CHAPTER 4

STUDY IMMUNOMODULATING POTENTIAL OF *LACTOBACILLUS* TRAINS ON PBMCs, PMNs AND MACROPHAGES

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4.1. Introduction

Human body hosts a diverse group of bacterial species including both aerobes and anaerobes. More than 400 amongst them are commensal bacteria that reside in the gastrointestinal tract (Falk *et al*. 1998), out of which some of them may potentially elicit pro-inflammatory responses. Under normal conditions, intestinal PBMCs, macrophages and dendritic cells are regulated to secrete low levels of pro-inflammatory cytokines, such as IL-12, in response to such bacteria (Kaji *et al*. 2010). Various mechanisms like constitutive expression of inhibitory transcription factors in the mucosal phagocytes and a supply of suppressive mediators by intestinal epithelial cells, have been proposed to explain the mechanism by which pro-inflammatory cytokine responses are controlled (Shibolet *et al*. 2007; Fritz *et al*. 2008). Upon inflammation, neutrophils migrate from the site of infection to neighboring lymph nodes where they undergo apoptosis and are taken up by DCs, thus ensuring that neutrophil derived antigens are presented to T cells (Miyazaki *et al*. 2004). Lactobacilli are probiotic commensal bacteria and also the potent modulators of immunity, when present in the gut or supplemented as probiotics, they beneficially modulate *ex vivo* immune responsiveness. Further, factors derived from several lactobacilli strains also act as immune regulators *in vitro* (Johansson *et al*. 2016). The primary effector arm of the immune system is the so-called innate immune system, which includes non-

specific immune protection mediated by monocytes, macrophages and dendritic cells. The cells of the innate immune system (PBMCs, macrophages, and dendritic cells) have an important role as antigen presenting cells (APC). The innate immune system further regulates the function of the antigen-specific adaptive immune system, such as the functional balance of immune response related to cytokine and chemokine profiles. Defective maturation of immune competence in association with poor microbial stimuli may lead to dysregulation of both innate and adaptive immune systems (Vaarala 2003). Lactobacilli strains and supplementation with different strains of lactobacilli can modulate stimulated responses *in vitro* (Pochard *et al*. 2002; Ghadimi *et al*. 2008; Ivory *et al*. 2008). Lactobacilli can elicit innate and adaptive immune responses in the host via binding to pattern recognition receptors (PRR) expressed on immune cells and many other tissues including the intestinal epithelium. PRR recognize conserved molecular structures known as microbe-associated molecular patterns (MAMPs) (Wells *et al.* 2011) and mediate their immune-modulatory effects through the induction of regulatory cytokines, such as IL-10 (de Moreno de LeBlanc *et al*. 2011), induction of T regulatory cells (Smelt *et al*. 2012; Liu *et al*. 2013), modulation of APC (Haileselassie *et al*. 2016), promotion of epithelial function and development (Yan *et al*. 2017) and by inhibition of pro-inflammatory cytokines (Kim *et al*. 2006). The most well understood signalling mechanisms involved the innate pattern recognition receptors such as Toll-like receptors (TLRs), nucleotide oligomerization domain-like receptors and C-type lectin receptors. Under *in vitro* conditions, the cytokine response of human peripheral blood mononuclear cells, macrophages and dendritic cells to lactobacilli can be strikingly different depending on both the bacterial species and the strain. Several factors have been identified in lactobacilli

that can influence the immune response (*in vitro* and *in vivo*) including cell surface carbohydrates, enzymes modifying the structure of lipoteichoic acids and metabolites (Wells *et al.* 2011). *Lactobacillus* can be able to reverse a series of molecular, cellular and immunological responses observed during inflammation process and then polarize stimulated M1 macrophages to M2 macrophages (Jang *et al*. 2013). M1 and M2 describe the two major and opposing activities of macrophages; M1 activity inhibits cell proliferation and causes tissue damage while M2 activity promotes cell proliferation and tissue repair. Pro-inflammatory "classically activated" subtype (M1) and anti-inflammatory "alternatively activated" subtype (M2) macrophages promote Th1 and Th2 responses, respectively (Jaguin *et al*. 2013; Wang *et al.* 2014). M1–M2 polarization of macrophage is a tightly controlled process entailing a set of signaling pathways, transcriptional and posttranscriptional regulatory networks. An imbalance of macrophage M1–M2 polarization is often associated with various diseases or inflammatory conditions (Wang *et al.* 2014). Activation of IRF/STAT signaling pathways by IFNs and TLR signaling will skew macrophage function toward the M1 phenotype (via STAT1), while activation of IRF/STAT (via STAT6) signaling pathways by IL-4 and IL-13 will skew macrophage function toward the M2 phenotype (Sica *et al*. 2012). As whole bacteria will most likely not enter the blood stream in large numbers, instead, bacterial metabolites have been shown to cross the epithelial barrier, retain their bioactive properties, and affect peripheral immunity *in vitro* and *in vivo* (Ménard *et al*. 2004; Ashraf *et al*. 2014). Secreted factors produced by lactobacilli have been extensively examined and factors like p40 and histamine, are discussed as potential effector molecules (Thomas *et al*. 2012; Yan and Polk 2012).

Lactobacillus rhamnosus GG (LGG) is clinically documented in relieving and preventing diarrhea, childhood infections, allergies and atopic eczema and also for its immunomodulatory properties on antigen-presenting cells (macrophage, monocytes and dendritic cells) (Fong *et al*. 2015). LGG-derived soluble factors also play a major role in immunomodulation on antigen presenting cells (Fong *et al*. 2016).

This study underlying effect of different lactobacilli on immune cells like PBMCs, macrophages and PMNs (from blood of healthy individual) on non-pathological conditions. Several strains were found to perform better in immunomodulatory properties than the established probiotic strain *L. rhamnosus* GG (LGG), which can be helpful for evaluating safety and further application of beneficial microorganisms in the prevention and treatment of different diseases.

4.2. Materials and methods

4.2.1. Isolation and stimulation of monocytes and polymorphonuclear neutrophils (PMNs) with lactobacilli

The method used for isolation and stimulation of monocytes was that reported by Haller *et al*. (2000) with a few modifications. Briefly, isolation of mononuclear cells was done from blood of healthy individual by using HISTOPAQUE®-1077 (Sigma-Aldrich). A buffy coat layer (PBMCs) obtained below the plasma and above the HISTOPAQUE®-1077 solution was aspirated out and washed with Dulbecco's PBS (DPBS). The cells were resuspended

in RPMI-1640 media (Sigma-Aldrich, Mumbai, India) with 10% (v/v) fetal calf serum (Gibco) and the cell-density was adjusted to 1×10^6 cells/mL in RPMI-1640 media. Further, the cells were transferred to a 24 well-plate. For macrophages, plate was kept at 37 °C with 5% CO_2 for 2 h to select for adherent macrophages. Following this, the PBMCs and macrophages were individually incubated with different lactobacilli cells (1×10^7 CFU/mL) as well as with an enteropathogen *E. coli* O26:H11 (1×10^7 CFU/mL) in the absence of gentamycin for 12-16 h at 37 °C in 5% CO_2. After incubation, the media was removed and the cells were washed twice with DPBS. PBMCs and differentiated monocytes i.e., macrophages were extracted, followed by lysing of cells using guanidine thiocyanate which was included in the total RNA extraction kit (RNAiso plus reagent; Takara, Japan). Further steps were performed as per manufacturer's instructions. Unstimulated PBMCs and macrophages were used as a control.

For polymorphonuclear neutrophils (PMNs), isolation was done from blood of healthy individual using HISTOPAQUE®-1077 and HISTOPAQUE®-1119 (Sigma-Aldrich). A buffy coat layer obtained between the HISTOPAQUE®-1119 and HISTOPAQUE®-1077 solution, was aspirated out and washed with Dulbecco's PBS (DPBS). For PMNs stimulation, the same procedure as described above was used and unstimulated PMNs were used as a control.

4.2.2. RNA isolation

Total RNA was isolated from control (to which no bacteria were added/unstimulated) cells (PBMCs/PMNs/macrophages) and those stimulated with various lactobacilli and *E. coli*

O26:H11 using RNAiso plus reagent (Takara, Japan). All the plastic wares and glass wares were first rinsed with chloroform to destroy any RNase present. All reagents used were prepared in diethyl pyrocarbonate (DEPC) treated autoclaved distilled water.

500 µL of RNAiso Plus reagent (Takara) was added to each well, containing the PBMCs/macrophages/PMNs, and mixed properly. The cells were transferred to a microfuge tube and left at room temperature for 10 min for lysis, followed by addition of 0.1 mL of chloroform. Then it was mixed until the solution turned milky and after this, the mixture was incubated at room temperature for 5 min and centrifuged at 12,000×*g* for 15 min at 4 ºC. The supernatant was then transferred into a fresh tube and equal volume of 100% isopropanol was added. The content was mixed and kept at 20 ºC for 10 min for precipitation followed by centrifugation at 12,000×g for 10 min at 4 ºC and then the supernatant was carefully removed without touching the pellet. 250 µL of ethanol (75%) was added to the pellet and centrifuged at 7,500×*g* for 5 min at 4 ºC. The supernatant was discarded and the pellet was dried at 42 ºC for 10 min and dissolved in 15 µL DEPC treated water. The quality of the RNA samples was assessed by inspecting the 28S and 18S bands following agarose gel electrophoresis and were also quantified using a nano spectrophotometer.

4.2.3. cDNA synthesis and quality confirmation

For cDNA synthesis, 1 µg of each total RNA sample was mixed with anchored oligo-dT in a 20 µL system using verso cDNA synthesis kit based on Moloney murine leukaemia virus (M MuLV) reverse transcriptase (Thermo Fisher Scientific, UK) following manufacturer's instructions. Briefly, the RNA was mixed with oligo-dT, RT enhancer

which contains DNAse I, dNTP mix and enzyme mix, followed by incubation at 50 ºC for 60 min and then at 95 ºC for 5 min to inactivate the enzyme in a thermal cycler (Eppendorf, Hamburg, Germany). Each of the cDNA preparations was then amplified for 35 cycles in a thermal cycler with β-actin specific primers by taking 2 µL of cDNA in a 25 µL system. This was used as control for synthesis of cDNA. PCR products were separated on a 0.8% agarose gel stained with ethidium bromide. For checking genomic DNA contamination, controls were set with amplification of the total RNA without reverse transcription which did not give any amplification (result not given).

