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RESEARCH PAPER

Immunomodulatory activity of two potential probiotic strains in LPS-stimulated HT-29 cells

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Abstract The relative expression of mucin, pro- and anti-inflammatory genes besides other signaling molecules in HT-29 cells by two test probiotic strains of *Lactobacillus plantarum* Lp9 and Lp91 and the reference strain *L. plantarum* 5276 was evaluated by RT-qPCR using Relative Expression Software Tool qBase-Plus under in vitro simulated gut conditions. Ten house keeping genes were evaluated by using geNorm 3.4 excel based application. The most stable genes were *RPL27*, *ACTB* and *B2M* which were subsequently used for calculating the normalization factor. Under pretreatment conditions (4 h probiotic treatment, followed by lipopolysaccharide challenge for 3 h), all the three strains evoked downregulation of IL-8 expression by $\sim$100 %, while in case of TNF-α, the downregulation of the relative gene expression was at the rate of 98.2, 93.8 and 98.0 % with Lp5276, Lp9 and Lp91, respectively, under the same set of conditions. Lp91 evoked maximum downregulation of IL12p35 and IFN-γ with corresponding fold reduction in relative expression of the two genes by 96.5 and 96.7 % during pre-treatment conditions. However, IL-10 and IFN-α were significantly upregulated to the extent of 8.13 ± 0.36 and 2.62 ± 0.14 fold by Lp91 under the same conditions. Lp9 and Lp91 were also quite effective in inducing the expression of *Cox*-1 and *Cox*-2 in HT-29 cells as can be reflected from their ratios, i.e., 5.90 and 6.50 (under pretreatment conditions); 3.79 and 4.36 (under co-culture conditions). Thus, the two putative indigenous *L. plantarum* strains Lp9 and Lp91 demonstrated immunomodulating functions in HT-29 cells at significant levels under different experimental conditions.

Keywords Lactobacilli · Gene expression · HT-29 · Lipopolysaccharide · Cytokines · Mucin

Introduction

The interest in probiotics has grown enormously during the last few years primarily due to immense health potentials and tremendous growth of probiotic based functional and health foods market across the world. The rapid growth and demand for probiotic foods and formulations are largely ascribed to growing awareness among the consumers about linkage of diet with general health, discovery of new probiotics with novel health functions and high commercial stakes involved in the probiotic marketing. It is now well recognized that diseases such as diarrhea, inflammatory bowel disease (IBD), ulcerative colitis (UC), peptic ulcers, crohn's disease (CD) and constipation, etc., associated with the dys or erratic functioning of human gut besides other chronic life threatening ailments can be alleviated through consumption of probiotic organisms directly or in the form of their food formulations (Brown and Valiere 2004; Hickson et al. 2007; Sheil et al. 2007; Maslowski and Mackay 2011; Mallappa et al. 2012). Probiotics can be explored either prophylactically or as biotherapeutics to manage inflammatory gut disorders and hence can serve as

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safe and cost effective alternative to drug treatment which invariably is associated with undesirable side effects besides being cost prohibitive.

Inflammatory mediators and cytokines have been particularly viewed as promising for both understanding the acute inflammation of the intestine and providing pathophysiological mechanisms to target for therapeutic interventions. In recent years, efforts have been made to explore dietary-based interventions to treat these diseases through modulation of cytokine production (Bassaganya-Riera and Hontecillas 2010; Adesokan and Neild 2012; Fung and Szilagyi 2012). The microbial environment of the intestine plays a major role in the development of chronic IBD, and hence targetting the microbiota presents an attractive option for therapeutic or preventive applications in such medical conditions. One such dietary strategy to manipulate the intestinal microbiota in disease management could be mediated through the administration of probiotics. Probiotics along with commensal bacteria residing in the gut are the main stimulators of immune and inflammatory response evoking cytokine production that plays a central role in influencing both innate and acquired immune responses in the host (Maslowski and Mackay 2011; Mallappa et al. 2012). However, most of the specific physiological functions of probiotics for therapeutic applications against the target diseases with regard to disease control are highly strain-specific (Crittenden et al. 2002). The mechanism of action of probiotic strains is multi-factorial specifically in the context of their immunomodulatory role with direct implications on health status. The immune system of mammals is comprised of a complex array of cells and molecules, which interact to provide protection from invading pathogenic microbes through mediation of T cells that include Th1 and Th2 (Ghadimi et al. 2008). Besides this, it is clearly demonstrated that other subpopulations such as Th17 and regulatory T cells (Treg) maintain immunological tolerance (Petersen et al. 2012). Th1 cells produce pro-inflammatory cytokines such as IFN-γ (interferon gamma), TNF-α (tumor necrosis factor-alpha), IL-1 (interleukin-1) and IL-2 (interleukin-2), IL-8 (interleukin-8) and *Cox*-2 (cyclooxygenase-2). Th2 cells, on the other hand, express anti-inflammatory cytokines, namely IL-4 (interleukin-4), IL-5 (interleukin-5), IL-6 (interleukin-6) and IL-10 (interleukin-10), etc. Certain probiotic strains of lactobacilli such as *Lactobacillus rhamnosus* Lr32 (*L. rhamnosus*) and *Lactobacillus salivarius* Ls33S were explored as immune-intervention therapy for the management of IBD by affecting specific pattern-recognition receptors and signaling pathways through stimulation of dendritic cells (DC) in mice model (Foligne et al. 2007b). The beneficial effects of probiotic interventions were also established previously in animal models of colitis and patients affected by IBD (Madsen et al. 1999; Fedorak et al. 2003; Sartor 2004; Lammers et al. 2005; Bibiloni et al. 2005; Duary et al. 2012b). The introduction of *L. plantarum* strain 299v into IL-10-deficient mice ameliorated colitis in these animals (Schultz et al. 2002). Controlled clinical trials have also shown that VSL#3 (cocktail of *Lactobacillus acidophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus casei, Lactobacillus plantarum, Bifidobacterium breve* (*B. breve*), *Bifidobacterium infantis, Bifidobacterium longum* and *Streptococcus thermophilus* cultures) was more effective than placebo for prevention of chronic pouchitis (Mimura et al. 2004; Holubar et al. 2010). Recently, a random meta-analysis based on published data with respect to various probiotic strains such as VSL#3, LGG, *Saccharomyces boulardii, B. infantis*, *Lactobacillus acidophilus, L. casei, Clostridium butyricum, Enterococcus faecum, L. plantarum* and *B. lactis* besides *L. acidophilus* combined with *B. infantis*, showed a significant effect against pouchitis, and IBD. However, their efficacy varied between different age groups or length of treatment or for single versus multiple species (Ritchie and Romanuk 2012). Similarly, *L. crispatus* CCTCC M206119 strain was found to be involved in the exacerbation of intestinal inflammation in DSS-colitis mice (Zhou et al. 2012), suggesting the safety and strain based specificity against specific diseases (Ritchie and Romanuk 2012; Van Neil 2005). In a previous study conducted in our laboratory, Sudhakaran et al. (2013) assessed the anti-inflammatory properties of one of the most potent indigenous *L. plantarum* strains Lp91 both in vitro in THP-1 cell lines and in vivo using mouse model under inflammatory conditions, demonstrating significant downregulation of TNF-α and increase in IL-6 expression.

Mucin 2 (*MUC*2) is the major mucin of the gastrointestinal tract along with other *MUC* proteins secreted by goblet cells and plays an important role in conferring the mucin barrier function of the gut. It has been hypothesized that probiotic bacteria can modify *MUC* gene expression and mucus secretion (Mack et al. 1999; Otte and Podolsky 2004; Duary et al. 2012b). Other important molecules belonging to Cox family include, *Cox*-1 (expressed constitutively in most tissues and maintains normal gastric mucosa and also influences kidney function) and *Cox*-2 (induced during inflammatory conditions in the gut). Since both *Cox*-1 and *Cox*-2 play a key role in gut-related inflammatory disorders, maintaining a perfect balance in *Cox*-1 and *Cox*-2 ratio expressed in GI tract is very crucial for the gut health. It is now well documented that probiotic effects are primarily mediated through the secretion of some metabolites that can enter the target cells to induce the expression of *Cox*-1/*Cox*-2 (Brudnak 2001a). The same authors (Brudnak 2001b), further reported that expression of *Cox*-1 gene was upregulated by bifidobacteria and recorded a three times increase in the ratio of *Cox*-1/*Cox*-2

which signified a shift of the cyclooxygenase expression toward a healthy direction. Furthermore, during the progression of gut-related inflammatory diseases associated with microbial infections, heat-shock proteins (Hsps) may also play an important role in cellular inhibition of pathogen invasion which may be possible through stabilization of the cell cytoskeleton. The mechanism of Hsp70 action involves stabilization of the actin filaments by cross-linking (Ren et al. 2001). In doing so, Hsps could prevent adherence and invasion of pathogens.

