was observed. While *Levilactobacillus brevis* IBARAKI-TS3 promotes IL-10 production via TLR2 engagement [46], and *L. reuteri* postbiotics shift IL-17/IL-10 balance toward regulation in inflamed HT-29 cells [47], our epithelial dataset did not exhibit excessive regulatory skewing suggestive of compromised vigilance. Barrier preservation provides an additional safeguard; *Lactobacillus plantarum* prevents tight junction disruption and endotoxemia through EGFR-dependent mechanisms [48]. Collectively, the differential modulation of pathogen-sensing modules in our system reflects structured immune recalibration rather than generalized immunosuppression.

Several limitations warrant consideration. First, the analysis was confined to a targeted RT² Profiler PCR Array centered on TLR-related genes; although this enables pathway-focused resolution, broader transcriptomic alterations beyond the predefined panel may have been overlooked. Second, our interpretations rely on mRNA-level modulation without parallel protein or functional validation, and transcript changes do not necessarily translate into proportional alterations in protein abundance or signaling activity, particularly for adaptor proteins and transcription factors subject to post-translational regulation. Third, the use of an in vitro HT-29 epithelial model, while providing controlled mechanistic insight, does not fully recapitulate the cellular complexity of the intestinal microenvironment, including immune cell interactions, microbiota dynamics, and barrier architecture. Future studies incorporating protein-level validation and more physiologically relevant in vivo or co-culture systems will be required to confirm the pathway-level inferences proposed here.

In conclusion, *L. brevis* CFS treatment of HT-29 epithelial cells induced coordinated, multi-layer reprogramming of the TLR signaling network rather than isolated cytokine suppression. Remodeling occurred across receptor complexes, adaptor routing, kinase tiers, JAK/STAT amplification loops, and intrinsic inhibitory checkpoints, collectively indicating structured attenuation of inflammatory signal propagation at multiple upstream nodes. Importantly, this pattern does not represent global immunosuppression. Interferon-associated pathways remained transcriptionally competent, TNF signaling was not pathologically suppressed, and IL-10 did not display disproportionate induction. Instead, pathogen-specific sensing modules were differentially tuned, suggesting preserved antiviral and antibacterial capacity alongside constrained inflammatory amplification. Together, these findings

support a mechanistic model in which probiotic-derived metabolites recalibrate epithelial immune thresholds through signal gating and reinforcement of endogenous regulatory circuits. Such multi-tier modulation may represent a rational strategy for restoring mucosal immune balance in IBD-associated inflammation without compromising core host defense functions.

Statement of Ethics

This study did not involve human participants, animal experiments, or clinical samples. All procedures were conducted using established human cell lines (HT-29) obtained from the Pasteur Institute of Iran, and therefore ethical approval was not required according to institutional and national guidelines.

Abbreviations

BTK	Bruton agammaglobulinemia tyrosine kinase
CASP8	Caspase 8, apoptosis-related cysteine peptidase
CCL2	Chemokine (C-C motif) ligand 2
CD14	CD14 molecule
CD180	CD180 molecule
CD80	CD80 molecule
CD86	CD86 molecule
CHUK	Conserved helix-loop-helix ubiquitous kinase
CLEC4E	C-type lectin domain family 4, member E
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)
CSF3	Colony stimulating factor 3 (granulocyte)
CXCL10	Chemokine (C-X-C motif) ligand 10
ECSIT	ECSIT homolog (Drosophila)
EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2
ELK1	ELK1, member of ETS oncogene family
FADD	Fas (TNFRSF6)-associated via death domain
FOS	FBJ murine osteosarcoma viral oncogene homolog
HMGB1	High mobility group box 1
HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog
HSPA1A	Heat shock 70kDa protein 1A
HSPD1	Heat shock 60kDa protein 1 (chaperonin)
IFNA1	Interferon, alpha 1
IFNB1	Interferon, beta 1, fibroblast
IFNG	Interferon, gamma
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
IL10	Interleukin 10
IL12A	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)
IL1A	Interleukin 1, alpha

IL1B	Interleukin 1, beta
IL2	Interleukin 2
IL6	Interleukin 6 (interferon, beta 2)
CXCL8	Interleukin 8
IRAK1	Interleukin-1 receptor-associated kinase 1
IRAK2	Interleukin-1 receptor-associated kinase 2
IRAK4	Interleukin-1 receptor-associated kinase 4
IRF1	Interferon regulatory factor 1
IRF3	Interferon regulatory factor 3
JUN	Jun proto-oncogene
LTA	Lymphotoxin alpha (TNF superfamily, member 1)
LY86	Lymphocyte antigen 86
LY96	Lymphocyte antigen 96
MAP2K3	Mitogen-activated protein kinase kinase 3
MAP2K4	Mitogen-activated protein kinase kinase 4
MAP3K1	Mitogen-activated protein kinase kinase kinase 1
MAP3K7	Mitogen-activated protein kinase kinase kinase 7
MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4
MAPK8	Mitogen-activated protein kinase 8
MAPK8IP3	Mitogen-activated protein kinase 8 interacting protein 3
MYD88	Myeloid differentiation primary response gene (88)
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
NFKBIL1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1
NFRKB	Nuclear factor related to kappaB binding protein
NR2C2	Nuclear receptor subfamily 2, group C, member 2
PELI1	Pellino homolog 1 (Drosophila)
PPARA	Peroxisome proliferator-activated receptor alpha