Table 4.1. RT-PCR (Reverse transcriptase) reaction system for cDNA synthesis.

Name	Volume
5X cDNA buffer	4 µL
dNTP mix (5 mM)	2 µL
RNA primer (500 ng/µL)	1 µL
RT enchancer	1 µL
Verso enzyme mix	1 µL
cDNA template	X
Autoclaved MilliQ water	Upto 20 µL
Total	**20 µL**

Table 4.2. RT-PCR (Reverse transcriptase) conditions for cDNA synthesis.

Temperature	Time
Take the template RNA	
70 ºC	5 min
Add the other components to it	
50 ºC (cDNA preparation)	60 min
95 ºC (inactivation)	5 min

Table 4.3. PCR reaction system for β-actin specific amplification.

Component	Volume
DNA	2 µL
10X Buffer	2.5 µL
10 mM dNTPs	0.5 µL
50 mM $MgCl_2$	0.75 µL
Forward Primer (10 mM)	1.25 µL
Reverse Primer (10 mM)	1.25 µL
Taq Polymerase (1 U/µL)	0.5 µL
Autoclaved MilliQ water	16.25 µL
Total	**25 µL**

Table 4.4. Conditions for β-actin specific amplification.

Steps	Temperature (°C)	Time	No. of cycles
Pre-cycle denaturation	94	6 min	1
Denaturation	94	30 sec	
Primer annealing	60	30 sec	34
Primer extension	72	30 sec	
Post-cycle elongation	72	10 min	1

4.2.4. Quantitative real time PCR (qRT PCR)

Quantitative PCR amplifications were then performed in CFX96TM real-time thermal cycler (Bio-Rad Laboratories, Hercules, USA) with specific primers for the M1 markers (CHI3L-1, CXCL10, IDO1, CD80, CXCL11) and M2 markers (CD36, CD200R, PPAR-γ, CCL22, SR-B1) (for both macrophages and PBMCs) and also for the pro-inflammatory cytokines; IL-2, IL-12, IFN-γ, TNF-α, IL-8 and anti-inflammatory cytokines; IL-4, IL-10, TGF-β (for PMNs). The amplification conditions were as follows:

Table 4.5. Conditions for qRT PCR amplification.

Steps	Temperature (°C)	Time	No. of cycles
Initial-denaturation	94	3 min	
Denaturation	94	10 sec	
Primer annealing	60	30 sec	45
Extension	72	1 min	

Table 4.6. Reaction system for qRT-PCR.

Component	Volume (µL)
DNA	0.5
Forward Primer (10 mM)	0.5
Reverse Primer (10 mM)	0.5
2x SYBr Green mix	5.0
Autoclave MilliQ water	3.5
Total	**10**

Each sample was run in triplicate and cycle threshold (Ct) was used for gene expression analysis. The transcripts expression of CHI3L-1, CXCL10, IDO1, CD80, CXCL11, CD36, CD200R, PPAR-γ, CCL22 and SR-B1 (for macrophages/PBMCs) and IL-2, IL-12, IFN-γ, TNF-α, IL-8, IL-4, IL-10 and TGF-β (for PMNs) in each sample was normalized to a Glyceraldehyde 3-phosphatedehydrogenase (GAPDH) transcript expression of the same sample using the CFX manager software (Bio-Rad Laboratories). Data were analyzed using the comparative Ct method. Relative quantity was defined as $2^{-\Delta\Delta Ct}$, in which

$$\Delta Ct = Ct\ (target)-Ct\ (reference),\ \Delta\Delta Ct = \Delta Ct\ (sample)-\Delta Ct\ (calibrator)$$

The calibrator was the unstimulated macrophages/PBMCs/PMNs mRNA used for normalization. Prism 6.01 (GraphPad Software, Inc., San Diego, CA, USA) was used for

statistical analysis. The strains were compared with two different controls (LGG and uninduced macrophages/PBMCs/PMNs) by means of two independent ANOVA tests. Significant ANOVAs were followed by Dunnett's test for multiple comparisons *vs.* the control group. The product specificity was confirmed by single peak in melt curve analysis (from 65 °C to 95 °C in 0.5 °C /5 s increments). The negative controls were set with the total RNA without reverse transcription (data not provided).

Table 4.7. M1 and M2 Primers used for qRT-PCR analysis.

M1 marker Primer	**Sequence (5'-3')**
CHI3L-1 F'P	GATAGCCTCCAACACCCAGA
CHI3L-1 R'P	AATTCGGCCTTCATTTCCTTGA
CXCL10 F'P	TTGTCCACGTGTTGAGATCATT
CXCL10 R'P	GATTTTGCTCCCCTCTGGTTT
IDO1 F'P	ATATGTGTGGGGCAAAGGTCA
IDO1 R'P	TCAGGGGCTTATTAGGATCCT
CD80 F'P	CAAGAGCATTTTCCTGATAACC
CD80 R'P	GGCGTACACTTTCCCTTCTC
CXCL11 F'P	CCTGGGGTAAAAGCAGTGAAA
CXCL11 R'P	TGGGATTTAGGCATCGTTGTC

M2 marker Primer	**Sequence (5'-3')**
CD36 F'P	ACAGATGCAGCCTCATTTCCA
CD36 R'P	GGCCTTGGATGGAAGAACAAA
CD200R F'P	TCCAAGTGTTAGTTACACCTGA
CD200R R'P	GCATGTACTCTTAACAGTCACT
PPAR-γ F'P	GTGATATCGACCAGCTGAATC
PPAR-γ R'P	TCAAGATCGCCCTCGCCTTT
CCL22 F'P	TTACGTCCGTTACCGTCTGC
CCL22 R'P	AGGCTCTTCATTGGCTCAGC
SR-B1 F'P	TGTGGGTGAGATCATGTGGG
SR-B1 R'P	GTTCCACTTGTCCACGAGGT

Table 4.8. Primers used for qRT-PCR analysis (for PMNs).

Primer (Pro-inflammatory)	Sequence (5'-3')
IL-2 F'P	AACTCACCAGGATGCTCACATTTA
IL-2 R'P	TCCCTGGGTCTTAAGTGAAAGTTT
IL-12 F'P	TGGAGTGCCAGGAGGACAGT
IL-12 R'P	TCTTGGGTGGGTCAGGTGTG
IFN-γ F'P	TCAGCTCTGCATCGTTTTGG
IFN-γ R'P	GTTCCATTATCCGCTACATCTGAA
TNF-α F'P	TCTTCTCGAACCCCGAGTGA
TNF-α R'P	CCTCTGATGGCACCACCAG
IL-8 F'P	GGCACAAACTTTCAGAGACAG
IL-8 R'P	ACACAGAGCTGCAGAAATCAGG

Primer (Anti-inflammatory)	Sequence (5'-3')
IL-4 F'P	CGAGTTGACCGTAACAGACAT
IL-4 R'P	CGTCTTTAGCCTTTCCAAGAAG
IL-10 F'P	GTGATGCCCCAAGCTGAGA
IL-10 R'P	CACGGCCTTGCTCTTGTTTT
TGF-β F'P	CAGCAACAATTCCTGGCGATA
TGF-β R'P	AAGGCGAAAGCCCTCAAATTT

Reference gene: GAPDH

Primer	Sequence (5'-3')
GAPDH F'P	TGAGCACCAGGTGGTCTCC
GAPDH R'P	TAGCCAAATTCGTTGTCATACCAG

4.2.5. Statistical analysis

Values are given as mean and standard deviations from three experimental replicates. Significant ANOVAs were followed by Dunnett's test in all the assays to compare the mean values of each lactobacilli group with two different controls (LGG and uninduced

PBMCs/macrophages/PMNs) ($P < 0.05$). All analysis was conducted using GraphPad Prism 6.01.

4.3. Results

4.3.1. RNA isolation and cDNA quality confirmation of PBMCs and macrophages exposed to different lactobacilli and *E. coli* O26:H11

The total RNA was isolated from the control and lactobacilli and/or *E. coli* O26:H11 exposed to PBMCs/macrophages using TaKaRa Isoplus kit (Takara, Japan). This was followed by 0.8% agarose gel electrophoresis, the presence of 18S and 28S rRNA on the gel showed the integrity of RNA sample. The quality of cDNA prepared by reverse transcription from these samples was confirmed by performing PCR with β-actin specific primers [Fig. 4.1A and Fig.4.1B, respectively (for PBMCs), Fig. 4.2A and Fig. 4.2B, respectively (for macrophages)]. The cDNAs prepared from these samples were further used to analyse the expression of cytokines and chemokines.

A

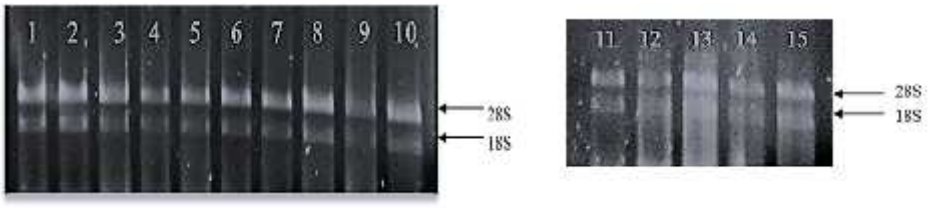


Figure 4.1. (A) 0.8% agarose gel stained with ethidium bromide with the total RNA from uninduced PBMCs, PBMCs stimulated with various lactobacilli and *E. coli*.