Although there is suggestive evidence for the functional and health claims linked with probiotics, the exact molecular mechanisms of their mode of action involved therein have largely remained unclear. Hence, genomics-based approaches are currently being explored to delineate the relevant molecular properties of probiotics and host cells and their interaction both under in vitro and in vivo conditions so as to reach some consensus. This information could be extremely valuable in understanding the functional efficacy of the currently available probiotic strains at molecular level and may eventually help in the mass screening and selection of novel probiotics. Although immunomodulatory properties of probiotics have been extensively studied in case of some well established strains, similar information on native strains of probiotic lactobacilli particularly of Indian gut origin is scanty. Therefore, it is necessary to investigate more number of native strains of probiotics in search for novel strains in terms of their effective colonization in the gut and protective immunomodulatory role in appropriate in vitro cell line models which mimic the gut environment to widen the scope of their therapeutic applications. This study was specifically undertaken with the objective of assessing the immunomodulatory efficacy of two potential indigenous probiotic strains of lactobacilli, viz. Lp9 and Lp91 in HT-29 cells subjected to lipopolysaccharide (LPS) treatment under different sets of conditions by looking at the expression of pro- and anti-inflammatory cytokines and other signaling molecules by RT-qPCR. The study was carried out using three different sets of conditions, i.e., pre-treatment, co-culture treatment as well as post-treatment of HT-29 cells subjected initially to LPS challenge, followed by probiotics so that eventually these strains could be explored as biotherapeutics in the management of inflammatory gut diseases.

Materials and methods

Bacterial strains and culture conditions

Two putative indigenous probiotic *Lactobacillus* isolates, i.e., *L. plantarum* Lp9 (National Collection of Dairy Cultures, National Dairy Research Institute, India; NCDC 344) and Lp91 (Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India; MTCC 5690) were selected as the subject of this study because these strains exhibited strong probiotic attributes and demonstrated cholesterol lowering effects as reported previously in our laboratory (Duary et al. 2010, 2011, 2012a, b; Kumar et al. 2011, 2012). The probiotic attributes and colonization potentials of these lactobacilli and their identity at species and strain level by molecular techniques were established previously as per FAO/WHO guidelines (FAO/WHO 2002). The study also included *L. plantarum* Lp5276 (also designated as CSCC5276, NCDO82 or VTTE-71034 which was procured from Dr. N. P. Shah from Victoria University, Australia) (Crittenden et al. 2002; Kaushik et al. 2009; Duary et al. 2010), a reference probiotic strain as a control for comparative analysis. The aforesaid strains were grown in MRS (de Man–Rogosa–Sharpe broth, pH 6.8, HiMedia, India) at 37 °C for 18 h and activated cultures were maintained in litmus milk (HiMedia, India) at 4 °C and as glycerol stocks (−70 °C).

Propagation and maintenance of cell lines

HT-29—human mucus secreting adenocarcinoma cell line (procured from Dr. Tapas Mukhopadhay, Punjab University; Chandigarh, India) was cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM, Sigma, USA) supplemented with 10 % (v/v) heat-inactivated (56 °C/30 min,) fetal bovine serum (Sigma, USA), 25 mM HEPES (Sigma, USA), 100 U/ml penicillin (Sigma, USA) and 100 µg/ml streptomycin (Sigma, USA) in 25 cm^2 culture flask (Falcon, Beckton Dickinson, Sparks, MD) at 37 °C in an atmosphere of 5 % CO_2/95 % air. The cultures were fed with fresh medium every alternate day and trypsinized using 0.25 % Trypsin–EDTA solution (Sigma, USA) at 37 °C. The final cell counts in suspension were measured with the help of hemocytometer (MBG, Germany). The cell suspension with 1×10^5 cells prepared in 4 ml complete DMEM medium was transferred to each well of six-well tissue culture plates (Falcon 6-well, Beckton Dickinson, Sparks, MD). The medium was changed every alternate day. When cells reached 80 % confluency, the spent medium was removed completely 24 h prior to challenge study, and cells were fed with fresh DMEM medium lacking antibiotics.

Viability of HT-29 cells challenged with lipopolysaccharide (LPS, Sigma USA, *E. coli*) was assessed by trypan blue assay (dye exclusion method). The assay was used for determining the concentration of LPS used for challenging the cell line. In brief, 10^6 cells/ml cell suspension were diluted in 1:1 ratio using a 0.2 % (w/v) trypan blue solution

(HiMedia, India) in saline water (mix 4 parts of 0.2 % trypan blue with 1 part of 5× Saline (4.25 % NaCl w/v). After incubation of 2 min at 37 °C under 5 % CO_2, the cells were counted using hemocytometer (MBG, Germany), and cell viability was calculated (viable cells will appear colorless). The viability of cell line was more than 95 % at 100 ng/ml LPS for 3 h based on trypan blue exclusion assay.

Challenge of HT-29 cells with probiotic strains and lipopolysaccharide (LPS)

After trypsinization, HT-29 cells at the level of 1×10^6 cfu/ml of cells were seeded in six-well plates in complete medium and incubated at 37 °C in 5 % CO_2. The growth medium was changed every 2 days. HT-29 cells were cultured until the attainment of complete confluency. As already mentioned, prior to challenge of the cell line with probiotic strains, complete medium was replaced with antibiotic free medium. The test probiotic strains were separately grown overnight in MRS broth at 37 °C and harvested at 5,500×*g* for 10 min. The bacterial cells were then washed twice in PBS buffer and OD_{600} adjusted to 1.5 which corresponded to 1×10^9 cfu/ml as determined by standard plate count technique. The bacterial cells were then resuspended in DMEM (1×10^9 cfu/ml). HT-29 cells were then subjected to probiotic/LPS treatment under three different conditions, i.e., pre, co (simultaneous) and post-treatments. For pre-treatment conditions, 1 ml of the probiotic culture (1×10^9 cfu/ml) was first added to the wells and incubated at 37 °C, 5 % CO_2 for 4 h. After incubation period, it was challenged with 100 ng/ml lipopolysaccharide (LPS, Sigma, USA from *E. coli*) (Vidal et al. 2002; Lee et al. 2005) and further incubated for additional 3 h. For co-treatment, HT-29 cells were challenged with both the test probiotic strain and LPS simultaneously for 7 h following the processing steps as described above. Lastly, for post-treatment, the HT-29 cells were initially challenged with LPS and incubated for 3 h. The spent medium was removed, and subsequently, the LPS-treated cells were subjected to respective probiotic lactobacillus test strains (1×10^9 cfu/ml) suspended in DMEM for additional 4 h by following the same protocol as described above.

Total RNA isolation and cDNA synthesis

After incubation of the treated HT-29 cells (Pre, co-culture and post-treatment), the growth medium was removed and trypsinized with 0.25 % trypsin–EDTA solution (Sigma, USA) and cells were pelleted by centrifugation at 1,000×*g* for 4 min. The total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, California, USA). The purity of the total RNA was determined based on OD 260/280 nm ratio, and the integrity was checked by electrophoresing on 1 % agarose gel. Residual DNA was removed by treating RNA with RNase free DNase I as per the manufacturer's instructions (Promega, USA). After determining the concentration of the RNA, it was stored at −80 °C until further use. An aliquot of 1 µg of the DNase-treated RNA was transcribed into cDNA, using ImPromII reverse transcriptase kit (Promega, USA) and random primers (Integrated DNA Technologies Inc., India). Untreated HT-29 cells grown in DMEM served as control.