PRKRA	Protein kinase, interferon-inducible double stranded RNA dependent activator
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
REL	V-rel reticuloendotheliosis viral oncogene homolog (avian)
RELA	V-rel reticuloendotheliosis viral oncogene homolog A (avian)
RIPK2	Receptor-interacting serine-threonine kinase 2
SARM1	Sterile alpha and TIR motif containing 1
SIGIRR	Single immunoglobulin and toll-interleukin 1 receptor (TIR) domain
TAB1	TGF-beta activated kinase 1/MAP3K7 binding protein 1
TBK1	TANK-binding kinase 1
TICAM1	Toll-like receptor adaptor molecule 1
TICAM2	Toll-like receptor adaptor molecule 2
TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR1	Toll-like receptor 1
TLR10	Toll-like receptor 10
TLR2	Toll-like receptor 2
TLR3	Toll-like receptor 3
TLR4	Toll-like receptor 4
TLR5	Toll-like receptor 5
TLR6	Toll-like receptor 6
TLR7	Toll-like receptor 7
TLR8	Toll-like receptor 8
TLR9	Toll-like receptor 9
TNF	Tumor necrosis factor
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A
TOLLIP	Toll interacting protein
TRAF6	TNF receptor-associated factor 6
UBE2N	Ubiquitin-conjugating enzyme E2N
ACTB	Actin, beta

B2M	Beta-2-microglobulin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HPRT1	Hypoxanthine phosphoribosyltransferase 1
RPLP0	Ribosomal protein, large, P0
RTC	Reverse Transcription Control
PPC	Positive PCR Control

Declarations

Conflict of Interest

Authors declare no conflict of interest.

CRedit authorship contribution statement

Alireza Moghanlou (Moghanlou@yahoo.com): Data curation. Shima Rasouli (shrs84@iau.ac.ir): Writing – original draft. Sheyda Asadi (asadish@gmail.com) : Investigation. Sarvenaz Falsafi (dr.sfalsafi@gmail.com): Investigation. Behrouzi Ava (avabehrouzi696463@iau.ac.ir): Writing – original draft.

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Author Contribution

Data curation: A.M. & A.B. & S.R. Investigation: S.A. & S.F. Writing – original draft: A.B. & S.R.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures

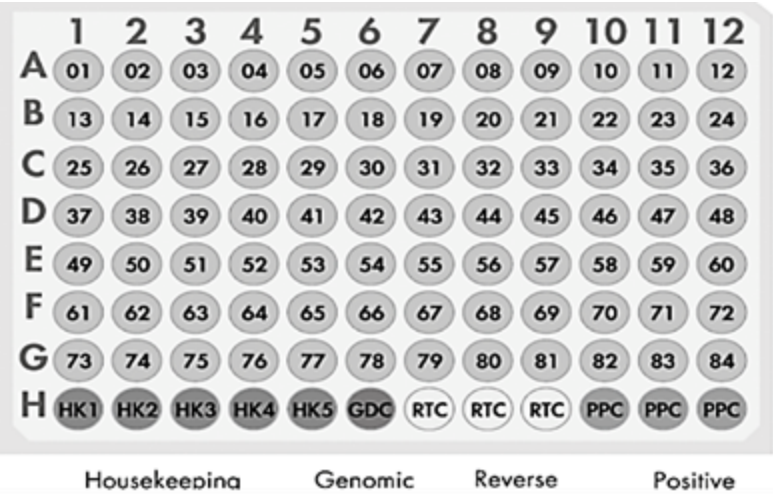


Figure 1

Fig 2.1. Schematic of the 96-well RT² Profiler™ PCR Array Human Toll-Like Receptor Signaling Pathway (Qiagen, Germany) used for gene expression analysis. The plate includes 84 TLR pathway-related genes, 5 housekeeping genes, and controls: genomic DNA control (GDC), reverse transcription controls (RTC), and positive PCR controls (PPC). Housekeeping genes (HK1 to HK5) were used for normalization of gene expression data. Each sample was loaded into the corresponding wells (A1–H12), as shown in the figure. The plate was used in conjunction with the.