Lane 1: uninduced PBMCs (Control)
Lane 2: induced with *E. coli*
Lane 3: induced with LGG
Lane 4: induced with GPI-4
Lane 5: induced with GPI-1(S)
Lane 6: induced with GRI-2
Lane 7: induced with GPI-1(B)
Lane 8: induced with GPI-6
Lane 9: induced with GPI-7
Lane 10: induced with GPI-3
Lane 11: induced with FA-1
Lane 12: induced with FA-5
Lane 13: induced with FA-7
Lane 14: induced with GKI-1
Lane 15: induced with IIS11.2

B

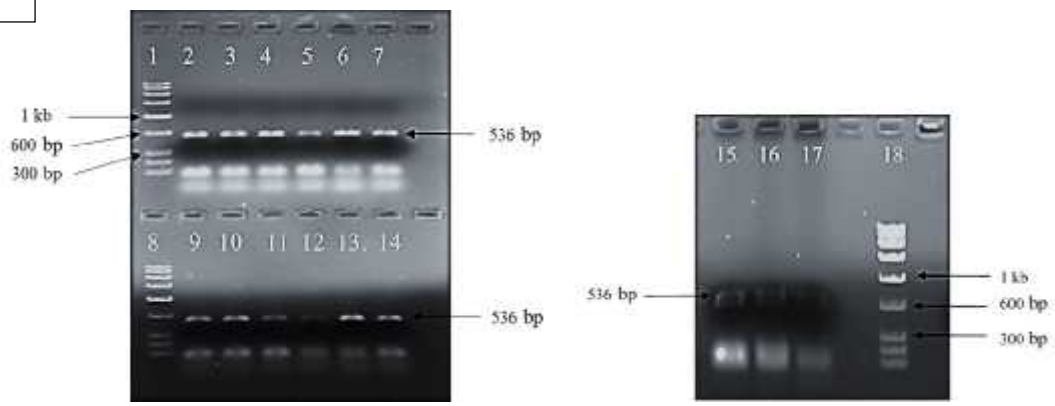


Figure 4.1. (B) 0.8% agarose gel stained with ethidium bromide with the β-actin specific region amplicons from cDNA prepared from uninduced PBMCs, PBMCs stimulated with various lactobacilli and *E. coli*.

β-actin amplicon
Lane 1 and 8: Low range DNA marker
Lane 2: uninduced PBMCs (Control)
Lane 3: PBMCs induced with *E. coli*
Lane 4: PBMCs induced with LGG
Lane 5: PBMCs induced with GPI-4
Lane 10: PBMCs induced with GPI-3
Lane 11: PBMCs induced with GPI-1(S)
Lane 12: PBMCs induced with FA-5
Lane 13: PBMCs induced with FA-7
Lane 14: PBMCs induced with GKI-1
Lane 15: PBMCs induced with IIS11.2

Lane 6: PBMCs induced with GRI-2
Lane 7: PBMCs induced with GPI-1(B)
Lane 9: PBMCs induced with GPI-7
Lane 16: PBMCs induced with GPI-6
Lane 17: PBMCs induced with FA-1
Lane 18: Low range DNA marker

A

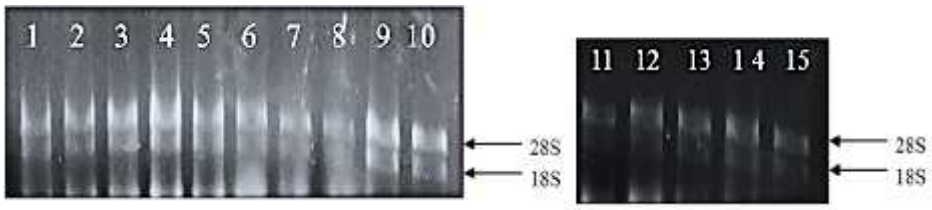


Figure 4.2. (A) 0.8% agarose gel stained with ethidium bromide with the total RNA from uninduced macrophages, macrophages stimulated with various lactobacilli and *E. coli*.

Lane 1: uninduced macrophages (Control)
Lane 2: induced with *E. coli*
Lane 3: induced with GPI-7
Lane 4: induced with GPI-4
Lane 5: induced with FA-5
Lane 6: induced with GRI-2
Lane 7: induced with IIS11.2
Lane 8: induced with GPI-3
Lane 9: induced with LGG
Lane 10: induced with GPI-6
Lane 11: induced with FA-7
Lane 12: induced with GPI-1(S)
Lane 13: induced with FA-1
Lane 14: induced with GKI-1
Lane 15: induced with GPI-1(B)

B

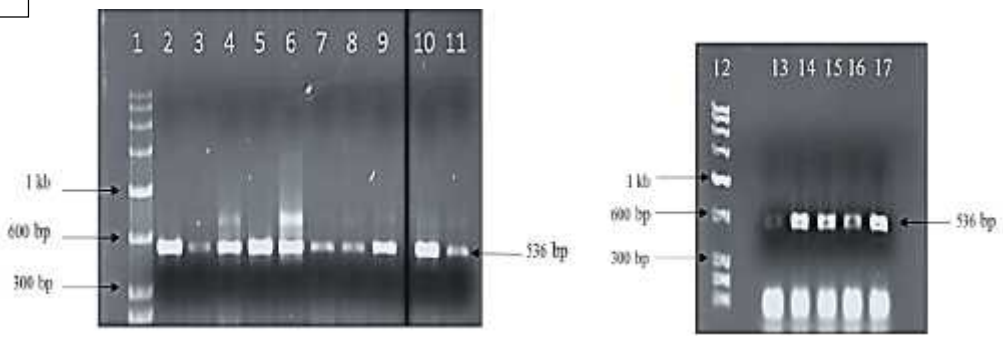


Figure 4.2. (B) 0.8% agarose gel stained with ethidium bromide with the β-actin specific region amplicons from cDNA prepared from uninduced macrophage, macrophages stimulated with various lactobacilli and *E. coli*.

β-actin amplicon
Lane 1 and Lane 12: Low range DNA marker
Lane 2: uninduced macrophage (Control)
Lane 3: macrophage induced with *E. coli*
Lane 4: macrophage induced with GPI-3
Lane 5: macrophage induced with GPI-4
Lane 9: macrophage induced with GPI-7
Lane 10: macrophage induced with LGG
Lane 11: macrophage induced with GPI-1(S)
Lane 13: macrophage induced with FA-5
Lane 14: macrophage induced with GPI-6

Lane 6: macrophage induced with IIS11.2
Lane 7: macrophage induced with GPI-1(B)
Lane 8: macrophage induced with GRI-2
Lane 15: macrophage induced with GKI-1
Lane 16: macrophage induced with FA-1
Lane 17: macrophage induced with FA-7

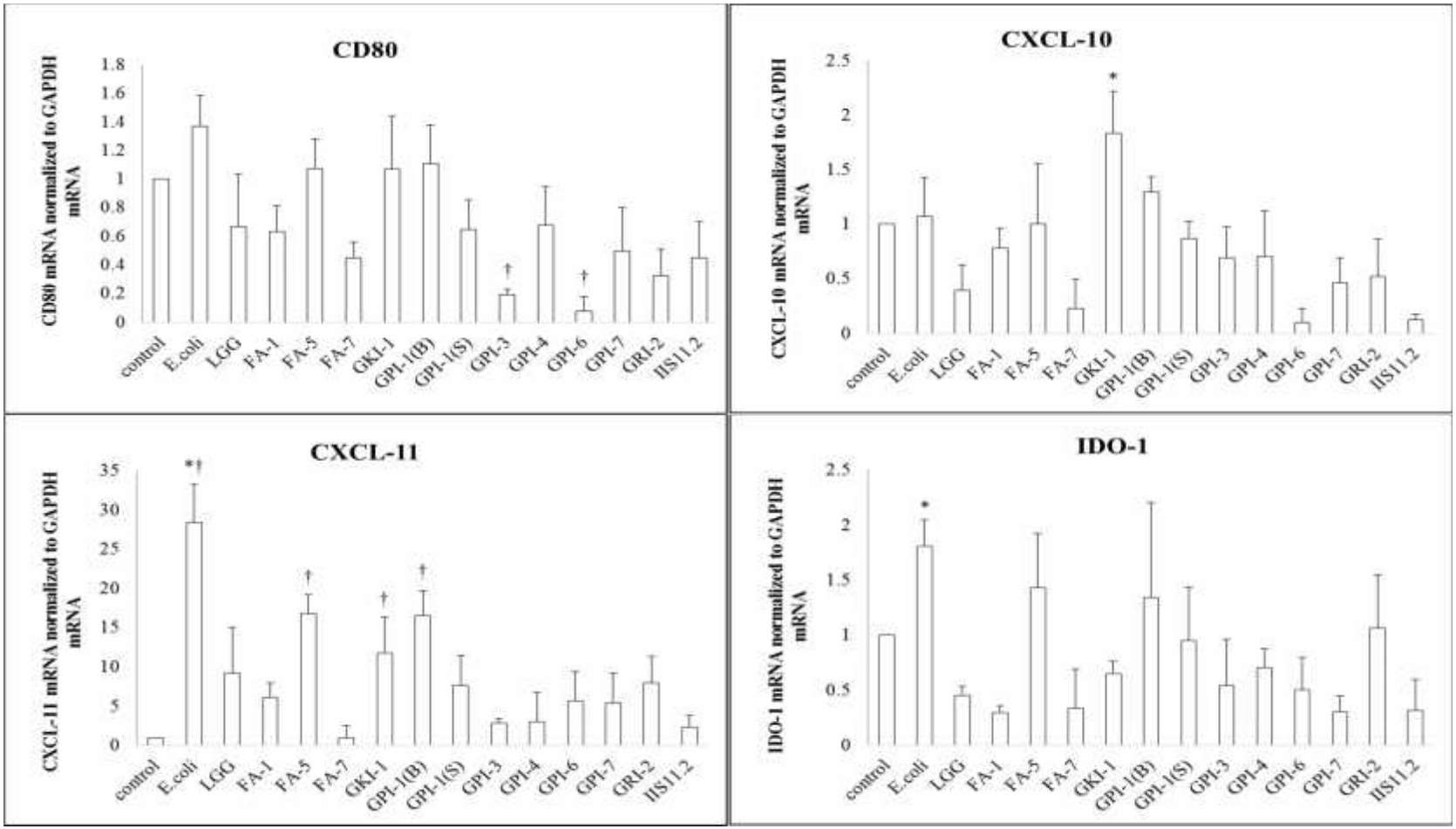
The expected amplicon size of 536 bp was obtained for all the cDNA prepared from their respective RNA. This demonstrates the integrity of the cDNA preparations.