Primer design

The primers used in this study are listed in Table 1. Ten house keeping genes namely glyceraldehyde 3-phospho dehydrogenase (*GAPDH*), beta-2-microglobulin (*B2M*), hydroxymethylbilane synthase (*HMBS*), actin-beta (*ACTB*), TATA box binding protein (*TBP*), succinate dehydrogenase complex (*SDHA*), ribosomal protein S18 (*RPS18*), ribosomal protein L27 (*RPL27*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) and aminolevulinate delta synthase 1 (*ALAS1*) were used to check stability of reference genes at different in vitro experimental conditions. Primers targeted against *Cox*-1, *Cox*-2, IL-10 (interleukin-10), *hsp*70 (heat-shock protein 70), IFN-α (interferons alpha) and IFN- γ (interferon gamma) were designed using Primer3plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) for real-time PCR analysis based on their available gene sequences. BLAST searches were performed against other genomes to determine the specificity of the primers. The primers were got custom synthesized from Imperial Biomed (Integrated DNA Technologies Inc.; www.idtdna.com). The appropriate selection criteria followed in primer designing included small amplicon size (100–200 bp), a GC content ranging between 40 and 60 % and melting temperature (Tm) from 55 to 60 °C. The amplicons obtained with each sets of primers in their respective RT-qPCR assays were checked on 2 % agarose gel, followed by melt curve analysis of the PCR amplified products to rule out the possibility of primer dimer formation.

LightCycler quantitative real-time PCR

Real-time PCR reactions were set up in LightCycler 480 (Roche, Switzerland) preloaded with relative quantification software (version LCS480 1.5.0.39, Roche) along with fluorescence signal detection based on SYBR Green after each amplification cycle. Each PCR reaction was performed in a 10.0 µl reaction mixture containing 5.0 µl of 2× SYBR Green PCR Master Mix (Roche), 2.5 µl of properly diluted cDNA (30 ng/µl of cDNA for all the genes used for RT-qPCR), 0.5 µl of each primer at 10 µM and

Table 1 Primer sequences of target and reference genes as well as their biological function

Name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)	Tm (°C)	References
ALAS1	aac ttg cca aaa tct gtt tc	ggt gat gag gga gtc tga at	159	81.47	Lallemant et al. (2009)
B2 M	cag cgt act cca aag att ca	gaa tgc tcc act ttt tca at	240	81.45	Lallemant et al. (2009)
HMBS	gaa aga caa cag cat cat gag	acc aag gag ctt gaa cat gc	145	85.56	Lallemant et al. (2009)
HPRT1	ctg acc tgc tgg att aca	gcg acc ttg acc atc ttt	256	81.77	Lallemant et al. (2009)
RPL27	tcg cca aga gat caa aga taa	ctg aag aca tcc tta ttg acg	121	80.04	Lallemant et al. (2009)
RPS18	agc ttg ttg tcc aga cca tt	tga gga aag cag aca ttg ac	187	84.28	Lallemant et al. (2009)
SDHA	agc aag ctc tat gga gac ct	taa tcg tac tca tca atc cg	200	88.68	Lallemant et al. (2009)
TBP	cac gaa cca cgg cac tga tt	ttt tct tgc tgc cag tct gga c	89	82.74	Lallemant et al. (2009)
ACTB	tgg ctg ggg tgt tga agg tct	agc acg gca tcg tca cca act	238	88.62	Lallemant et al. (2009)
GAPDH	caa cga cca ctt tgt caa gc	ttc ctc ttg tgc tct tgc tg	140	85.88	This study
MUC2	cag cac cga ttg ctg agt tg	gct ggt cat ctc aat ggc ag	140	86.5	Hatayama et al. (2007)
IL12p35	ttc acc act ccc aaa acc tgc	gag gcc agg caa ctc cca tta	225	84.5	Maaser et al. (2004)
IL-8	tgg ctc tct tgg cag cct tc	tgc acc cag ttt tcc ttg gg	238	83.0	Saegusa et al. (2007)
Cox1	gca acc tca aca cca cct tc	ccg aag cct ggt agg ata ag	118	82.0	This study
Cox2	caa ttc ccg gac gtc taa acc	cta gga cga tgg gca tga aac	114	83.5	This study
IL-10	aaa gaa ggc atg cac agc tc	aag cat gtt agg cag gtt gc	132	90.0	This study
TNF-α	agc cca tgt tgt agc aaa cc	tga ggt aca ggc cct ctg at	134	89.5	Saegusa et al. (2007)
hsp70	gat cat ggg ttg ctt ctt cc	gaa gcc aaa tac tgc cat cc	128	83.5	This study
IFN- α	ctg aaa cca tcc ctg tcc tc	cac agg ctt cca ggt cat tc	147	83.5	This study
TGF-β_2	atg ccc gta ttt atg gag tt	att gtc att ttg gtc ttg cc	234	83.0	Saegusa et al. (2007)
IFN-γ	atc cag tta ctg ccg gtt tg	gaa gca cca ggc atg aaa tc	135	82.0	This study

1.5 µl of nuclease-free water. The negative controls (with no DNA template, only primer pair, water and 2× SYBR Green PCR Master Mix) for each primer set were included in each run. The thermal cycling conditions included initial denaturation at 95 °C for 5 min., followed by 40 cycles each of denaturation (10 s at 95 °C), annealing (20 s at 64 °C for *MUC*2; 60 °C for TNF-α and IL-8; 57 °C for IL12p35 (interleukin-12p35); 56 °C for *Cox*-1, *Cox*-2, IL-10, *hsp*70, IFN- α and TGF-β_2 (transforming growth factor beta2); 50 °C for IFN-γ) and extension (15 s at 72 °C with a single fluorescence measurement), melt curve program (60–95 °C with a heating rate of 0.11 °C/s and a continuous fluorescence measurement) and finally a cooling step at 40 °C. The RNA extraction and measurement of gene expression by RT-qPCR were performed in triplicate, and the mean of all these values was considered for final analysis.

Statistical analysis of relative target gene expression

Quantification cycle value (the *Cq*) generated by real-time PCR for respective genes constitute the initial quantitative data for relative or absolute expression study (Bustin et al. 2009). Pfaffl used following mathematical model for calculating relative expression ratio of the individual transcripts (Pfaffl 2001):

$$\text{Ratio} = \left(E_{\text{target}}\right)^{\Delta Cq}_{\text{target}}(\text{control} - \text{sample}) \,/\, \left(E_{\text{ref}}\right)^{\Delta Cq}_{\text{ref}}(\text{control} - \text{sample})$$

The relative expression ratio of each of the target gene was computed based on its real-time PCR efficiencies (*E*) and the crossing point difference (ΔCq) for an unknown sample versus a control (untreated HT-29 cells grown in DMEM). Real-time PCR amplification efficiencies were determined for each set of primers from the slope of a linear regression model, i.e., *E* (reaction efficiency) $= 10^{[-1/\text{slope}]}$ (Pfaffl 2001). The standard curves were generated by plotting the log cDNA values versus Cq values obtained over the range of the dilutions of cDNA samples (100, 50, 25, 5, 1 and 0.25 ng/µl). The stability of the mRNA expression of the reference genes was checked by using freely distributed geNorm 3.4 an MS-Excel-based application (Vandesompele et al. 2002). This software is used to select the most stably expressed reference or housekeeping genes in a particular experimental condition, assuming that minimally regulated and stably expressed genes stay in a constant ratio to each other. The expression stability measure *M* (which must be lower than 1.5) for each reference gene is the average pairwise variation for a particular reference gene with all the other reference genes tested. The highest *M* value reflects the gene with the least stable expression

and vice versa. Stepwise exclusion of genes with the highest *M* values allows ranking of the genes tested according to their expression stabilities. Expression stability of ten housekeeping/reference genes was measured, by determining the control gene stability measure *M* (average expression stability) and *V* (pairwise variation). Proposed cut-off value for the pairwise variation is 0.15, below which inclusion of additional gene is not required.

The total expression ratio of the *MUC*2, pro-(IL-8, TNF-α, IL12p35 and IFN-γ) and anti-inflammatory cytokines (IL-10, IFN- α and TGF-β) along with other signal molecules such as *Cox*-1, *Cox*-2 and *hsp*70 under different in vitro experimental inflammatory conditions on probiotic interventions were tested for significance by the Relative Expression Software Tool qBase-Plus (www.gene-quantification.info) which is the modification of the classic delta–delta Ct method and takes multiple reference genes and gene-specific amplification efficiencies into account, as well as the errors on all measured parameters along the entire calculation track (Hellemans et al. 2007). Statistical significance was considered significant at $P \leq 0.05$. The data obtained were expressed as mean ± standard deviation values and analyzed statistically with the Statistical Package for the Social Science (SPSS for Windows, version 10.1, SPSS, Chicago).