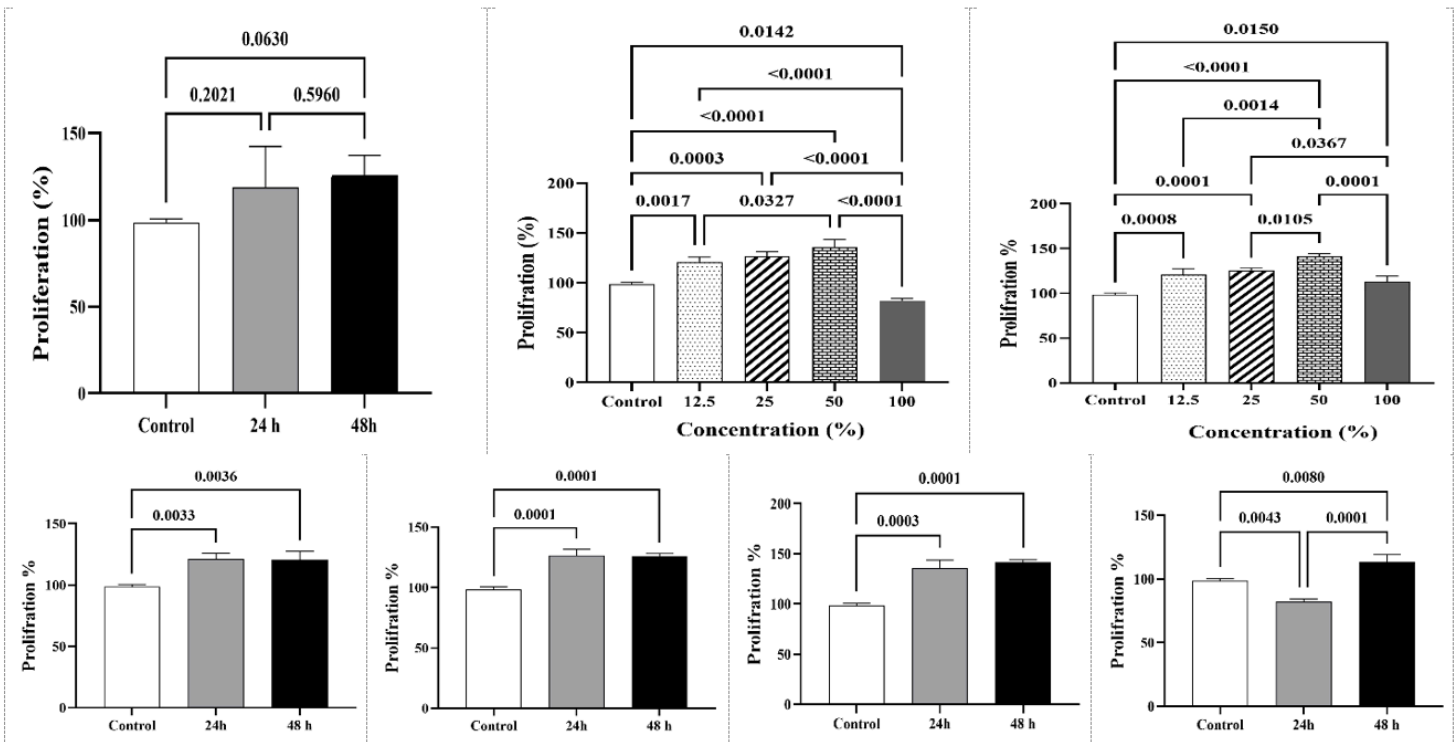


Figure 2

Fig 3.1. Effects of *L. brevis* FCS on HT-29 cell proliferation (MTT assay). Data are expressed as percentage of the unstimulated control (set at 100%, indicated by dashed line). (A) Time-dependent effects of CFS on proliferation at 24 h and 48 h compared with untreated control. (B) Concentration-dependent response at 24 h following treatment with 12.5%, 25%, 50% and 100% CFS. (C) Proliferation profile at 48 h across the same concentration range. (D1–D4) Direct comparison of 24 h and 48 h exposure within each concentration: (D1) 12.5%, (D2) 25%, (D3) 50%, and (D4) 100%.

Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test, with significance accepted at $p < 0.05$.