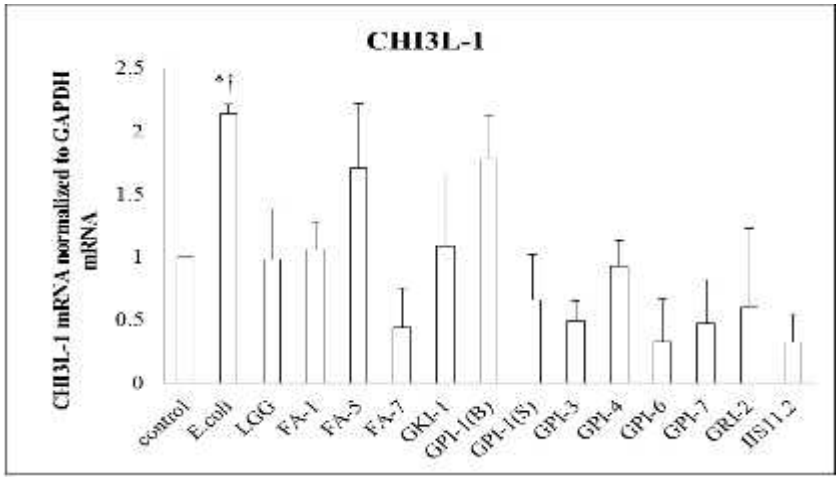
4.3.2. Quantification of various macrophage markers (M1 and M2) in PBMCs and macrophages through real-time PCR

Monocytes differentiate into M1 and M2 macrophages depending on the external stimuli or environment present. M1 and M2 macrophages express different kinds of markers and hence depending on expression of the markers, it may be predicted that which *Lactobacillus* culture will lead to generation of M1 or M2 macrophages. The cultures which after stimulating PBMCs and macrophages lead to expression of M1 markers will lead to generation of pro-inflammatory cytokines, whereas those cultures which will lead to expression of M2 macrophages will lead to generation of anti-inflammatory cytokines. Thus, the expression level of various M1 and M2 markers in both PBMCs and macrophages have been analysed through real-time PCR (Fig. 4.3A and Fig.4.3B, respectively).

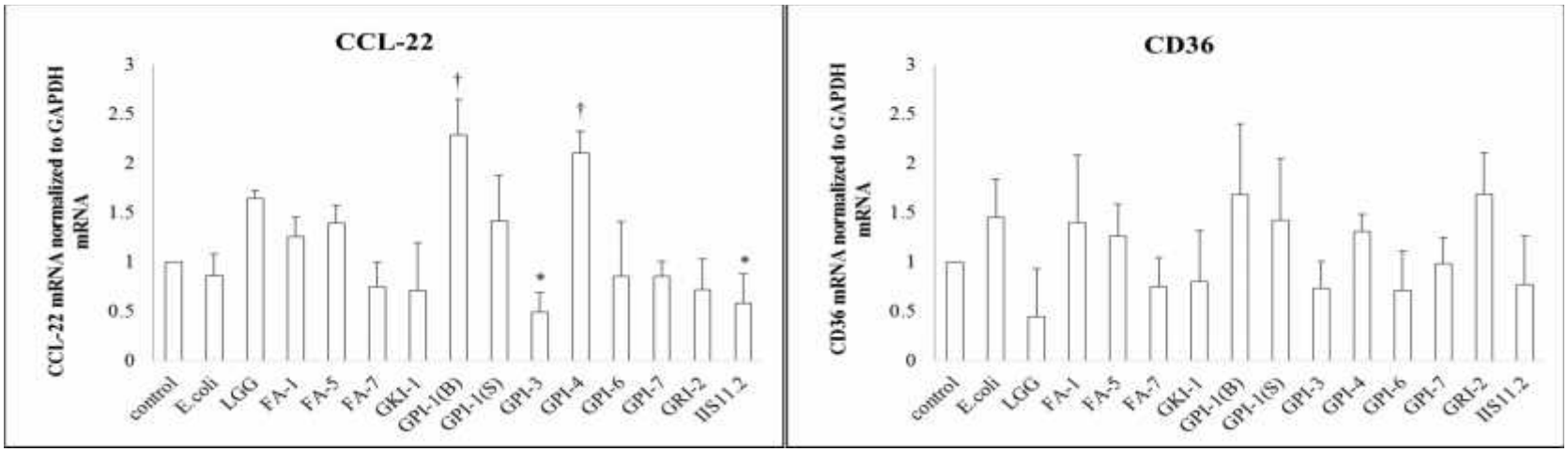
A

M1 markers (CD80, CXCL-10, CXCL-11, IDO-1 and CHI3L-1) expression in PBMCs co-cultured with lactobacilli and *E. coli* O26:H11‡





M2 markers (CCL-22, CD36, PPAR-γ, CD200R and SR-B1) expression in PBMCs co-cultured with lactobacilli and *E. coli* O26:H11‡



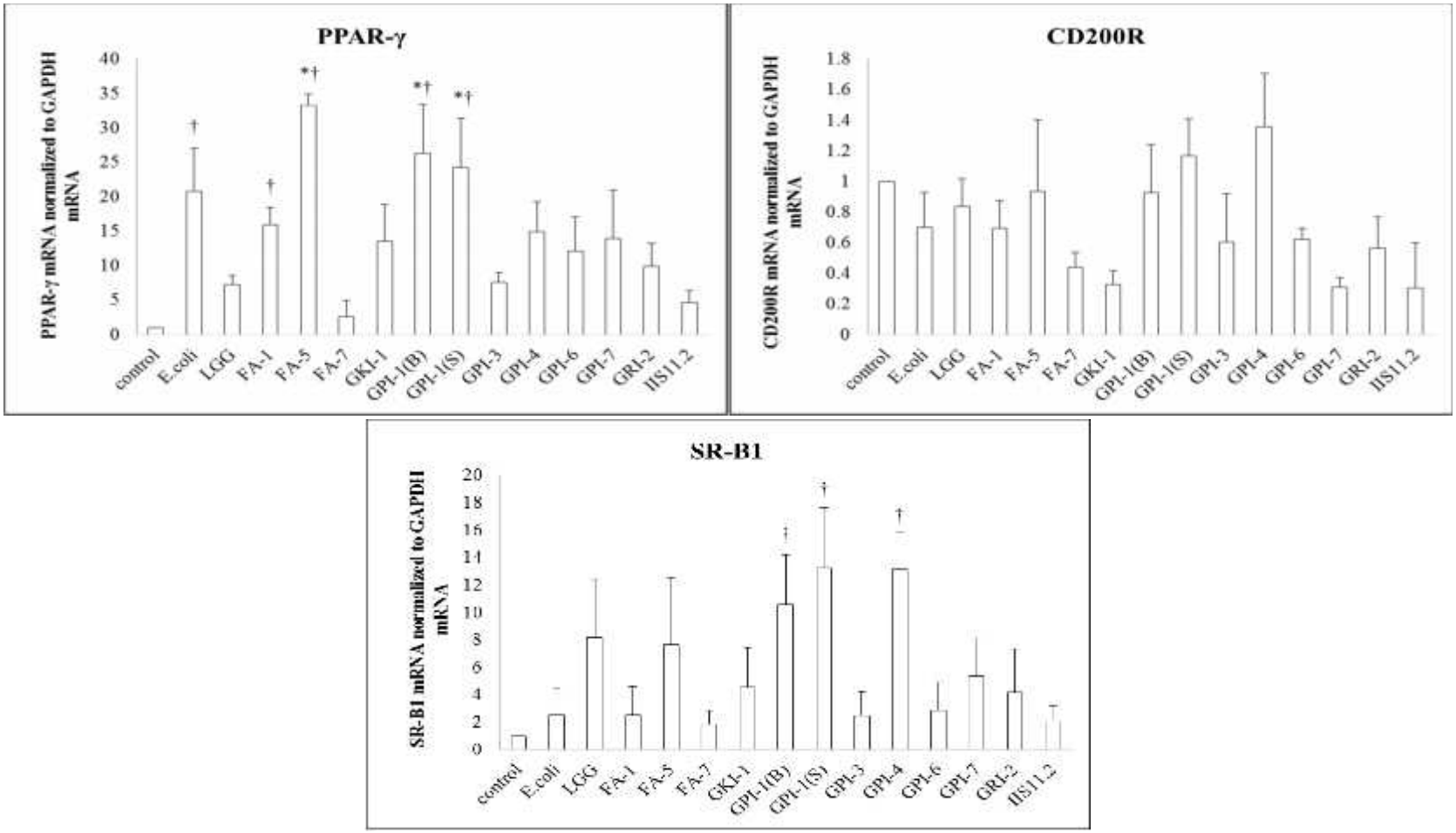


Figure 4.3. (A) M1 and M2 marker expression level in PBMCs after co-incubated with different lactobacilli. *Mean value of isolates was significantly different from that of LGG ($P < 0.05$). †Mean value of isolates was significantly different from that of control (uninduced PBMCs) ($P < 0.05$). ‡Results were obtained from two independent experiments performed in triplicates. The strains were compared with two different controls (LGG and uninduced PBMCs) by means of two independent ANOVA tests. Significant ANOVAs were followed by Dunnett's test for multiple comparisons *vs.* the control group.