Results

Of the many health benefits associated with probiotics, modulation of both innate and adaptive immune system in the gut has received the most attention. Since the pro and anti-inflammatory cytokines and other stress-related signal molecules play a vital role in maintaining the homeostasis of immune and inflammatory responses, many of the key molecules were targeted in this study to investigate the effect of the two indigenous strains Lp9 and Lp91 on their expression in HT-29 cells under three different in vitro conditions simulating the affected gut from inflammatory disease perspective. However, before assessing the immunemodulatory efficacy of these probiotic strains in HT-29 cells, the viability of the latter under stressed conditions evoked by LPS treatment was checked and found to be more than 95 % at 100 ng/ml LPS for 3 h. Similarly, for normalizing the relative expression of the target genes in HT-29 cells induced with probiotic strains, initially, the expression stability of ten house keeping genes, viz. *GAPDH, B2M, HMBS, ACTB, TBP, SDHA, RPS18, RPL27, HPRT1* and *ALAS1* was assessed using geNorm to select the most stable genes under all the three treatment conditions. In this context, the *RPL27* and *ACTB* were found to be the two most stable genes under the experimental conditions (Fig. 1). However, the pairwise variation was at 0.15 cut-off value, thereby, suggesting that a third most stable gene (*B2M*) was required as the additional internal control to normalize the gene expression optimally. Hence, the most stable reference genes selected in this study were *RPL27*, *ACTB* and *B2M* which were eventually used for calculating the normalization factor.

Relative expression of pro-inflammatory cytokines

Challenge of HT-29 cells with LPS for 3 h led to a significant upregulation of IL-8, TNF-α, IL12p35 and IFN-γ by 28.73 ± 0.53, 4.49 ± 1.30, 3.72 ± 0.80 and 2.76 ± 0.70 folds, respectively, relative to control [Fig. 2 and Table 1S (Supp. Data)]. On the other hand, when HT-29 cells were either pretreated or subjected to simultaneous treatment, the expression level of IL-8 gene was significantly downregulated to the extent of 0.01 ± 0.00 (with both Lp9 and Lp91) and 0.09 ± 0.01 (with Lp5276) as compared to LPS stimulation. The reduction in the expression of this cytokine in HT-29 cells was as high as 100 % induced with all the three probiotic strains under investigation in this study in the aforesaid sets of conditions. Almost a similar trend was witnessed in the expression of TNF-α under the same conditions with fold reduction values of 0.08 ± 0.01, 0.28 ± 0.01 and 0.09 ± 0.03 for Lp5276, Lp9 and Lp91, respectively. Relative to LPS control, the corresponding percent reduction values were 98.2, 93.8 and 98.0 % for Lp5276, Lp9 and Lp91 at pre-treatment condition. The co-treatment of HT-29 cells simultaneously with probiotic and LPS also led to more or less a similar reduction in the TNF-α expression, i.e., 94.9 (Lp5276), 97.8 (Lp9) and 95.6 % (Lp91). Similarly, with regard to expression of IL12p35, the fold reduction values induced with probiotic treatment were 0.14 ± 0.01 (Lp5276), 0.18 ± 0.03 (Lp9) and 0.15 ± 0.01 (Lp91) with corresponding percent decrease values of 96.2, 95.1 and 96.5 % in case of pre-treatment. These results further revealed almost the same trend in the expression of IL12p35 and IFN-γ under pre and co-treatment studies.

Contrary to the pre and co-treatments, when HT-29 cells were post-treated, the IL-8 expression was upregulated to the extent of 49.78 ± 2.20 (Lp5276), 37.60 ± 1.51 (Lp9) and 74.08 ± 3.77 (Lp91) fold relative to control, and the corresponding percent increase values were 73.3, 30.9 and 157.9 % relative to LPS challenge alone. Similarly, the corresponding values for TNF-α expression were 11.46 ± 1.10, 5.67 ± 0.12 and 10.23 ± 1.18 fold with respective percent increase values of 155.2, 26.3 and 127.8. However, the trend was slightly divergent in respect of IL12p35 expression as indicated by upregulation of the gene with Lp5276 (37.9 %) and downregulation in the gene expression to 16.1 and 20.2 % with Lp9 and Lp91, respectively. The post-treatment led to a significant upregulation of the IFN-γ gene, i.e., 5.65 ± 0.38,

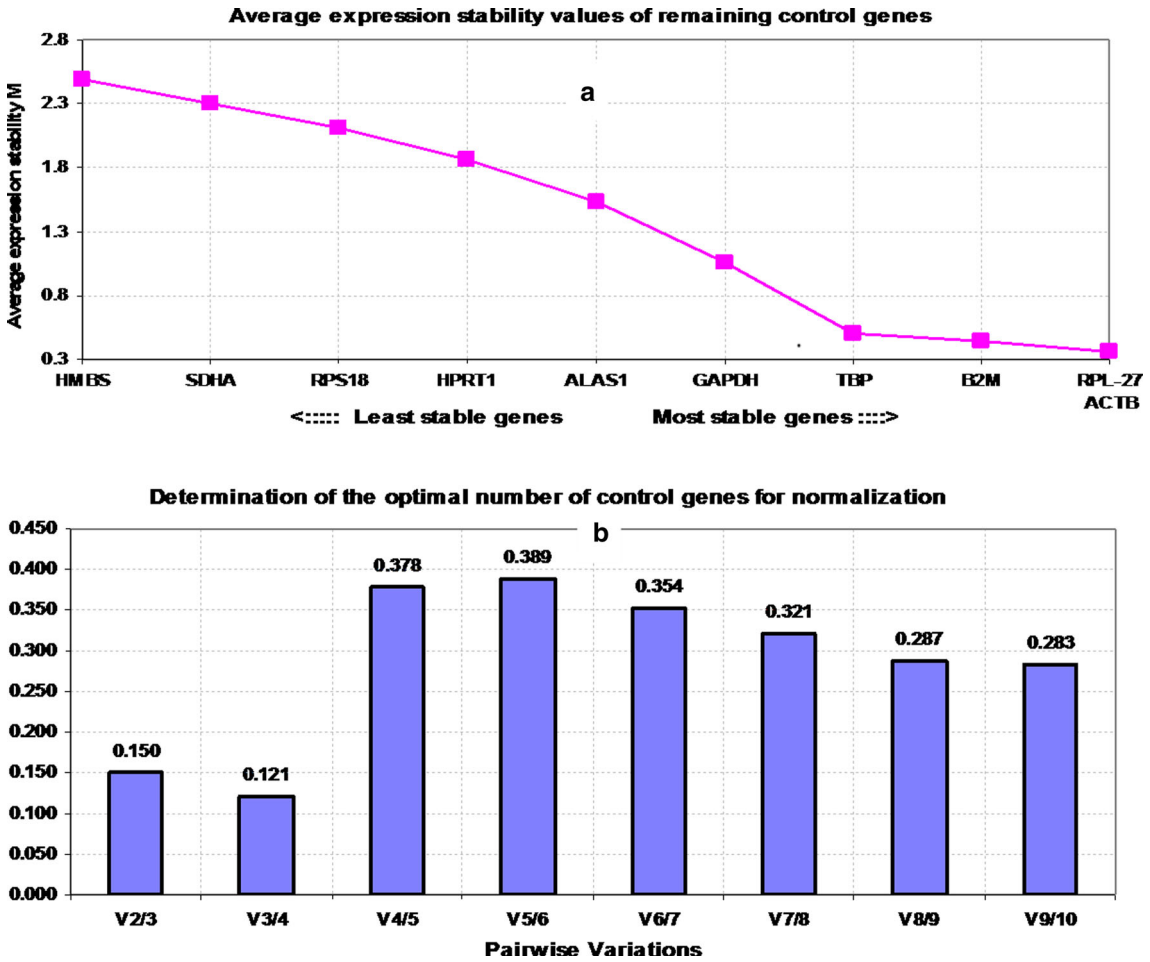

Fig. 1 Stability of reference genes by geNorm. Average expression of stability values of reference genes by geNorm analysis (**a**) and determination of the optimal number of reference genes for normalization by geNorm analysis (**b**) pairwise variations in expression ratios

2.18 ± 0.18 and 3.08 ± 0.81 obtained with Lp5276, Lp9 and Lp91 respectively.