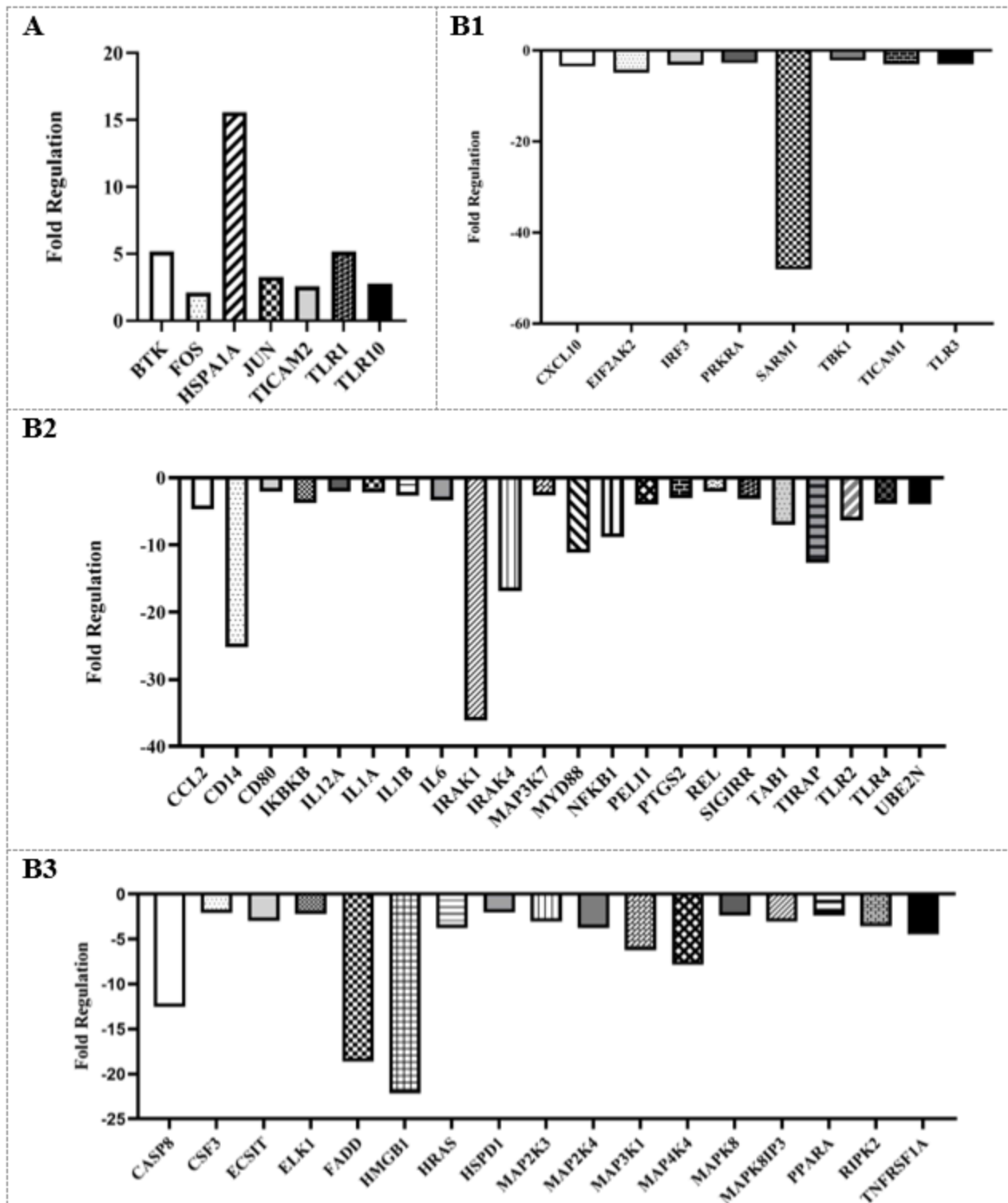


Figure 3

Fig 3.2. Transcriptional response of HT-29 cells to 50% *L. brevis* CFS treatment. Genes were categorized into an upregulated panel (A) and three downregulated panels (B1-B3) based on their primary role in TLR signaling pathways: the TLR/MyD88-dependent pro-inflammatory pathway (B1), the TRIF-dependent antiviral pathway (B2), and an integrated modulation network (B3). Expression data are presented as log2 fold change normalized to the geometric mean of five housekeeping genes (*ACTB*,

Figure 5

Fig 3.4. MYD88-dependent TLR signaling pathway in HT-29 cells following treatment with *L. brevis* cell-free supernatant (CFS). Differentially expressed genes identified by RT² Profiler PCR Array were mapped onto the MYD88-dependent TLR signaling cascade. Genes are color-coded according to expression changes: dark red, significantly upregulated ($p < 0.05$, fold regulation ≥ 2); light red, significantly upregulated ($p < 0.05$, fold regulation < 2); dark blue, significantly downregulated ($p < 0.05$, fold regulation ≥ 2); light blue, significantly downregulated ($p < 0.05$, fold regulation < 2); gray, no statistically significant change ($p \geq 0.05$). The pathway illustrates modulation of adaptor molecules, kinases, transcription factors, and downstream inflammatory mediators.