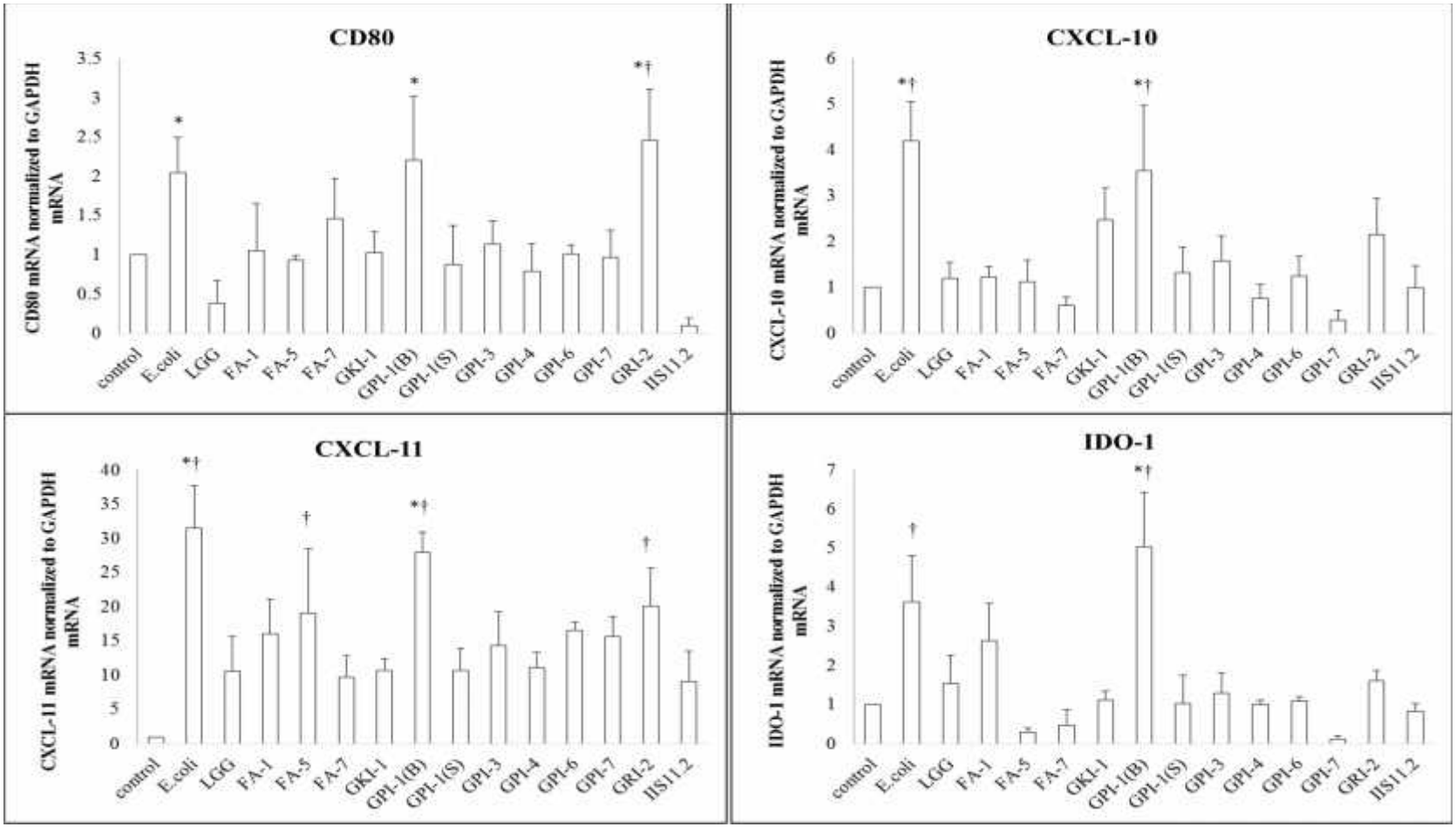
Table 4.9. Summary of M1 and M2 marker expression in PBMCs after co-incubated with different lactobacilli*

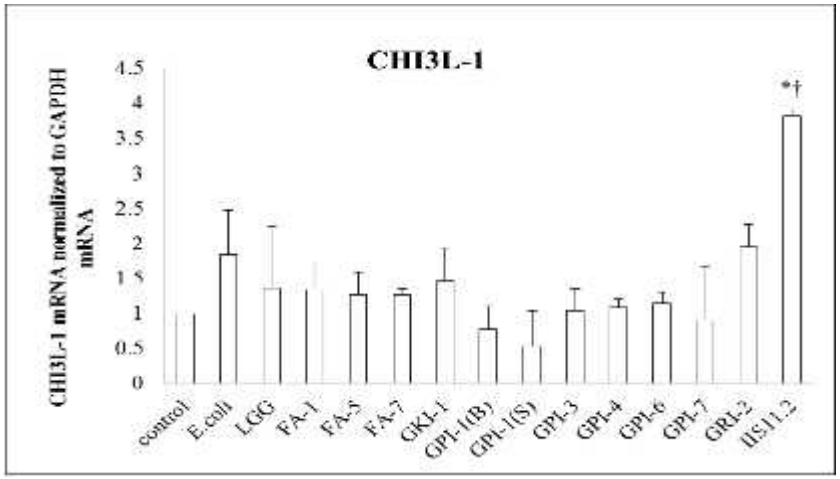
Culture	M1↑	M2↑	M1↓	M2↓	M1↑ & M2↑	M1↓ & M2↓	M1↓ & M2↑
FA-1							
FA-5	+	+			+		
FA-7			+	+		+	
GKI-1	+						
GPI-1(B)	+	+			+		
GPI-1(S)		+	+				+
GPI-3			+	+		+	
GPI-4		+	+				+
GPI-6			+	+		+	
GPI-7							
GRI-2							
HS11.2			+	+		+	

*Symbols refer to change in the expression level of M1 and M2 markers as compared to respective controls (uninduced PBMCs): ↑, up regulation; ↓, down regulation; +, respective isolates. None inclusion of a marker means the marker message was not either detected or significantly affected in that particular co-cultivation.

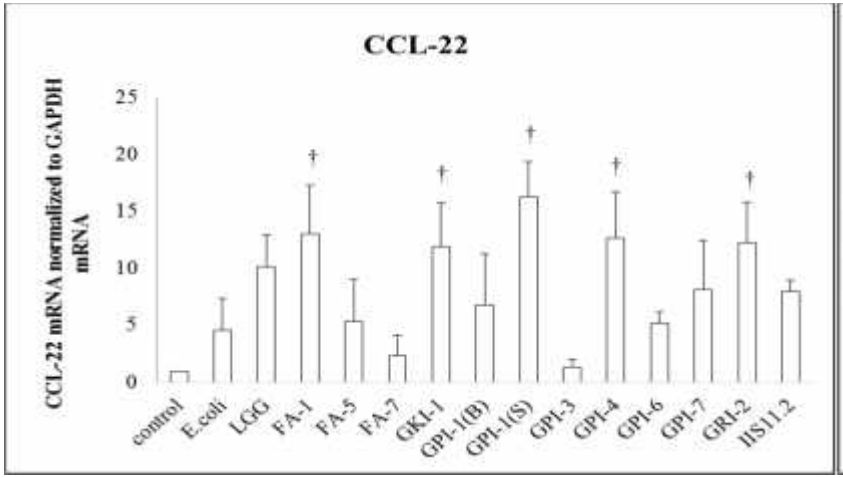
B

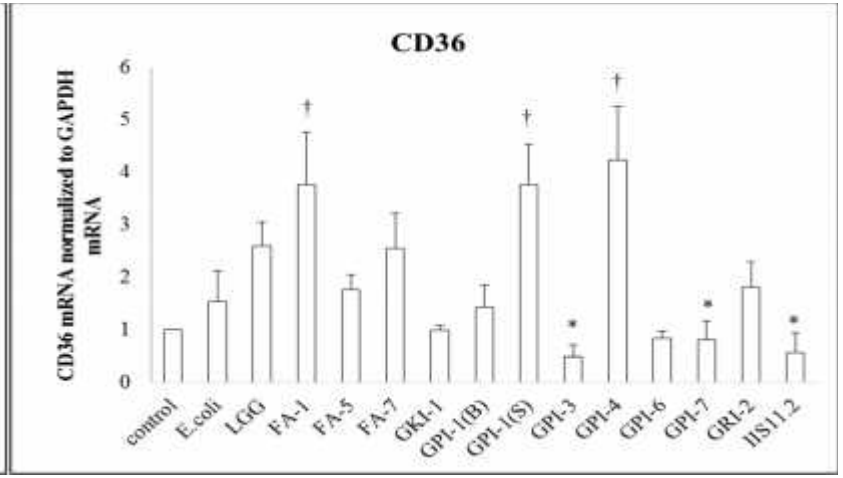
M1 markers (CD80, CXCL-10, CXCL-11, IDO-1 and CHI3L-1) expression in macrophages co-cultured with lactobacilli and *E. coli* O26:H11‡





M2 markers (CCL-22, CD36, PPAR-γ, CD200R and SR-B1) expression in macrophages co-cultured with lactobacilli and *E. coli* O26:H11‡