Hence, based on the comparative analysis of the consolidated data on the influence of probiotic treatments in HT-29 cells on the expression of the target genes presented in Table 1S (Supp. Data), it can be concluded that Lp91 was the most effective immunosuppressant as it was able to significantly downregulate the expression of all the four cytokines, i.e., IL-8, TNF-α, IL12p35 and IFN-γ in HT-29 cells under pre- and simultaneous treatment conditions. The percent reduction in the expression of all the four cytokines recorded with all the three strains ranged from 95.6 to 100 with IL-8 being the most affected cytokine registering nearly 100 % reduction in gene expression. On the other hand, the expression of IFN-γ was affected the least with all the three probiotic strains, although the downregulation of the gene was still significant. However, when the HT-29 cells were subjected to post-treatment with probiotics after LPS stimulation, the trend in expression of the four pro-inflammatory cytokines in the cells was reversed. On treatment with Lp5276, the upregulation of the expression of all the target genes was recorded with maximum percent increase in the level of IL-8 (173.3 %), followed by TNF-α (155.2 %) and least in case of IL12p35 (37.9 %). Contrary to this, the effect of Lp9 and Lp91 differed considerably from that of Lp5276 in the sense that the former two were able to enhance the expression of IL-8 by 30.9 and 157.9 % and 26.3 and 127.8 % for TNF-α, whereas the expression of the other two cytokines, i.e., IL12p35 and IFN-γ was downregulated by 16.1 and 20.2 (IL12p35) and 21.0 and 11.6 % (IFN-γ) with Lp9 and Lp91, respectively. These results clearly indicate that Lp5276 demonstrated significant immunostimulatory response in HT-29 cells by upregulating all the four cytokines under post-treatment. On the other hand, Lp9 and Lp91 evoked both the immunostimulating effect on IL-8 and TNF-α by enhancing their expression and immunosuppressing response by reducing the expression of IL12p35 by 16.1 (Lp9) and 22.2 (Lp91) and IFN-γ by 21.0 % (Lp9) during post-treatment. However, under the

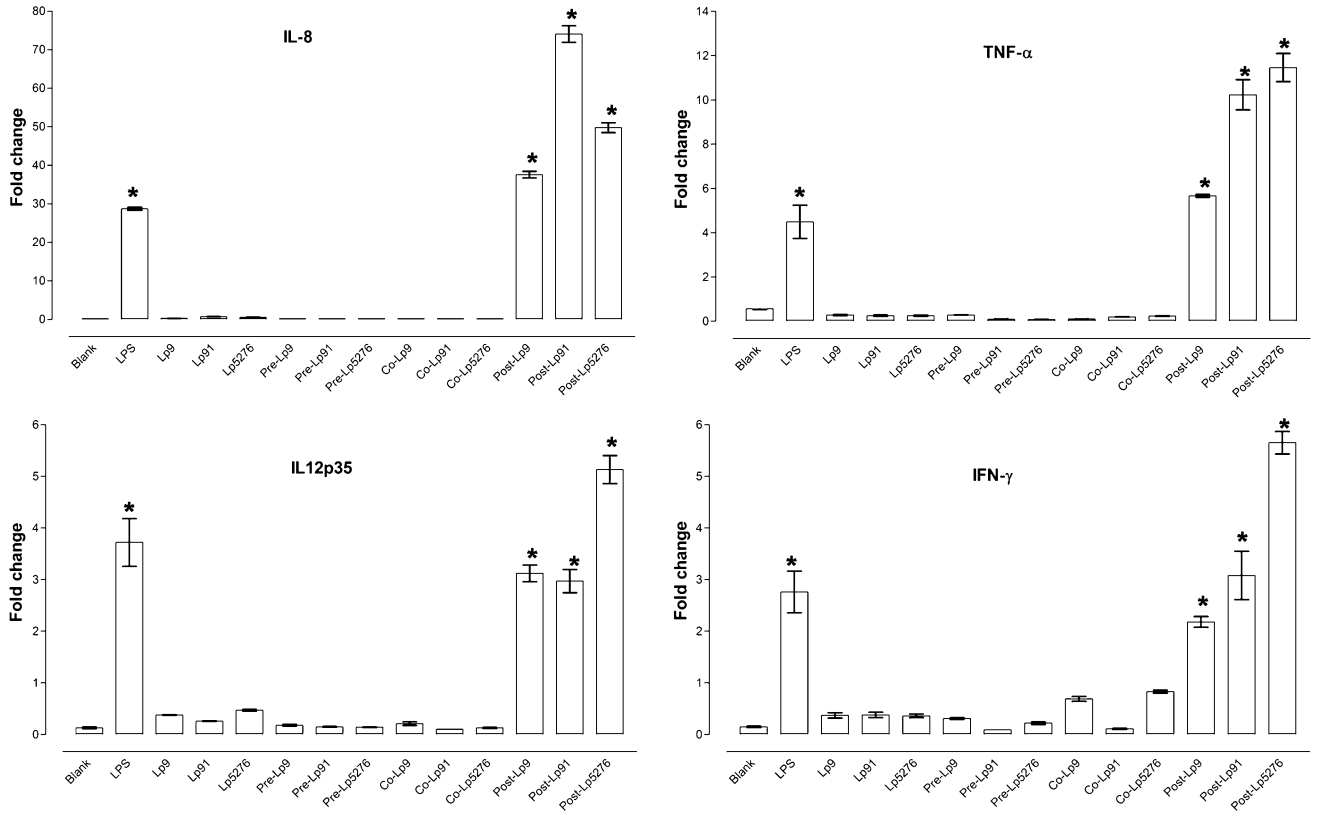

Fig. 2 Induction of pro-inflammatory cytokines expression in HT-29 cells on treatment with LPS and putative probiotic lactobacilli. Data are represented as mean ± SD; number of RT-qPCR experiments (n) = 3. *$P < 0.001$ compared with blank

same conditions Lp91 induced upregulation of IFN-γ by 11.6 %. These results further reveal strain to strain variation in the immunomodulatory functions of probiotics.

Relative expression of anti-inflammatory cytokines

The values for the basal level expression of IL-10, IFN-α and TGF-β in HT-29 cells on LPS stimulation for 3 h were 0.03 ± 0.00, 0.09 ± 0.01 and 2.05 ± 0.36, respectively. The treatment of HT-29 cells with Lp5276, Lp9 and Lp91 separately for 7 h resulted into an expression level of 0.45 ± 0.01, 0.33 ± 0.02 and 0.36 ± 0.04 in the context of IL-10, 0.54 ± 0.01, 2.30 ± 0.07 and 1.80 ± 0.31 for IFN-α and 0.46 ± 0.01, 0.57 ± 0.06 and 0.50 ± 0.01 for TGF-β, respectively (Fig. 3 and Table 2S). However, pre-treatment of HT-29 led to significant increase ($P \leq 0.05$) in the expression of IL-10 to 2.89 ± 0.09, 3.99 ± 0.43 and 8.13 ± 0.36 fold with Lp5276, Lp9 and Lp91, respectively. The corresponding values for IFN-α were 2.03 ± 0.40, 2.33 ± 0.26 and 2.62 ± 0.14 for Lp5276, Lp9 and Lp91, respectively, under the pre-treatment conditions. Contrary to these, there was a decreasing trend in the relative expression of TGF-β, i.e., 0.22 ± 0.01, 0.51 ± 0.04 and 0.12 ± 0.00 fold obtained with Lp5276, Lp9 and Lp91, respectively.

Under simultaneous treatment, the level of expression of IL-10 further increased to 5.11 ± 0.13 and 4.99 ± 0.65 with Lp5276 and Lp9, respectively. Contrary to this, under the same conditions, there was a drastic reduction in IL-10 expression induced with Lp91 as can be reflected from the lowered fold expression value, i.e., 1.54 ± 0.20, thereby, indicating that this effect was also strain-specific. The level of expression of IFN-α was 2.45 ± 0.25, 2.24 ± 0.16 and 1.02 ± 0.09 fold with Lp5276, Lp9 and Lp91, respectively. All the three strains behaved more or less the same in influencing the expression of TGF-β under co- treatment.

The post-treatment, on the other hand, did not influence the gene expression of IL-10 and IFN-α in HT-29 cells with any of the three probiotic strains with expression values showing almost the same trend as recorded with LPS treatment alone. However, there was significant upregulation of the TGF-β with maximum response evoked by Lp5276 (2.36 ± 0.25), followed by Lp9 (2.11 ± 0.24) and Lp91 (1.41 ± 0.11), thereby, suggesting that with regard to this particular gene, all the three probiotic cultures were quite effective.