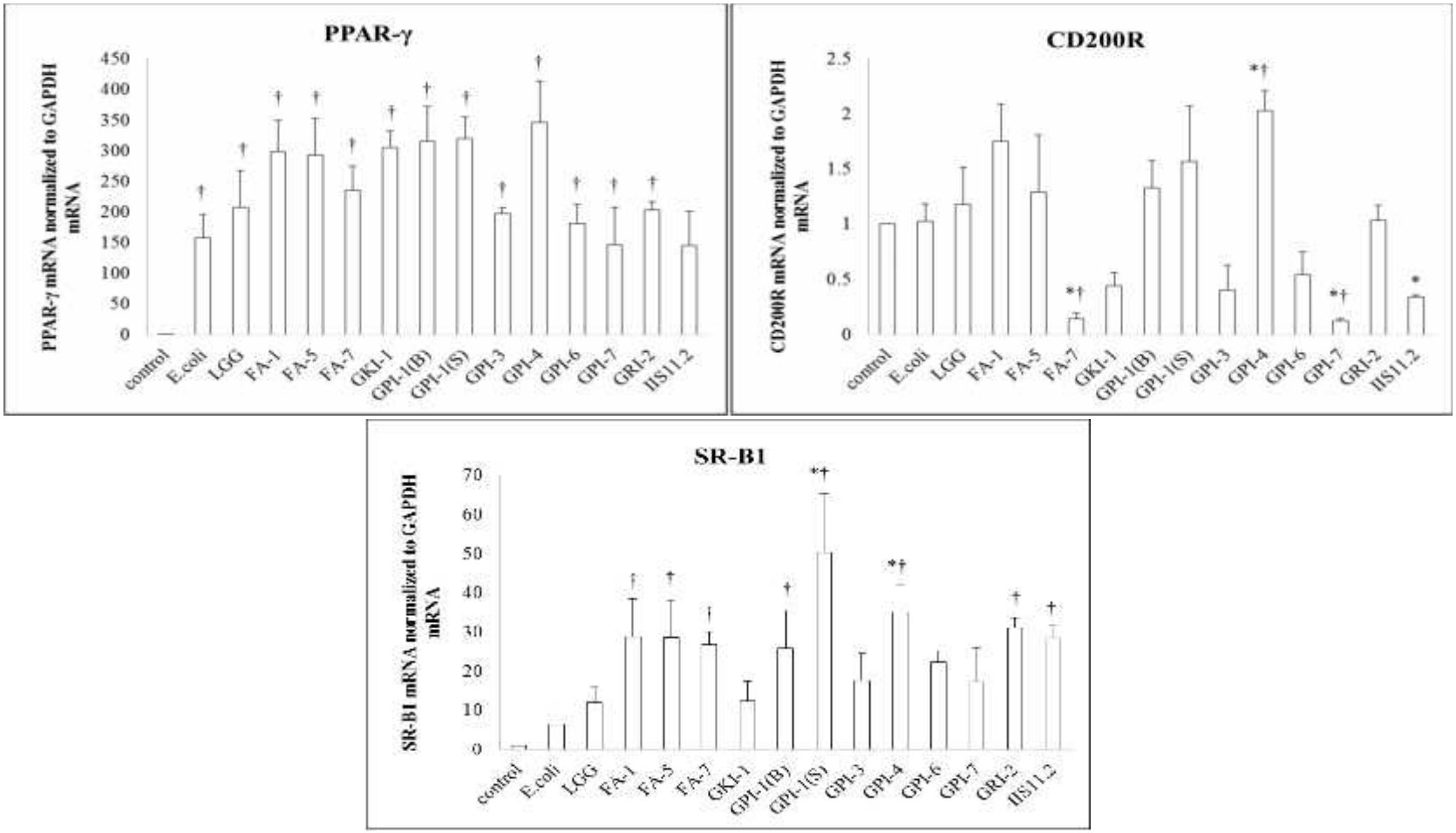


Figure 4.3. (B) M1 and M2 marker expression level in macrophages after co-incubated with different lactobacilli. *Mean value of isolates was significantly different from that of LGG ($P < 0.05$). †Mean value of isolates was significantly different from that of control (uninduced macrophages) ($P < 0.05$). ‡Results were obtained from two independent experiments performed in triplicates. The strains were compared with two different controls (LGG and uninduced macrophage) by means of two independent ANOVA tests. Significant ANOVAs were followed by Dunnett's test for multiple comparisons *vs.* the control group.

Table 4.10. Summary of M1 and M2 marker expression in macrophages after co-incubated with different lactobacilli*

Culture	M1 ↑	M2 ↑	M1 ↓	M2 ↓	M1 ↑ & M2 ↑	M1 ↓ & M2 ↓	M1 ↓ & M2 ↑
FA-1		+					
FA-5		+					
FA-7							
GKI-1							
GPI-1(B)	+	+			+		
GPI-1(S)		+	+				+
GPI-3			+	+		+	
GPI-4		+	+				+
GPI-6			+	+		+	
GPI-7			+	+		+	
GRI-2	+	+			+		
IIS11.2			+	+		+	

*Symbols refer to change in the expression level of M1 and M2 markers as compared to respective control (uninduced macrophages): ↑, up regulation; ↓, down regulation; +, respective isolates. None inclusion of a marker means the marker message was not either detected or significantly affected in that particular co-cultivation.

L. salivarius strains GPI-1(S) and GPI-4 treated PBMCs and macrophages both expressed significantly ($P < 0.05$) higher level of anti-inflammatory M2 marker and low level of pro-inflammatory M1 marker (Table 4.9 and Table 4.10, respectively). Thus, it indicated that GPI-1(S) and GPI-4 strains will lead to high production of anti-inflammatory cytokines and less production of pro-inflammatory cytokines. Generation of anti-inflammatory cytokines is considered to be beneficial at the state of an infection and most of the strains (*L. salivarius* GPI-1(S) and GPI-4, LGG, *L. plantarum* GRI-2, *L. fermentum* FA-1 and GPI-1(B)) induced PBMCs and macrophages both showed significant higher "anti-inflammatory" M2 profile ($P < 0.05$). Whereas *E. coli* O26:H11 infected PBMCs and

macrophages both showed higher “pro-inflammatory” M1 profile. This study showed that the M1 and M2 markers expression level in both human PBMCs and macrophages to lactobacilli can be different depending on the strains. From this study it was observed that the M2 marker expression levels were found to be higher in macrophages as compared to PBMCs. In healthy individual also, M1 marker expression levels in both PBMCs and macrophages were not significantly ($P < 0.05$) different as compared to control (uninduced PBMCs/macrophages).

4.3.3. RNA isolation and cDNA quality confirmation of PMNs exposed to different lactobacilli and *E. coli* O26:H11

The total RNA was isolated from the control and lactobacilli and/or *E. coli* O26:H11 exposed to PMNs using TaKaRa Isoplus kit. This was followed by 0.8% agarose gel electrophoresis and the presence of 18S and 28S rRNA on the gel shows the integrity of RNA sample. The quality of cDNA prepared by reverse transcription from these samples was confirmed by performing PCR with β-actin specific primers (Fig. 4.4A and Fig. 4.4B, respectively).

A

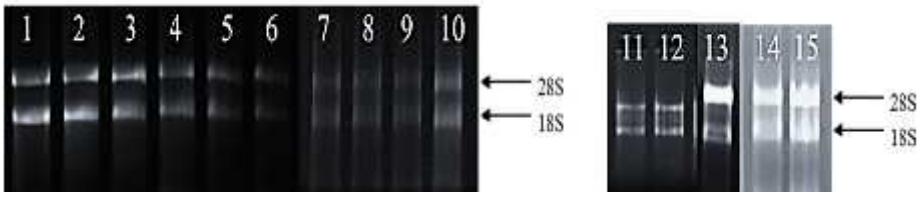


Figure 4.4. (A) 0.8% agarose gel stained with ethidium bromide with the total RNA from uninduced PMNs, PMNs stimulated with various lactobacilli and *E. coli*.

Lane 1: uninduced PMNs (Control)
Lane 2: induced with GPI-1(B)
Lane 3: induced with GPI-4
Lane 4: induced with LGG
Lane 5: induced with FA-5
Lane 6: induced with IIS11.2
Lane 7: induced with *E. coli*
Lane 8: induced with FA-1
Lane 9: induced with GKI-1
Lane 10: induced with FA-7
Lane 11: induced with GPI-6
Lane 12: induced with GPI-1(S)
Lane 13: induced with GPI-3
Lane 14: induced with GPI-7
Lane 15: induced with GRI-2

B

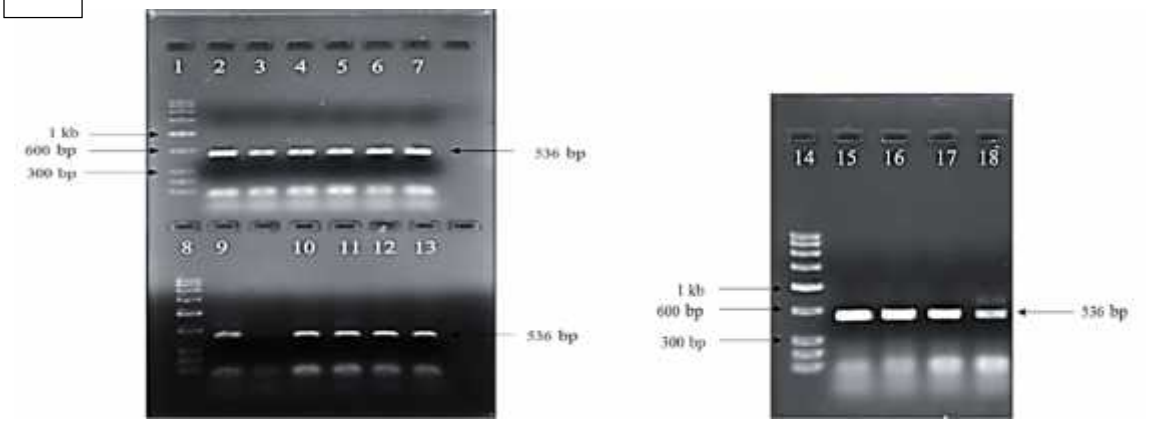


Figure 4.4. (B) 0.8% agarose gel stained with ethidium bromide with the β-actin specific region amplicons from cDNA prepared from uninduced PMNs, PMNs stimulated with various lactobacilli and *E. coli*.

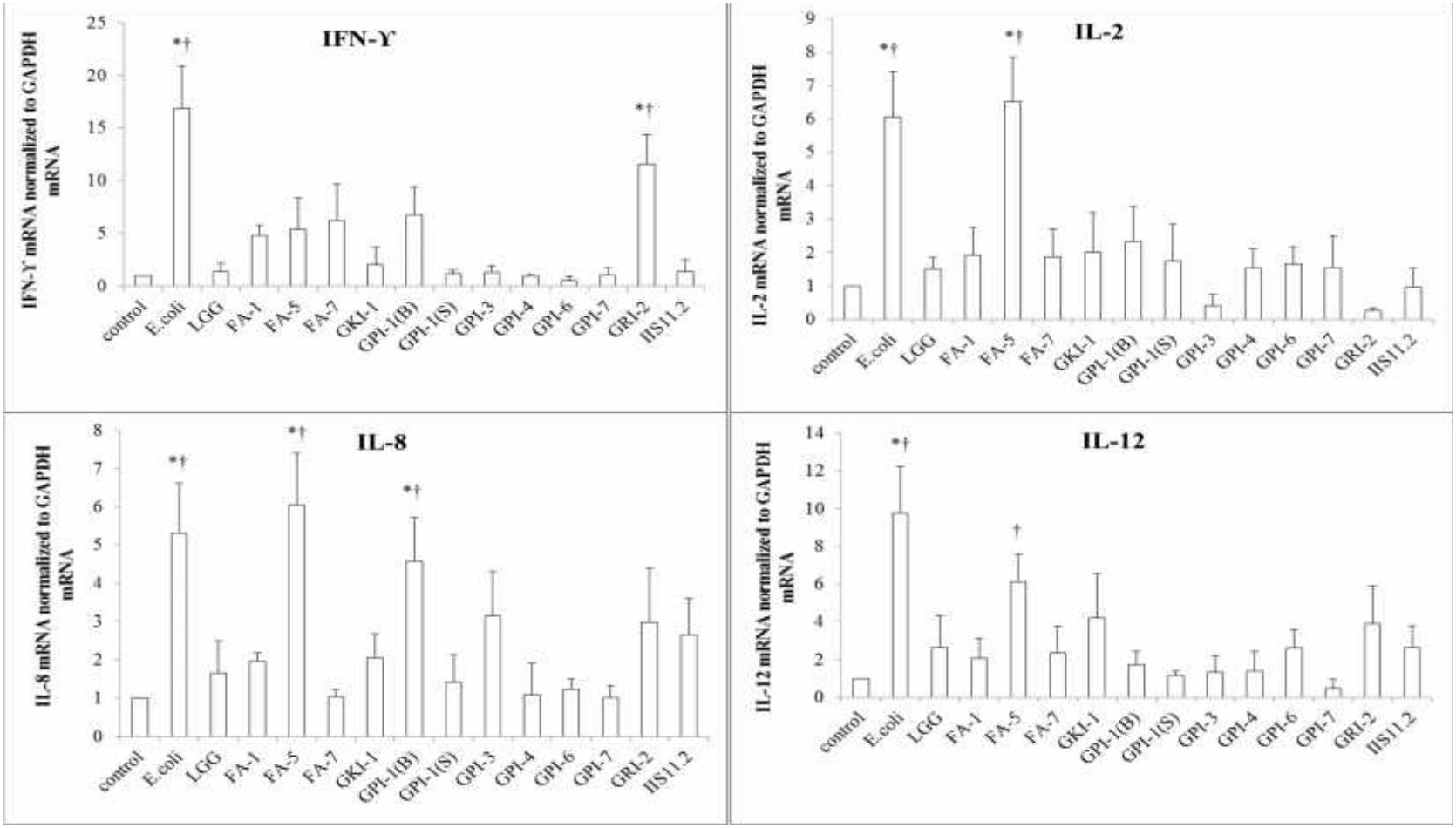
β-actin amplicon
Lane 1, 8 and 14: Low range DNA marker
Lane 2: uninduced PMNs (Control)
Lane 3: PMNs induced with FA-7
Lane 4: PMNs induced with GRI-2
Lane 5: PMNs induced with GKI-1
Lane 6: PMNs induced with GPI-7
Lane 7: PMNs induced with GPI-1(B)
Lane 9: PMNs induced with GPI-3
Lane 10: PMNs induced with IIS11.2
Lane 11: PMNs induced with FA-5
Lane 12: PMNs induced with GPI-6
Lane 13: PMNs induced with FA-1
Lane 15: PMNs induced with LGG
Lane 16: PMNs induced with GPI-1(S)
Lane 17: PMNs induced with GPI-4
Lane 18: PMNs induced with *E. coli*

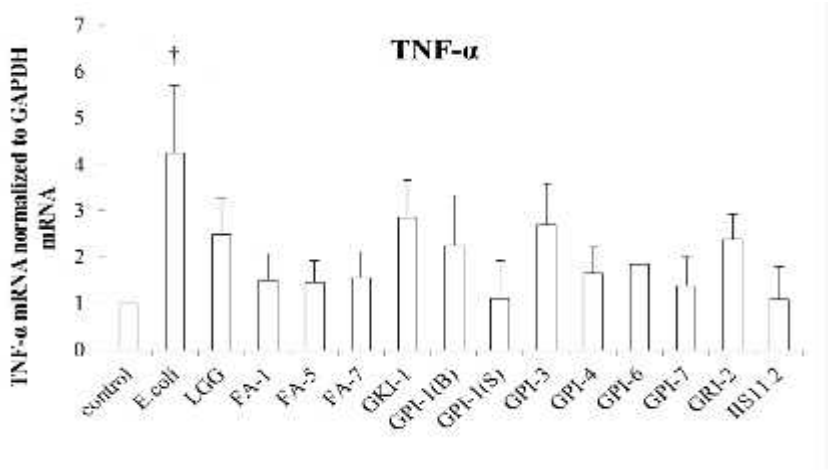
The expected amplicon size of 536 bp was obtained for all the cDNA prepared from their respective RNA which demonstrated the integrity of the cDNA preparations.

Quantification of various cytokine through real-time PCR

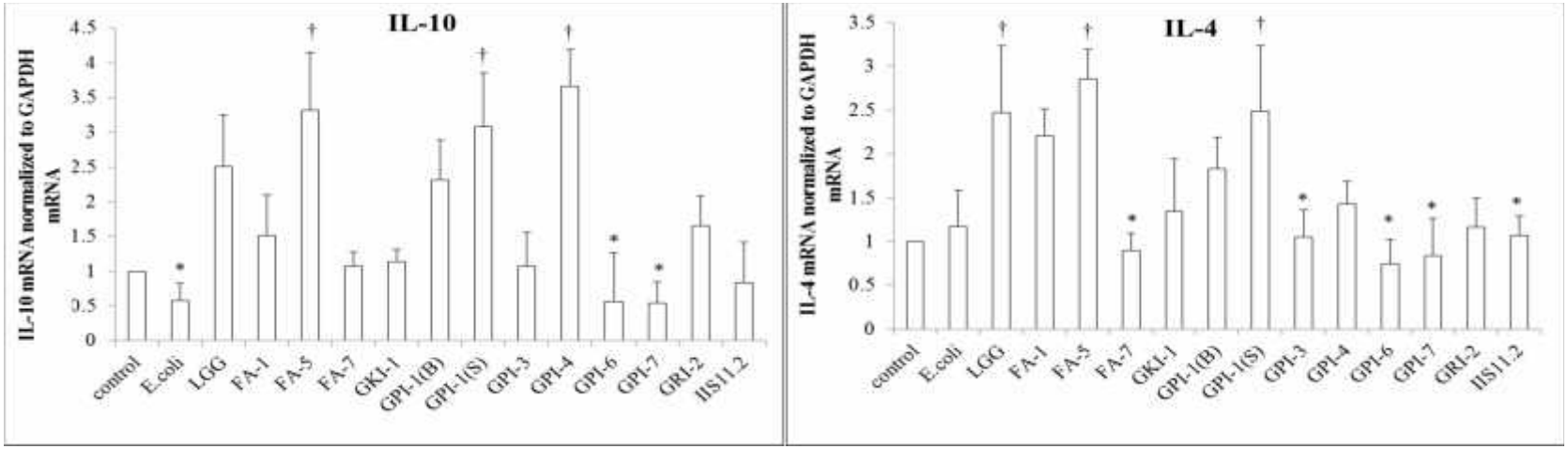
The cDNAs prepared from these samples were further used to analyse the expression level of pro and anti-inflammatory cytokines (Fig. 4.5).

Pro-inflammatory cytokines (IFN-γ, IL-2, IL-8, IL-12 and TNF-α) expression in PMNs co-cultured with lactobacilli and *E. coli* O26:H11‡





Anti-inflammatory cytokines (IL-10, IL-4 and TGF-β) expression in PMNs co-cultured with lactobacilli and *E. coli* O26:H11‡



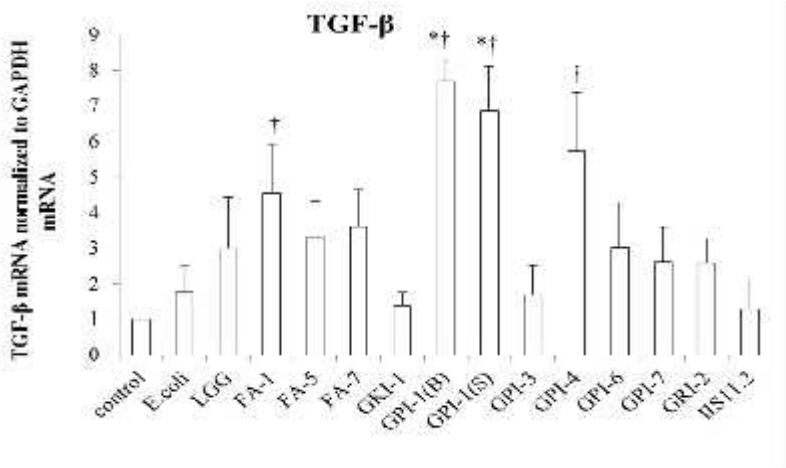


Figure 4.5. Cytokine expression level in PMNs after co-incubated with different lactobacilli. *Mean value of isolates was significantly different from that of LGG ($P < 0.05$). †Mean value of isolates was significantly different from that of control (uninduced PMNs) ($P < 0.05$). ‡Results were obtained from two independent experiments performed in triplicates. The strains were compared with two different controls (LGG and uninduced PMNs) by means of two independent ANOVA tests. Significant ANOVAs were followed by Dunnett's test for multiple comparisons *vs*. the control group.

Table 4.11. Summary of pro- and anti-inflammatory cytokine expression in PMNs after co-incubated with different lactobacilli*

Culture	Pro-inflammatory ↑	Anti-inflammatory ↑	Pro-inflammatory ↓	Anti-inflammatory ↓	Pro- & Anti-inflammatory ↑↑	Pro- & Anti-inflammatory ↓↓	Pro & Anti inflammatory ↓↑
FA-1		+	+				+
FA-5	+	+			+		
FA-7				+			
GKI-1	+			+			
GPI-1(B)	+	+			+		
GPI-1(S)		+	+				+
GPI-3				+			
GPI-4		+	+				+
GPI-6			+	+		+	
GPI-7			+	+		+	
GRI-2	+	+		+	+		
IIS11.2			+	+		+	

*Symbols refer to change in the expression level of pro and anti-inflammatory cytokine as compared to respective control (uninduced PMNs): ↑, up regulation; ↓, down regulation; +, respective isolates. None inclusion of a cytokine means the cytokine message was not either detected or significantly affected in that particular co-cultivation.