On comparative evaluation of the efficacy of probiotic strains based on the ratio of IL-10/IL12p35 recorded with HT-

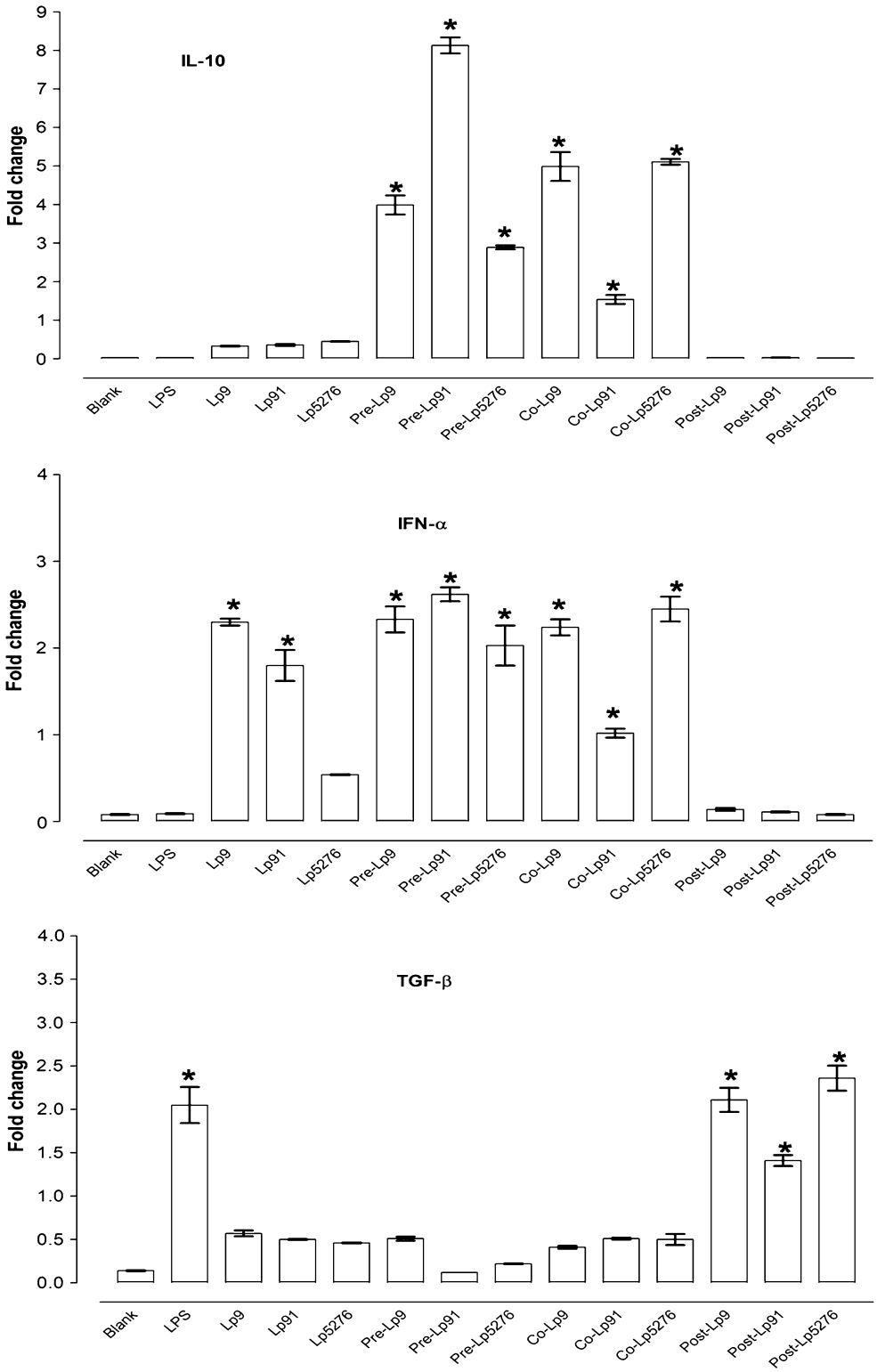

Fig. 3 Induction of anti-inflammatory cytokines expression in HT-29 cells on treatment with LPS and putative probiotic lactobacilli. Data are represented as mean ± SD; number of RT-qPCR experiments (n) = 3. *$P < 0.001$ compared with blank

29 cells under the three treatments, it was revealed that the LPS stimulation for 3 h resulted into a value of 0.01. While during pre-treatment, these ratios were increased to 22.17, 54.20 and 20.64 for Lp9, Lp91 and Lp5276, respectively. The corresponding values with regard to IL10/IL12p35 were 23.76, 15.40 and 39.31 for Lp5276, Lp9 and Lp91 under co-treatment. Contrary to this, there was a decreasing trend in both the cytokine ratio during post-treatment.

On critical evaluation, Lp91 was rated as the most effective as it significantly upregulated both IL-10 and

Table 2 Efficacy of putative probiotic lactobacilli in inducing the relative expression of *Cox*-1 and *Cox*-2 genes in HT-29 cells under different experimental conditions

Treatment	Fold change				
	*MUC*2	*hsp*70	*Cox*-1	*Cox*-2	*Cox*-1/*Cox*-2
Blank	0.24 ± 0.02	0.18 ± 0.01	0.40 ± 0.01	1.18 ± 0.05	0.34 ± 0.02
LPS	0.37 ± 0.01	2.10 ± 0.13*	0.21 ± 0.02***	14.77 ± 0.63*	0.01 ± 0.001
Lp9	1.49 ± 0.16*	0.97 ± 0.03*	0.61 ± 0.04***	0.20 ± 0.01	3.06 ± 0.35*
Lp91	0.54 ± 0.01	0.73 ± 0.02*	1.26 ± 0.02*	0.75 ± 0.0	1.68 ± 0.03
Lp5276	2.96 ± 0.19*	1.08 ± 0.05*	1.10 ± 0.07*	0.17 ± 0.00	6.47 ± 0.41*
Pre-treatment with probiotics prior to LPS stimulation					
Lp9	3.30 ± 0.16*	1.78 ± 0.07*	1.12 ± 0.06*	0.19 ± 0.01	5.92 ± 0.63*
Lp91	3.70 ± 0.39*	1.08 ± 0.03*	1.04 ± 0.03*	0.16 ± 0.01	6.51 ± 0.22*
Lp5276	4.33 ± 0.72*	1.36 ± 0.04*	0.77 ± 0.07*	0.07 ± 0.01	11.21 ± 2.43*
Co-culture treatment with probiotics and LPS					
Lp9	2.57 ± 0.08*	1.58 ± 0.12*	1.44 ± 0.15*	0.38 ± 0.01	3.78 ± 0.30*
Lp91	1.26 ± 0.05**	0.76 ± 0.01*	1.83 ± 0.04*	0.42 ± 0.01	4.36 ± 0.01*
Lp5276	5.59 ± 0.21*	1.98 ± 0.05*	1.15 ± 0.01*	0.25 ± 0.01	4.60 ± 0.14*
Post-probiotic treatment after LPS stimulation					
Post-Lp9	0.37 ± 0.05	1.09 ± 0.12*	0.20 ± 0.00***	17.87 ± 3.08*	0.01 ± 0.002
Post-Lp91	0.43 ± 0.03	1.20 ± 0.18*	0.28 ± 0.04	15.98 ± 2.74*	0.02 ± 0.01
Post-Lp5276	0.29 ± 0.01	1.52 ± 0.14*	0.16 ± 0.01**	14.05 ± 1.07*	0.01 ± 0.002

Data are represented as mean ± SD; number of RT-qPCR experiments (n) = 3

* $P < 0.001$ compared with blank; ** $P < 0.01$ compared with blank; *** $P < 0.05$ compared with blank

IFN-α expression in HT-29 cells under pre-treatment conditions. Lp9 on the other hand was the most potent by evoking the maximal IL-10 expression in HT-29 cells under co-treatment conditions. These findings indicate that the efficacy of probiotic strains in terms of expression of anti-inflammatory cytokines in HT-29 cells differed considerably between different treatments.