L. fermentum FA-1, *L. salivarius* strains GPI-1(S) and GPI-4 treated PMNs expressed significantly ($P < 0.05$) higher level of anti-inflammatory cytokines and low level of pro-inflammatory cytokines (Table 4.11). Whereas *E. coli* O26:H11 infected PMNs showed higher pro-inflammatory cytokines level (IFN-γ, IL-12, IL-2, TNF-α and IL-8). It was observed that in healthy individual, pro-inflammatory cytokine expression levels in PMNs was not significantly ($P < 0.05$) different as compared to control (uninduced PMNs). This study also showed that the cytokine expression level in human PMNs to lactobacilli can be different depending on the strains, as same as earlier results with PBMCs and/or

macrophages. The reasons are not clear but it may be related to species and strain dependent differences in LTA (lipoteichoic acid) and WTA (wall teichoic acids) composition.

Overall it was observed that *L. salivarius* strains GPI-1(S) and GPI-4 treated PBMCs, macrophages and PMNs expressed significantly ($P < 0.05$) higher level of anti-inflammatory cytokine or M2 markers and low level of pro-inflammatory cytokine or M1 markers. Hence these two strains can be considered as a beneficial at the state of an infection.

4.4. Discussion

Intestinal microflora and probiotics, may influence the immune mechanisms of the host by effects on mucosal barrier mechanisms and on the functional maturation of the immune system. Cells of innate immune system like PMNs, monocytes, macrophages or the most professional antigen presenting cells and dendritic cells, has an important effect on the functional maturation of the immune system (Bennouna *et al*. 2003; Silva 2010). Several lactobacilli have been shown to activate monocytes, macrophages, and PMNs under different *in vitro* co-culture systems, which are important in antigen processing, presentation and activation of antigen-specific immune response, i.e. cell-mediated immunity (Meijerink and Wells 2010). *Lactobacillus* strains are known to impart immunomodulatory effect to the host by generation of pro- or anti-inflammatory cytokines by various immune cells like monocytes, macrophages, and PMNs (Stadlbauer *et al*. 2008; Taverniti *et al*. 2012). Generation of anti-inflammatory cytokines is considered to be beneficial at the site of extensive inflammation. Specific probiotic characteristics of

lactobacilli have been associated with the presence of particular surface molecules or structures, such as peptidoglycan, teichoic acids, exopolysaccharides and surface proteins, to evoke different host responses (Lebeer *et al*. 2008). Additionally, Johansson *et al*. (2016) reported immune-modulatory nature of factors derived from several lactobacilli, suggesting that molecules present in the lactobacilli-CFS such as lactate are able to directly dampen *in vitro Staphylococcus aureus*-induced activation of conventional and unconventional T cells and NK cells. Effect of cell-surface fractions (LPS) and secreted metabolites (in the lactobacilli-CFS) on cytokine production and induction of CD25 expression in human PBMCs was also discussed by Ashraf *et al*. (2014). Thomas *et al*. (2012) suggested that *L. reuteri* derived histamine suppresses TNF via modulation of PKA and ERK Signaling. Furthermore, LGG-derived soluble protein p40, ameliorated cytokine induced intestinal epithelial apoptosis through activation of the EGF receptor, thereby preventing and treating intestinal inflammation in mouse models of colitis (Yan and Polk 2012). Further investigation by Fong *et al*. (2016), suggested that the soluble factors (metabolites, proteins, DNA and cell-wall constituents) released by LGG during its growth exert similar immunomodulatory effects as the intact bacterial cells in monocytes and macrophages.

In the present study different lactobacilli were evaluated for their ability to modulate the immune system after co-incubated with different immune cells like PBMCs, macrophages and PMNs. *L. salivarius* strains GPI-1(S) and GPI-4 treated both PBMCs and macrophages expressed high level of “anti-inflammatory” M2 marker and low level of “pro-inflammatory” M1 marker. In the literature there are many reports that suggest anti-

inflammatory role of *L. salivarius* strain, for example, Kigerl *et al*. (2009) demonstrated that their strains *L. salivarius* Ls33 and *L. rhamnosus* Lr32 showed more anti-inflammatory profile in PBMCs then other strains, as similar to present study. Peran *et al*. (2005) observed that *L. salivarius* strain was not only able to reduce the LPS-induced TNF-α, IFN-γ and IL-12 production, but also to increase the levels of IL-10, similar results were observed with the *L. salivarius* strains used in this study. Most of the strains (*L. salivarius* GPI-1(S) and GPI-4, LGG, *L. plantarum* GRI-2, *L. fermentum* FA-1 and GPI-1(B)) induced PBMCs and macrophages both showed higher "anti-inflammatory" M2 profile. Whereas *E. coli* O26:H11 infected PBMCs and macrophages both showed higher "pro-inflammatory" M1 profile.

LGG, *L. fermentum* FA-1, FA-5, *L. salivarius* GPI-1(S) and GPI-4 induced PMNs were also showed higher expression level of anti-inflammatory cytokines (IL-10, IL-4 and TGF-β), whereas *E. coli* O26:H11 infected PMNs showed higher pro-inflammatory cytokines level (IFN-γ, IL-12, IL-2, TNF-α and IL-8). Earlier reports (Ambarus *et al*. 2012; Sica *et al*. 2012; Wang *et al*. 2014) suggested that LPS and the pro-inflammatory cytokine IFN-γ promote the differentiation of classically-activated "M1" macrophages (via STAT1 signaling pathway) and anti-inflammatory cytokine IL-4 or IL-10 and TGF-β promotes an alternatively activated "M2" phenotype (via STAT6 signaling pathway). Also more IL-10 was produced after exposure to LGG and other *Lactobacillus* strains (FA-5, GPI-1(S) and GPI-4) with neutrophils in the present study, which is similar as earlier report by Cai *et al*. (2016). IL-10 plays a central role in down-regulating inflammatory cascades and maintaining gut homeostasis (Mohamadzadeh *et al.* 2005). Further, Jang *et al*. (2013)

reported that IL-10 expression-inducing LAB can ameliorate colitis by inhibiting NF-κB, MAPK and AKT pathways and by polarizing M1 macrophages to M2-like macrophages. Several studies indicated that selective probiotics induce IL-10 production in the intestine or the development of IL-10 producing T cells *in vitro*, depending on the TLRs pathways (Rachmilewitz *et al*. 2004; Jeon *et al*. 2012). In earlier reports it was also suggested that, metabolites from lactic acid producing bacteria have been able to reduce TLR-induced inflammatory responses (Menard *et al*. 2004; Johansson *et al*. 2012). Moreover, Kaji *et al*. (2010) suggested that production of IL-10 and IL-12 by macrophages in response to lactobacilli requires the activation of common signal transduction pathways and that additional potent activation of TLR2-dependent ERK leads to IL-10 production but that insufficient ERK activation leads to IL-12 production. Initiation of TLR signaling is tightly regulated because prolonged and excessive activation of TLRs can lead to uncontrolled inflammation detrimental to the host. Varied mechanisms appear to contribute to control of TLR activation in the intestinal epithelium. These include the collective effects of several negative regulators that include IRAK-M, TOLLIP, SIGIRR, A20, Nod2, and PPARγ (Shibolet *et al*. 2007). Veckman *et al*. (2003) reported that *L. rhamnosus* GG (LGG) showed low CXCL-10 (M1) marker expression, while pathogenic bacteria showed high CXCL-10 expression, which is also in line with present result. This study has shown that the M1 and M2 marker expression level in human PBMCs and macrophages to lactobacilli can be different depending on the strain and different cell types used, which is also reported by others (Foligne *et al*. 2007; Kigerl *et al*. 2009). Strain-dependent differences in microbe-associated molecular patterns (MAMPs) such as lipoteichoic acids (LTA), the peptidoglycan structure and non-methylated CpG motifs of lactobacilli can modulate pro-

inflammatory or anti-inflamatory immune responses (Wells 2011). Rong *et al*. (2015) results suggested that the S-layer protein isolated from *L. helveticus* NS8 mainly attenuated LPS induced IL-12 levels in mouse macrophage cell line RAW264, while didn't influence the expression levels of IL-10. In contrast, the S-layer protein of *L. helveticus* MIMLh5 induced a pro-inflammatory effect in human U937 macrophages and macrophages isolated from mouse bone marrow (BMDMs) (Taverniti *et al*. 2013). Such different data can be explained by considering the difference in surface proteins and different cell types used.

Other than pro- or anti-inflammatory cytokines regulation, lactobacilli have also been shown to mediate their immune-modulatory effects through other mechanisms including, (i) induction of T regulatory cells: Smelt *et al*. (2012) demonstrate that in healthy mice, *L. plantarum*, *L. salivarius*, and *L. lactis* strains can balance T cell immunity in favor of a more regulatory status, via both regulatory T cell dependent and independent mechanisms in a strain dependent manner, (ii) modulation of APC: Haileselassie *et al*. (2016) reported that *L. reuteri*-CFS modulates the phenotype and function of retinoic acid imprinted mucosal-like DC *in vitro*, (iii) promotion of epithelial function and development: *Yan et al.* (2017) suggested that neonatal colonization of mice with LGG promotes intestinal development through intestinal epithelial cell proliferation, differentiation, tight junction formation and mucosal IgA production and decreases susceptibility to colitis in adulthood. From the results in this study it was observed that in healthy individual, M1 marker and pro-inflammatory cytokine expression levels in PBMCs and/or macrophages and in PMNs respectively, were not significantly different as compared to control (uninduced PBMCs/macrophage/PMNs), suggesting that the "physiological state of inflammation"

remains maintained. In accordance with results from this study, Galdeano *et al*. (2007) demonstrated that the positive influence of probiotics on gut homeostasis is achieved by bacterial antagonism and immunomodulation, which help the healthy host to maintain a "physiological state of inflammation" or to control several infectious, inflammatory and immunologic reactions. From this study, it was also observed that M2 marker expression levels were found to be higher in macrophages as compared to PBMCs. These results conclude that the molecular mechanisms of immunomodulatory capacity of lactobacilli depends on the specific interactions between bacterial ligand and host receptor/different cell types. This study has therefore been able to select some *Lactobacillus* strains that have been demonstrated to behave better than standard probiotic strain LGG in terms of their immunomodulatory properties and can be useful in the development of nutraceutical products for the prevention or treatment of inflammation-associated diseases.