Relative expression of *MUC*2, *Cox*-1, *Cox*-2 and *hsp*70

The relative expression of *MUC*2, *Cox*-1, *Cox*-2 and *hsp*70 in HT-29 cells after different probiotic treatments under different conditions has been presented in Table 2. When HT-29 cells were stimulated with LPS (*E. coli*, 100 ng/ml) alone for 3 h, the fold increase in *MUC2* gene expression was 0.37 ± 0.01 relative to blank/control (0.24 ± 0.02). However, when the cell line was subjected to probiotic treatment as such with Lp5276, Lp9 and Lp91 individually for 7 h (1×10^9 cfu/ml), the fold expression was enhanced to 2.96 ± 0.19, 1.49 ± 0.16 and 0.54 ± 0.01, respectively, indicating that the fold increase obtained with Lp5276 treatment was significant. On the other hand, when the HT-29 cells were pre-challenged, the fold increase in *MUC*2 gene expression achieved was highly significant with all the three strains, viz. Lp5276 (4.33 ± 0.72), Lp9 (3.30 ± 0.16) and Lp91 (3.70 ± 0.39). The influence of Lp5276 on the *MUC*2 gene expression in HT-29 cells was further increased to 5.59 ± 0.21 under simultaneous treatment conditions in comparison with Lp9 and Lp91. However, none of the cultures was able to influence *MUC*2 gene expression in HT-29 cells when inflammatory conditions were induced by post-treatment.

The LPS stimulation of HT-29 cells, on the other hand, demonstrated opposite response on *Cox*-1 and *Cox*-2 expression as indicated in Table 2 and Fig. 2S. The expression of *Cox*-1 was decreased to 0.21 ± 0.02, whereas the same treatment led to increased *Cox*-2 expression, i.e., 14.77 ± 0.63 relative to control. However, when HT-29 cells were treated either with probiotic alone or with probiotic and LPS (pre and simultaneous treatment), there was no significant variation in the level of *Cox*-1 gene expression. Among the different strains, Lp91 induced relatively higher *Cox*-1 expression, i.e., 1.83 ± 0 in simultaneous treatment. The post-treatment of HT-29 cells, on the other hand, revealed considerable reduction in *Cox*-1 expression which was nearly the same as recorded with LPS treatment alone. Under the same conditions, *Cox*-2 expression was just the opposite. There was significant reduction in the level of expression under both pre- and co-treatments as well as direct probiotic treatment. The percent reduction values ranged from 97.2 to 99.5 %. Contrary to this, under post-treatment conditions, the level of expression of Cox-2 gene increased to 17.87 ± 3.08, 15.98 ± 2.74 and 14.05 ± 1.07 fold brought about with

Lp5276, Lp9 and Lp91. These results are further supported by the corresponding *Cox*-1 and *Cox*-2 ratios obtained with these three probiotic strains under all the defined experimental conditions.

The expression of *hsp*70 in LPS-treated HT-29 cells increased to 2.10 ± 0.13 fold as compared to blank. However, no significant difference in the level of *hsp*70 expression was recorded when HT-29 cells were treated individually with the three probiotic cultures. On the other hand, when HT-29 cells were pre-treated with probiotics prior to LPS, there was only a marginal increase, i.e., 1.36 ± 0.04, 1.78 ± 0.07 and 1.08 ± 0.03 fold in the level of expression with Lp5276, Lp9 and Lp91, respectively, and corresponding fold reduction values were 35.2, 15.2 and 48.6 % relative to LPS alone indicating superiority of Lp9 over other strains. Contrary to this, during simultaneous treatment of HT-29 cells with Lp5276, Lp9 and Lp91 separately in conjunction with LPS, the expression of *hsp*70 reduced to 5.7 (1.98 ± 0.05 fold), 24.8 (1.58 ± 0.12) and 63.8 % (0.76 ± 0.01), respectively, indicating that effect was significant only with Lp5276. During post-treatment, the trend of *hsp*70 profile was nearly the same as recorded with pre and co-treatments, although the changes induced with post-probiotic treatments in *hsp*70 expression were not significant relative to LPS. Thus, the expression of *hsp*70 was much more influenced by the stressed condition induced by LPS alone and all the probiotic lactobacilli including Lp5276 did not show any significant changes in the *hsp*70 expression under the defined experimental conditions.

Discussion

Since low-grade inflammation is the hall mark of some chronic autoimmune and inflammatory intestinal disorders including IBDs, the efficacy of Lp9 and Lp91 to contain hostile inflammatory reaction in LPS-stimulated HT-29 cells under three different probiotic treatments was investigated in this study by looking at the expression of the key pro- and anti-inflammatory cytokines. The anti-inflammatory efficacy of these strains can be assessed by their ability to either downregulate the pro-inflammatory cytokines or upregulate the anti-inflammatory cytokines or the combined effect of both. Both Lp9 and Lp91 strains under investigation were able to downregulate the expression of the key pro-inflammatory cytokines, i.e., IL-8, TNF-α, IL-12p35 and IFN-γ and also upregulated the anti-inflammatory cytokines Il-10, IFN- α and TGF-β significantly relative to control under different conditions. However, the extent of the immunemodulatory effect varied between the strains and the experimental conditions. The inflammatory response with regard to pro- and anti-inflammatory cytokines in enterocytes may differ markedly between different probiotic strains as recorded in this study. Most of the genes encoding pro-inflammatory cytokines, chemokines and other inflammatory mediators were upregulated by LPS but markedly reduced by all the three *L. plantarum* strains used in this study. The upregulation of all the four cytokines by LPS could be attributed to T cell proliferation and activation. The reduction in the expression of those genes after treatment with *L. plantarum* strains could be evoked probably by counteracting the molecular events leading to T cell activation as reported for a probiotic *L. casei* strain in a previous study (Carol et al. 2006) and lends support to the concept that probiotics can influence immune homeostasis (Hill and Artis 2010). For example, all the three probiotic strains behaved more or less same in terms of suppressing the four pro-inflammatory cytokines under pre- and co-treatment conditions with highest response coming from Lp91 which was either comparable to the reference strain or even better than that. However, under post-treatment conditions, the trend was just reversed since all the three strains recorded upregulation of the four pro-inflammatory cytokines instead of their downregulation indicating that the time of probiotic treatment could also influence the cytokine expression differently in HT-29 cells. These opposite effects of the probiotic strains with regard to expression of pro-inflammatory cytokines in the enterocytes specifically under post-treatment conditions could be attributed to involvement in different signal pathways triggered by the same probiotic strains under different treatment conditions. However, in the absence of any published report on these lines, we are not in a position to support the validity of this contention.

Both Lp9 and Lp91 along with Lp5276 were found to be effective in our study in reducing the expression of IL-8 in the enterocytes under pre- and co-treatment conditions. Our results appear to be consistent with the findings of several other investigators who too reported considerable reduction in IL-8 expression using probiotic treatment under in vitro studies using different strains of probiotics and different inflammatory agents (Eun et al. 2007; Candela et al. 2008; Liu et al. 2010). However, our findings with regard to significant increase in IL-8 expression levels in the enterocytes recorded with indigenous probiotic *L. plantarum* strains under post-treatment conditions are corroborated by the outcome of a study carried out by Pinto et al. (2009) which also demonstrated significant increase in IL-8 expression in LPS-treated HT-29 cells on probiotic interventions with LGG and *L. plantarum* BFE 1685.

Recent studies also suggest that some lactobacilli can elicit an anti-inflammatory response (Kotzamanidis et al. 2010). Like our strains, *L. plantarum* 299v also increased IL-8 mRNA levels in HT-29 cells previously stimulated with TNF-α, but IL-8 production required the presence of

live bacteria and was not observed when adhesion between *L. plantarum* 299v, and HT-29 cells was inhibited (McCracken et al. 2002). However, this study could not provide any explanation for the evidence that although the IL-8 mRNA clearly increased in the presence of TNF-α, the secretion of IL-8 in the supernatant actually decreased. Nevertheless, it turned out to be a landmark study as it suggested that probiotic adhesion to intestinal cells was an important attribute for unraveling their mode of action.

The expression of other pro-inflammatory cytokines, viz. TNF-α, IL12p35 and IFN-γ in enterocytes on probiotic treatments under different conditions followed more or less the same trend as recorded for IL-8 in our study. The altered expression of these pro-inflammatory cytokines under different conditions might be due to following the same routes as described for IL-8, and hence the same explanation for different inflammatory responses under the experimental conditions used in the study as discussed previously may hold good for these cytokines as well. The downregulation of TNF-α in HT-29 cells by Lp9 and Lp91 along with Lp5276 recorded in our study is in agreement with those of previous studies which also showed a significant decrease in the release of TNF-α after administration of probiotics (Haller et al. 2000; Morita et al. 2002) suggesting its immunosuppressing ability. However, our results in this regard are at variance with those of Fitzpatrick et al. (2008) and Lee et al. 2008, who reported reduction in TNF-α level in RAW 264.7 macrophage cell line when exposed to LPS, followed by treatment with different probiotic strains including *L. plantarum* HY115 and *L. brevis* HY7401, thereby, indicating that the anti-inflammatory efficacy of these probiotics worked under post-treatment conditions also. Similarly, Lee et al. (2008) demonstrated enhanced TNF-α production in Caco-2, HT-29 and SW480 cells on challenge with probiotic derived components. Pagnini et al. (2010), on the other hand, reported that beneficial effects of VSL#3 probiotics were associated with immunostimulatory effects rather than immunosuppression and suggested that probiotics prevented the onset of experimental ileitis by mechanisms involving stimulation of TNF-α which can restore the breach of the innate immunity system, and thereby, prevent the onset of the inflammatory disease. In our study, we found that Lp9 and Lp91 led to an increase in TNF-alpha which was elicited when the two probiotics were given after LPS challenge. This may be due to stimulatory response of epithelial innate responses by cumulative effects of both LPS and probiotic lactobacilli, whereas it exerted suppressive effects on the innate immune system, when probiotic bacteria were added before and simultaneously to cell line with LPS.

As far as the effect of probiotic treatment on the expression of IL12p35 in HT-29 cells is concerned, our results indicate downregulation of the gene under the first two conditions as recorded with the previous cytokines. These results are consistent with those of Hart et al. (2004) and Peran et al. (2005), who reported reduction in IL-12 expression in LPS-stimulated human DC and rodent bone marrow-derived macrophages on treatment with VSL#3 and *L. salivarius* ssp. *salivarius,* respectively. Contrary to this, Cammarota et al. (2009) reported no differences between experimental groups for IL12p40 expression with the potential probiotic strain, viz. *L. plantarum* DSMZ 12028 on monocytic cell line (THP-1) against *E. coli* K4.

All these findings point toward the probiotic heterogeneity and strain specificity. The ratios of IL-10/IL12p35 were increased at pre- and co-treatment conditions for both Lp9, Lp91 along with the standard Lp5276 strain. These results further suggest that some of the probiotic cultures could have immunestimulatory role rather than immunosuppressive to protect the gut from external pathogen infections.

Reduction in IFN-γ expression in enterocytes (HT-29 cells) observed in our study on probiotic treatment could be a very interesting probiotic feature. Besides its crucial role in innate and adaptive immune response against intracellular pathogens and other inflammatory diseases, prolonged high levels of IFN-γ can lead to chronic autoimmune and inflammatory diseases such as IBDs and multiple sclerosis with serious health implications. Hence, probiotic interventions having the ability to downregulate pro-inflammatory cytokines could be a quite promising strategy to manage these chronic inflammatory diseases (Rovedatti et al. 2009; Schoenborn and Wilson 2007).

Upregulation of IL-10 expression in HT-29 cells evoked with the two indigenous probiotic lactobacilli recorded in our study are consistent with the findings of Christensen et al. (2002) who also reported that lactobacilli induced production of IL-10 in a dose-dependent fashion in DC. Similarly, the study conducted by Niers et al. (2005) in PBMCs demonstrated that incubating the cell lines with *Lactobacillus* cultures induced IL-10 release and was highly strain-specific (Foligne et al. 2007a). However, Ma et al. (2004) reported that pre-incubation with *L. reuteri* (10 organisms/cell) did not affect IL-10 mRNA in T84 and HT-29 cells. It was further reported that the expression of IL-10 was dose dependent. Thus, therapeutic potential of oral administration of a combination of probiotics may provide a complete understanding of the host–commensal interactions that may contribute to beneficial effects in autoimmune diseases.

The increased expression profile of IFN-α in our study in different conditions suggests its role as an important anti-inflammatory marker. Cross et al. (2004) earlier reported an increase in IFN-α production of ~35 and ~20 pg/ml in supernatant when both live and killed cells

of *L. casei* Shirota were cultured on murine monocyte/macrophage cell line. The pleiotropic nature of TGF-β varied the expression profile of this cytokine in most cells under different conditions. It was also reflected from the outcome of our study wherein it was downregulated in HT-29 cells under pre- and co-culture conditions with Lp9 and Lp91. However, an increasing trend of this cytokine was recorded with Lp9 when the HT-29 cells were post-cultured, whereas Lp91 showed just the opposite effect, i.e., downregulated TGF-β under the same conditions. These findings are further supported by the data of Mileti et al. (2009) who evaluated the efficacy of different lactobacilli in influencing this cytokine and found that among all the probiotic cultures tested, *L. paracasei* B21060 induced maximal level of TGF-β.

Our results with regard to *MUC*2 expression in HT-29 cells induced by Lp9, Lp91 and Lp5276 are in close agreement with the findings of Mack et al. (1999) who too reported enhanced expression of both *MUC*2 and *MUC*3 genes in HT-29 cells by probiotic *L. plantarum* 299v and *L. rhamnosus* GG under different conditions. Our results in this regard are further substantiated by more or less similar observations made by Otte and Podolsky (2004), who assessed the effects of two widely used probiotic preparations, a single strain *E. coli* Nissle 1917 and a multi strain VSL#3 on naive intestinal epithelial cells (IEC) T84 including HT-29 cells on *MUC*2, *MUC*3 and *MUC*5AC gene expression by RT-qPCR assays. Both Lp9 and Lp91 were quite effective in producing appreciable amount of mucin resulting in strengthening the mucosal barrier function.

Lp9 and Lp91 were quite effective under pre-treatment conditions as indicated by increased expression of *Cox*-1 and decreased *Cox*-2 expression. Nurmi et al. (2005) demonstrated that *Bifidobacterium spp.* 420, which produced acetate and lactate downregulated *Cox-2* expression and increased *Cox*-1 transcription. However, contrary to our results, *L. acidophilus* used in their study had no effect on the *Cox*-1/*Cox*-2 ratio, thereby, confirming the strain-specific properties of probiotic in response to *Cox* gene regulation. The outcome of a different study conducted by Putaala et al. (2008) provides a conclusive evidence that bioactive metabolites produced by probiotics demonstrated cyclooxygenase expression and maintained the tight junction integrity of Caco-2 cells. The metabolites formed by two probiotic strains, *L. rhamnosus* HN001 and *L. acidophilus* NCFM on Caco-2 cell lines increased *Cox*-1 and *Cox*-2 ratio (Makelainen et al. 2009). Hence, as can be evidenced from our study as well as from other groups that, increase in expression ratio of both *Cox*-1 and *Cox*-2 are very important for maintaining the homeostasis of normal gut functioning.

None of the probiotic strains used in our study could induce any significant changes in *hsp*70 gene during different experimental conditions. In this regard, the results obtained from this study are in contradiction to those reported by Nemeth et al. (2006), who used probiotic bacteria or their spent culture supernatant on Caco-2 cells and demonstrated that these bacteria and their microbial products had additional beneficial effects with regard to the expression of *hsp*70. The variation in our results with regard to expression of *hsp*70 on probiotic intervention from those of other groups is difficult to explain at this juncture. However, the strain specificity of the probiotics with regard to induction of *hsp*70 expression cannot be ruled out.

Conclusion

It can be concluded from the outcome of this study that both the indigenous probiotic strains Lp9 and Lp91 demonstrated adequate immunomodulating and anti-inflammatory functions in HT-29 cells under different experimental conditions. The functional efficacy of both these strains was even better than the reference strain. By virtue of possessing these therapeutic attributes, these probiotics can be explored both as potential immunosuppressants in the management of inflammatory gastric disorders including IBD and also as adjunct therapeutics through their immunestimulatory action. By expressing immunotherapeutic effects, these bacteria can provide adequate protection to the gut against infectious agents. The association of Lp9 and Lp91 with highly immunomodulating potentials besides other health promoting functions makes them the ideal target for more intensive research to harness their bioactive effects optimally from human health perspectives after establishing their efficacy in vivo and clinical trials in human subjects.

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